

Isolating novel antibodies against *Eimeria* infections of chickens using Next Generation Phage Display and conventional Phage Display

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DVM & MSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

January 2020

School of Veterinary Medicine and Science
The University of Nottingham, UK

Author's Declaration

I declare that, except where reference is made to the contribution of others, that this thesis is the product of my own work and has not been submitted for any other degree at the University of Nottingham or any other institution.

Signature: _____  _____

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Abstract

Poultry coccidiosis caused by the protozoan parasite *Eimeria* is a major cause of economic losses in the poultry industry. The resilience and short life cycle of the parasite coupled with high stocking densities in modern day commercial poultry establishments ensure that this parasite is an ever-present pathogen on poultry farms. Traditional methods of diagnosis such as oocyst morphology, counting oocysts per gram of faeces and lesion scoring are still heavily relied upon in spite of them being highly subjective and prone to under-diagnose subclinical disease. PCR based molecular diagnostics have been available since the late 1990's but have not gained widespread application likely due to the relative expense and the need for specialist equipment. LAMP assays have recently been developed that can distinguish between the seven *Eimeria* species of chicken and they offer the advantage of being less subjective and not requiring expensive laboratory equipment. However, these assays still require template DNA to be obtained from scrapping mucosal cells of the intestines where the different *Eimeria* species are likely to be present and this requires some expertise knowledge. There is therefore a need to develop a cheap, rapid test that will help differentiate infecting *Eimeria* species and ideally that can eventually be applied in the field. This is particularly needful because of the tight economic margin prevalent in present day poultry production. Phage display is a powerful technology for isolation of diagnostic antibodies that bypasses the use of animals and links the phenotype of a displayed ligand to its genotype. The coupling of phage display and the screening power of next generation sequencing (NGS) has the potential to transform the discovery of novel antibodies against any target. It was hypothesized that NGPD will allow comparative screening of antibody binding events to identify novel antibodies to species-specific epitopes of *Eimeria* oocysts and such ligands could then be used to develop immunoassays to differentiate infecting species of *Eimeria* in chickens. Consequently, in this study, next generation phage display (NGPD) strategies were used to identify novel antibodies against whole *Eimeria* oocysts. Using a naïve scFv library and panning against whole oocysts in solution, 6 binders to *Eimeria* oocysts were identified. Whole oocysts could not be immobilised readily on solid surfaces and binding properties were established in fluorescent microscopy (FM) and a novel spin-down ELISA (SDA). The spin down assay on occasions gave different binding specificities compared to fluorescent microscopy but on the whole, the FM gave less positive results than the SDA. The inability to develop a high throughput assay for the antibodies as well as difficulties encountered in rescuing clones from polyclonal phage sub libraries were major challenges in this

approach. In later panning experiments, the use of the camelid V_HH library that uses a single antibody framework resolved the difficulty in rescuing phage candidates and conducting panning experiments on 96 well plates using oocyst lysates and distinct peptide regions of *Eimeria* oocyst surface displayed peptides had the potential to be applied in high throughput assays. For these panning experiments, the NGS approach was again used in comparison with a conventional colony picking method and the NGS data analysis involved ranking candidates using Z scores and removing non-specific binders. Subtractive phage panning was also conducted in later panning experiments to maximise the chances of isolating species-specific clones. This might have led to the removal of high affinity binders as no ELISA positive clones could be identified. The final part of this study assessed the bioinformatics analysis used in this study to try and develop strategies to identify V_HH binders. The V_HH NGS data was reassessed based on candidate frequency in the sequenced data and cohort analysis and candidates that were likely to be binders were highlighted.

Future approaches suggested include targeting oocyst homogenates or Gam56 peptides, the use of an antibody library with a single region of diversity and the application of less stringent subtractive steps in the panning. Finally, bioinformatics approach that identifies clones that are enriched in selection (not growth) stages and either have relatively high frequencies or high species-specificity as analysed by Z score analysis should be employed.

Conferences attended

- British Veterinary poultry association spring meeting 2019. *Progress and Politics in protozoal diseases*. Cedar Court Hotel, Harrogate, HG1 5AH. March 14th-15th 2019 **(Oral)**.

- 4th World Veterinary poultry association Asia Meeting. *Detection and control of poultry diseases*, Pullman Hotel 4 Jalan Conlay 50450 Kuala Lumpur, Malaysia October 1st – 2nd, 2018.

- Link 18 conference, postgraduate students' network. East Midlands conference centre, University of Nottingham, 31st May, 2018 **(Poster)**.

- 22nd Woods Hole immunoparasitology meeting, 7 Marine biological laboratory St, Woods Hole, MA Barnstable United States. April 15th-18th 2018 **(Poster**).

- British society of Parasitology Autumn meeting. '*The multidisciplinarity of parasitology: Host-parasite evolution and control in an ever-changing world'*. The Linnean society, Burlington House, London. 28th September 2017:

- Symposium School of Biosciences and Veterinary Medicine and science, Sutton Bonington campus, University of Nottingham, United Kingdom 16th-17th May 2017 **(Oral).**

- World's Poultry Science Association 2017 Annual spring meeting. *Animal science for a sustainable future*. University of Chester, Parkgate Road, Chester CH1 4AR 25th-27th April, 2017.

- Annual seminar, school of Veterinary Medicine and science 3rd July, 2016 **(oral)**

Acknowledgements

I dedicate this thesis to my faith in Jesus Christ, family, purpose and friendships; my very existence revolves around these.

I would like to express my utmost sense of gratitude to my supervisors, Professor Kevin Gough (School of Veterinary Medicine and Science, Nottingham University) and Dr Ben Maddison (ADAS Biotechnology group). They have been inspirations throughout the period of this study. Their support, knowledge, enthusiasm and attitude are unparalleled and I am grateful to have worked with them.

I would like to thank Dr Colin Crouch (MSD Animal health) for providing all the oocysts used in this study.

I am saying a big thank you to the members of ADAS; Dr Jon Owen, Keith Bishop, Claire Baker and Dr Helen Rees for their friendship, help, support and guidance in the course of these years. Special thanks to Jon for his help with my bioinformatics data.

Thank you to my fellow postgrads in the lab Matt, Rob, Rose, Kate, Brittany, Maria, Anitha, Juan and Kieran and to Ali and Amy their friendships have made the whole experience worthwhile. The contributions of Ruby and Meagan are acknowledged with gratitude.

A special thanks to my family especially my parents; Raymond N Angani and Angelina J Angani (of blessed memory) for their love and support throughout my life and for setting a solid foundation for me. To my sisters Jess, Agatha, Agnes, Linda, Tonia, Pauline and Miriam, brothers in law, nieces and nephews thanks for their concern, love and prayers.

I am grateful to some dear friends that have been inspirations at different times during the course of this PhD and to my chuch communities and people whom have inspired me one way or the other in the course of the PhD, thanks for being there. I would also like to express my gratitude to staff, technicians and postgraduate communities within the School of Veterinary Medicine and Science/ University of Nottingham for their friendship and support.

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List of abbreviations

ANOVA	analysis of variance
AP	alkaline phosphatase
BSA	Bovine serum albumin
CDR	Complementary determining region
CH	Constant region
CV	Coefficient of variation
dsDNA	Double stranded deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen binding fragment
FM	Fluorescent microscopy
FW	Framework region
HCDR3	Heavy chain complementary determining region 3
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAMP	Loop mediated isothermal amplification
LCDR3	Light chain complementary determining region 3
MBP	Maltose binding protein
MOI	Multiplicity of infection
NGPD	Next generation phage display
NGS	Next generation sequencing

OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque forming unit
PVDF	Polyvinylidene difluoride
scFv	Single chain variable fragment
SD	Standard deviation
SDA	Spin down ELISA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
TAE	Tris-acetate-EDTA
TMB	3,3',5,5'-Tetramethylbenzidine
VH	Variable heavy chain
VL	Variable light chain
V_{NAR}	Single variable new antigen receptors

1. Literature review

1.1 Overview of *Eimeria* in Poultry

As the human population keeps growing, there is increasing pressure to maximise food production to meet demands. There is also an increased consumer demand for higher quality and better welfare animal products. The poultry industry is hence under pressure to maximise productivity and profit while maintaining good welfare, high quality meat and affordability of produce to meet consumer demand. Poultry production is one of the fastest growing aspects of animal production in the world accounting for over one-third of protein for human consumption (Muthamilselvan *et al*., 2016, Quiroz-Castañeda and Dantán-González, 2015). The greatest increases over recent years have occurred in developing countries in Africa and Asia with prospects of continuing expansion (Grace *et al*., 2012, Ojimelukwe *et al*., 2018). It is predicted that this expansion in poultry production will continue for at least 30 years, posing enormous challenges for pathogen control and production practices (Blake and Tomley, 2014).

Poultry production is of high economic importance in many countries particularly in Africa and Asia were backyard poultry production is the mainstay of many households and has helped in poverty alleviation, food production, food security, employment opportunities and gender equality (Ojimelukwe *et al*., 2018, Alemayehu *et al*., 2018). Poultry production is often confronted by various infectious diseases such as avian coccidiosis, avian influenza, salmonellosis, infectious bronchitis and others (Quiroz-Castañeda and Dantán-González, 2015). Amongst these diseases, enteric diseases are an important concern to the poultry industry not only because of losses accrued from increased production costs, mortalities, and reduced welfare of birds but there is also the risk of contamination of poultry products for human consumption (Kaboudi *et al*., 2016). Poultry coccidiosis is one of the most common and economically important diseases of poultry worldwide (Shirley *et al*., 2005, Olanrewaju and Agbor, 2014). The causative agent, *Eimeria* is present wherever poultry are kept and through a combination of the parasite fecundity, ubiquity and pathogenicity has been reported to be one of the ten most economically significant endemic livestock diseases in the UK and developed world (Bennett and IJpelaar, 2005). The parasite infects a wide variety of hosts including bats (Duszynski *et al*., 1999), seals (Munro and Synge, 1991), turtles (McAllister *et al*., 1994), fish (Kocylowski *et al*., 1976), rodents (Levine, 2018), dogs, cats (Barutzki and Schaper, 2011) and livestock (Bennett and IJpelaar, 2005). *Eimeria* was first recognised by Antony van Leeuwenhoek who made a short but identifiable reference to oocysts of rabbit *Eimeria stiedea* in 1674 but not knowing what they were, his findings were never published (Dobell, 1922). (Hake, 1839)

published the first account of the oocysts of *Eimeria stiedae* while (Carswell, 1838) attempted to describe the lesions seen in hepatic coccidiosis caused by *Eimeria stiedae* (Dobell, 1922). *Eimeria* in poultry was initially described by (Fantham, 1910) who studied coccidiosis in wild grouse and he believed that coccidiosis was caused by only one species in birds and he called it *Eimeria avium* (coccidian). As the years went by, more research was done on the parasite and it was established that other different species existed that affected different birds at different predilection sites of the intestine (Ruff, 1999).

Poultry coccidiosis is a world-wide major protozoan disease caused by several *Eimeria* species (Wakelin *et al*., 1990, Kumar *et al*., 2014). They are obligatory intracellular apicomplexan protozoan parasites that belong to the family *Eimeridae* (Shirley *et al*., 2005). They are predominantly host-specific with each species occurring in a single host (Chapman *et al*., 2013). Experimentally, (McLoughlin, 1969) demonstrated that an *Eimeria* parasite of turkeys; *Eimeria meleagrimitis* was able to infect chickens that were immunosuppressed by daily injections of dexamethasone. However, a similar treatment of turkeys did not succeed in making them susceptible to *Eimeria tenella*, another species that infects chickens. Host specificity to *Eimeria* infections are thought to include genetic (Mayberry *et al*., 1982, Mathis and McDougald, 1987) and biochemical/nutritional factors (Fry *et al*., 1984, Smith and Lee, 1986) even though the underlying mechanisms are not well understood (Yun *et al*., 2000).

The parasite usually targets, invades and proliferates in the epithelial tissues of the intestine of its host resulting in severe damage and disruption of nutrient absorption. This compromising of the function of the gut leads to a decline in utilization of feed in poultry (Reid *et al.,* 1976, Ojimelukwe *et al.,* 2018) leading to poor growth and economic losses (Li *et al*., 2015, Amerah and Ravindran, 2015).

Coccidiosis is associated with high morbidity, high mortality and high production losses (Karaer *et al*., 2012, Györke *et al*., 2013) and is a major challenge for the poultry industry worldwide (Tewari and Maharana, 2011). This disease has a high impact on the poultry industry mostly because parasite transmission is favoured by modern-day poultry production involving the rearing of large numbers of birds in small confined areas at high densities (Blake and Tomley, 2014, Lal *et al.,* 2009, McDonald and Shirley, 2009, Sharma[1] *et al.,* 2009). It is not uncommon for 200-500 birds to be housed under one roof with a stocking density of up to 33 kg per m^2 (Chapman *et al*., 2013). Intensive poultry rearing practices have led to an increase in poultry stress levels and incidence of the disease (Yun *et al*., 2000).

Chickens between 3-18 weeks of age are mostly affected by *Eimeria* species and infection can cause high mortality in young chicks (Kaboudi *et al*., 2016, Prakashbabu *et al*., 2017). Oocyst excretion has been reported to be highest in chickens between 4 and 8 weeks of age (Williams, 1998) reinforcing the susceptibility of young birds to infection and the spreading of the parasite. Chickens that have recovered from coccidiosis or those with subclinical infections usually perform less optimally throughout their life (Arabkhazaeli *et al*., 2013). Seven species of *Eimeria* have been recognised to infect *chickens* at various locations along the intestinal tract: *Eimeria tenella* (Railliet and Lucet, 1891) *Eimeria brunetti,* (Levine, 1942) *Eimeria necatrix, Eimeria praecox* (Johnson, 1930) *Eimeria acervulina, Eimeria mitis* and *Eimeria maxima* (Tyzzer, 1929). Mixed infections are commonly found in field conditions with species and strains looking similar (McDougald and Fitz-Coy, 2008) but having considerable differences in their biology and pathogenicity (McDougald and Reid, 1997). This occurrence of mixed infections complicates the diagnosis and treatment of coccidiosis in poultry.

Some species; *E. maxima, E. necatrix, E. tenella, E. brunetti* and *E. acervulina* are commonly considered to be the most economically important as a result of certain factors such as their higher occurrence rate (Jadhav *et al*., 2011), fecundity and pathogenicity, even though all species can undermine the overall health and well-being of chickens (Blake and Tomley, 2014, Jadhav *et al*., 2011). *Eimeria tenella* is the most economically important and amongst the best studied species of *Eimeria* in chickens (Clark *et al*., 2017). *Eimeria mitis* and *E. praecox* have not been associated with severe disease in poultry but have been reported to reduce feed efficiency in chickens (Conway *et al*., 1993, Fitz-Coy and Edgar, 1992, Gore and Long, 1982). *Eimeria necatrix, E. brunetti*, and *E. tenella* have been reported to be associated with haemorrhagic coccidiosis while *E. acervulina, E. mitis, E. praecox* and *E. maxima* are largely associated with malabsorptive pathologies (Long *et al*., 1976, Blake *et al*., 2015). (Jenkins *et al*., 2017) in their study to determine if there was a correlation between *Eimeria* oocyst levels and broiler performance reported that there is a correlation between the abundance of *E. maxima* oocyst and chick mortality but found no observable correlation in the performance parameters, daily weight gain and feed conversion ratio with oocyst levels (Jenkins *et al*., 2017). It has been reported that *E. necatrix* is the most pathogenic *Eimeria* species which infects chickens, but *E. tenella* is more common and has a greater impact on poultry production (Long *et al*., 1976) Blake *et al*., 2015). *Eimeria* infections in poultry have been documented to hinder the digestion of many nutrients especially fat soluble vitamins status and they decrease bone mineralization (Sakkas *et al*., 2018, Ravindran *et al*., 2016).

Disease transmission is through faecal-oral route and it occurs only after ingestion of sporulated oocysts in susceptible hosts. Clinical manifestations of the disease can vary significantly but it is generally characterized by diarrhoea, emaciation, enteritis, drooping wings, poor growth, increased morbidity and mortality. Certain poor management practices such as high stocking density and wet litter can exacerbate the clinical signs.

The global poultry industry produces approximately 67 billion chickens annually and poultry coccidiosis poses a serious threat to production (Lal *et al*., 2009, McDonald and Shirley, 2009). It has been reported that poultry coccidiosis results in annual global economic losses of more than 3 billion US dollars which includes production losses as a result of morbidities, mortalities, poor growth rates in broilers, delayed sexual maturity and poor egg production in layers, poor feed conversion ratios as well as disease prevention and treatment costs (Williams, 1999, Blake and Tomley, 2014, Lobago *et al*., 2005). Drug related costs account for 24% of losses (Shirley *et al*., 2005, Ahmad *et al*., 2016, Zhang *et al*., 2013). The UK poultry industry has been reported to loose in excess of £38 million annually due to coccidiosis (Williams, 1999).

Even though each *Eimeria* species affecting chickens and the associated pathology has been understood since the 1930's (Long *et al*., 1976, Tyzzer *et al*., 1932, Chapman, 2014), the extent of genetic diversity and parasite population structures are only recently emerging. These new developments are as a result of interest in parasite epidemiology and diversity fuelled by a world-wide need for affordable and effective vaccines as alternatives to anticoccidial drugs (Clark *et al*., 2017). Reduced cost and greater efficiency in genome sequencing and the resulting collation of reference genomes has served to provide enormous opportunities for genomics studies on these parasites. For *Eimeria*, genome sequencing started in 2002, beginning with the reference *E. tenella* Houghton strain (Chapman and Shirley, 2003). There has now been successful publication of genome sequences for all seven species of *Eimeria* which are associated with coccidiosis in chickens after more than a decade of research from a consortium of twenty Institutions across ten countries (Reid *et al.,* 2014) (Blake, 2015). These studies are essential in helping to develop better means of control of this disease (Chapman *et al*., 2013). Negative views and pressure about the use of chemotherapeutic agents by consumers over recent years had led to the enactment of legislations reducing drug use in the food chain and has encouraged the search for alternative control measures for coccidiosis (Shirley *et al*., 2007, Godfray *et al*., 2010).

Recently, genotyping large numbers of field samples has revealed the existence of three new cryptic and divergent *Eimeria* operational taxonomic units (OTU),

genotypes termed OTUx, OTUy and OYUz which were first described in Australia in 2007 (Cantacessi *et al*., 2008, Godwin and Morgan, 2015, Clark *et al*., 2016). OTUx, OTUy and OYUz are most related to *E. maxima, E. brunetti* and *E. mitis,* respectively. It has not been established if these variants can circumvent immune protection offered by the current live vaccines (Clark *et al*., 2017) even though (Morris *et al*., 2007a) reported an instance of one of the variants being present in 100% of a flock in one of two groups of chickens that had been vaccinated with a live vaccine but had a persistent recurring coccidiosis problem, thereby suggesting a possible evasion of vaccine protection. The importance of understanding the population structure and distribution of coccidiosis cannot be overemphasized because it forms the basis of explaining the epidemiology of the disease and will aid in the development of sustainable control measures (Blake *et al*., 2015). The knowledge of antigenic diversity of circulating field species and strains, probability of coinfection by genetically distinct strains, and understanding the efficacy of cross protection will inform the prediction of the longevity and possible efficacy of future subunit vaccines in field conditions (Blake *et al*., 2015)

1.2 Taxonomy and classification of *Eimeria*

Eimeria belong to the protistan phylum Apicomplexa. Most livestock are infected by one species or more and they are species-specific. Chickens, geese, ducks, turkeys and other types of poultry are all infected by different types of coccidian (Table 1.1).

Table 1:1: *Eimeria* species that infect poultry

Infecting *Eimeria* species	**Host**
E. acervulina	Chickens
E. adenoeides	Turkeys
E. brunetti	Chickens
E. dispersa	Turkeys
E. gallopavonis	Turkeys
E. maxima	Chickens
E. meleagrimitis	Turkeys
E. mitis	Chickens
E. necatrix	Chickens
E. praecox	Chickens
E. tenella	Chickens
E. truncata	Geese

1.3 Life cycle of *Eimeria* in poultry

Eimeria species usually infect only a single host during their life cycle; they are monoxenous parasites (Fayer, 1980). The *Eimeria* life cycle (1.1) typically involves three stages; sporogony, schizogony and gametogony (Rasheed and Shameer, 2016) and the infective stage of this parasite is the sporulated oocyst which is oval or spherical and double walled when released from the host, it can persist in the environment for a long time (Williams, 1995). The process of sporogony occurs when the oocysts containing a zygote undergoes a reduction division to form four haploid sporoblasts and each sporoblast develops to form sporocysts, each with a distinct cell wall. The process of sporulation is affected by relative humidity, temperature and oxygen levels in the poultry house (Chapman *et al*., 2013). In the host cells, parasite development involves both asexual (schizogony) and sexual stages (gametogony) of development. As the parasite develops and multiplies, destruction of the host tissue is observed leading to various clinical manifestations observed whenever there is a disease outbreak (Conway and McKenzie, 2007). Development of the different species of *Eimeria* in poultry includes slight variations but a basically similar pathogenesis (Conway and McKenzie, 2007). The cycle begins with a susceptible host ingesting a sporulated oocyst from its environment, each sporulated oocyst contains four sporocysts and each sporocyst contain two sporozoites. Upon ingestion of sporulated oocysts, due to mechanical and biochemical action in the digestive tract of the chicken, oocysts excyst in the intestine each releasing 8 motile sporozoites (Figure 1.1). The process of excystation has been reported to be aided by trypsin, chymotrypsin and bile salts in the intestines (Chapman, 1978). The sporozoites then penetrate the epithelial cells in the gastrointestinal tract at an *Eimeria* species-specific location, encapsulate themselves within a parasitophorous vacuole, transform within 12 to 48 hours into a feeding stage called a trophozoite and replicate. The trophozoite replicates by enlarging and the nucleus dividing by a process of asexual division known as shizogony and at this stage, it is known as a schizont. After a series of nuclear division then cytokinesis, large numbers of merozoites are produced. Rupturing of the infected cell occurs and each merozoite penetrates a new epithelial cell. It has been observed that each *Eimeria* species is programmed genetically for a specific number of merogonic divisions (schizogony) but it is mostly between 1-4 times. Through these cycles of replication, when the parasite reinvades the cells it damages the intestine and causes severe pathology. After the last species-specific merogonic generation, merozoites invade the enterocytes to differentiate into male

(Microgametocytes) and female (Macrogametocytes). Microgametes are produced from microgametocytes, which burst from the infected cell and fertilize macrogametes upon entering the cells that contain them. Zygotes are then produced which lay down an impervious cyst wall around themselves, forming oocysts. After fertilization, the oocyst ruptures from the host's enterocytes and leaves the host within faeces (Lillehoj *et al*., 2004, Lillehoj and Trout, 1996, Shirley *et al*., 2005). Initially, the oocyst is unsporulated, containing only a zygote that is not yet infective to a new host. At optimal environmental conditions of oxygen, warmth and humidity, the oocyst becomes sporulated and 8 sporozoites are formed within the oocyst. It is now infective and when a susceptible host ingests the sporulated oocyst, the life cycle continues.

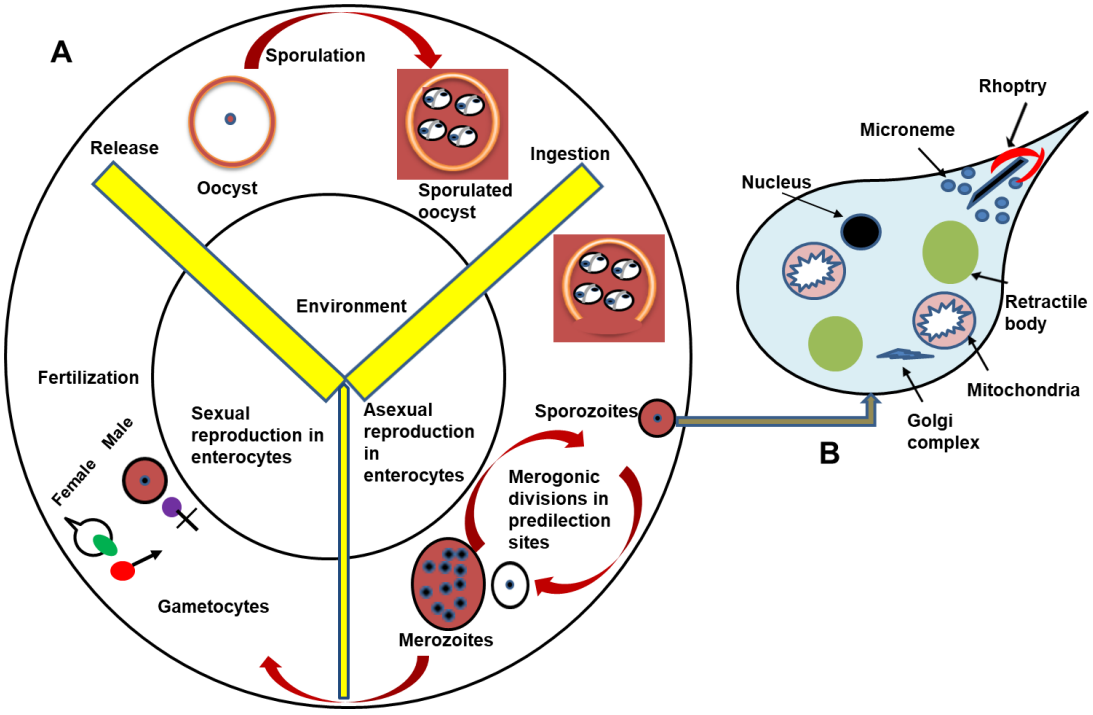


Figure 1:1: (A) Diagrammatic representation of the life cycle of *Eimeria* and (B) simplified structure of *Eimeria* spp. Sporozoite redrawn from (Ahmad *et al*., 2016)

1.4 Morphology of *Eimeria* oocysts

The oocyst is the most accessible stage of the coccidium and some species are known only by the size, shape and colour of their oocysts (Wan *et al*., 2017, McDougald *et al*., 1997). Oocysts are spherical in shape and not embryonated at the point they are expelled in faeces. Morphometric analysis of oocysts has been used to distinguish between species of *Eimeria*; oocysts of *E. maxima* are large and yellowish in colour and distinct from the oocysts of other species (McDougald *et al*., 1997). (Amer *et al*., 2010) in their study on isolation and identification of *Eimeria* from field coccidiosis in chickens were able to identify oocysts of *E. tenella, E. necatrix, E. acervulina* and *E. praecox* using morphological features. Some species have small oocysts; *E. mitis* (14 X 15.5 µm) and *E. acervulina* (14 X 18 µm). Some species can be classified as medium-sized oocysts; *E. necatrix* (20 X 17 µm), *E. tenella* (22 X 19 µm) and *Eimeria praecox* (17 X 21 µm) and some can be classified as large oocysts; *E. brunetti* (26 X 22 µm) and *E. maxima* (20 X 30 µm) (Long and Reid, 1982, Haug *et al*., 2008b, Reid and Long, 1979, McDougald *et al*., 1997). Broadly, *E. maxima* is the largest while *E. mitis* is the smallest (McDougald *et al*., 1997). However, relying completely on the oocyst size and or morphology to differentiate between species has been shown to be misleading (Reid and Long, 1979). It has been determined that variations are seen with the stage of patency and this has been reported in at least three species; *E. tenella* (Fish, 1931) *E. brunetti* (Becker *et al*., 1955) *E. necatrix* (Becker *et al*., 1956).

1.5 The *Eimerian* oocyst wall

The oocyst wall is a robust structure that is resistant to diverse chemical and environmental insults and this resilience accounts for the ability of oocysts to survive for long periods (Mai *et al.,* 2009). The oocyst wall is impermeable to water soluble substances which includes many disinfectants and detergents (Monné and Hönig, 1954, Hammond and Long, 1973) and is also resistant to proteolysis. It is however permeable to some small molecules and lipid/soluble substances such as methyl bromide and ammonia (Kuticic and Wikerhauser, 1996). Across different species of coccidian parasites, the oocyst wall is essentially a consistent in structure (Belli *et al.,* 2006). The ease of acquiring large numbers of oocysts of the *Eimeria* parasite has led to more studies on oocyst wall being performed on this genus. The oocyst wall is bilayered consisting of a 10 nm thick outer lipid layer covering a 90 nm thick inner layer of glycoprotein (Stotish *et al.,* 1978). The contents of two specific organelles,

wall forming bodies Type 1 (WFB1) and Type 2 (WFB2) which are exclusively found in the sexual macrogametocyte stage of coccidian parasites form the oocyst wall (Scholtyseck *et al.,* 1969, Ferguson *et al.,* 1975, Pittilo and Ball, 1980, Ferguson *et al.,* 2003, Belli *et al.,* 2006). The protein content of the oocyst wall is over 90% but only a few of these proteins have been studied. This is because of the difficulty encountered in extracting them from the wall (Eschenbacher *et al.,* 1996, Karim *et al.,* 1996). There are two groups of proteins; cysteine rich proteins and tyrosine rich proteins. These proteins range from 8-31 kDa and are derived from the large precursor proteins Gam56 and Gam82 kDa found in the wall forming bodies (Mai *et al.,* 2009, Xu *et al.,* 2013, Belli *et al.,* 2003). The processing of the precursors into tyrosine-rich peptides is catalysed by proteases and the peptides are then oxidatively crosslinked in a reaction catalysed by peroxidases. Therefore, the oocyst wall has high levels of di-tyrosine crosslinked protein bonds which provide a structural matrix for assembly of the oocyst wall, contributing to its resilience (Mai *et al.,* 2009). As mentioned, Gam56 and Gam82 are processed into small tyrosine-rich wall proteins of between 8 to 31 kDa hence antibodies to Gam56 and Gam82 can also react with these smaller oocyst wall proteins.

Gam56 has been shown to have good immunogenicity and antigenicity (Belli *et al.,* 2004) and its extraction from *E. maxima* constitutes one of the main components in the chicken coccidiosis killed parasite vaccine CoxAbic®. A 50-80% reduction in oocyst production has been reported after immunization with this vaccine in clinic applications (Wallach *et al.,* 2008). It has also been reported that this vaccine can improve body weight and reduce intestinal lesions (Xu *et al.,* 2009). Monoclonal antibody has also been successfully raised against the 56-kDa gametocyte antigen of *E. maxima* following immunisation of mice with enriched gametocyte extracts. In experimental challenged 2-week old chicks that had been passively immunised with sera containing the anti-56kDa protein IgG antibodies, oocyst shedding was reduced by 40%-50% (Sharman *et al.,* 2010).

In *E. maxima*, *E. tenella* and *E. acervulina*, homologous genes for the two major gametocyte proteins Gam56 and Gam82 were found and alignment of the predicted protein sequences of these genes revealed a strong homology in their amino terminal regions, where protective antibodies bind, as well as areas of shared tyrosine richness (Belli *et al.,* 2009). This accounts for the cross reactivity of antibodies raised against the 56 kDa gametocyte antigen in *E. maxima* to the same protein from *E. acervulina* and *E. tenella* (Belli *et al.,* 2009). Localisation studies of Gam56 and Gam82 antigens show that they are found in the wall forming bodies in macrogametocytes in the inner region of the oocyst wall (Belli *et al.,* 2002a).

1.6 Pathogenesis and pathology of *Eimeria* in poultry

The pathology seen in *Eimeria* infections is caused entirely by the intracellular asexual and sexual reproductive stages of the life cycle (Rasheed and Shameer, 2016). The various species of *Eimeria* invade different sites in the gut; *E. maxima* (jejunum, and ileum), *E. mitis* (ileum) and *E. necatrix* (jejunum, ileum and caeca) (Table 1.2). *Eimeria acervulina* infects the duodenum extending to the ileum while *E. praecox* invades the duodenum extending to the jejunum mid-intestine in heavy infections (Chapman, 2014). *Eimeria tenella* affects the caecum (Joyner, 1978) and *E. brunetti* affects the rectum (Table 1.2). The magnitude of the infection and the pathogenicity of the infecting species directly determines the extent of damage caused to the intestine (Tyzzer *et al*., 1932). Species that are less pathogenic such as *E. mitis* and *E. praecox* may result in mild enteritis resulting in malabsorption of nutrients and loss of fluid. More pathogenic species such as *E. brunetti*, *E. acervulina* and *E. maxima* cause petechial haemorrhaging of the intestines, inflammation of the intestinal wall and sloughing of intestinal epithelia, while highly pathogenic species such as *E. tenella* and *E. necatrix* cause more damage to the intestinal epithelium resulting in complete destruction of intestinal villi, severe haemorrhage and death (Chapman, 2014).

There is a phenomenon known as ‘the crowding effect’ which occurs when there is competition within and between species for a particular region of the intestine which occurs when there is the presence of more than one species in an outbreak (Williams, 2001). Competition for enterocytes can be seen when the predilection sites of the competing species are in close proximity leading to the crowding effect, a state where all available cells are infected (Jenkins *et al*., 2008).

Coccidiosis infection may cause changes in the permeability of the intestinal mucosa resulting in leakage of plasma proteins into the lumen of the intestine (Preston-Mafham and Sykes, 1967), reduction of gut motility (Schildt, 1955) and increased pH (Stephens, 1965).

For *E. tenella* and *E. necatrix*, upon penetration of the surface epithelium of the intestines by the sporozoites, they are carried by the macrophages in the lamina propria deep into the crypts of Lieberkühn. Here, they reproduce and the second generation schizonts that are produced are responsible for severe haemorrhaging seen with infections with these two species (Rasheed and Shameer, 2016, Soulsby, 1968).

Eimeria tenella, the sole cause of caecal coccidiosis causes the caecum to be thickened, dilated and filled with blood. *Eimeria acervulina* affects the duodenum, which is where most nutrient absorption takes place hence several studies conducted on the effect of *Eimeria* infections on absorption of nutrients focused on the species (Chapman, 2014). (Preston-Mafham and Sykes, 1967) reported an impairment of glucose absorption and consequent weight loss as a result of infection with *E. acervulina*. There are several documentations of *E. acervulina* impairing the absorption of fats (Sharma and Fernando, 1975), zinc (Southern and Baker, 1983), amino acids (Parker *et al*., 2007, Amerah and Ravindran, 2015), carotenoids and vitamin A (Kouwenhoven and van der Horst, 1972). (Turk, 1973) reported a reduction in calcium in chickens infected with *E. acervulina, E. brunetti* and *E. necatrix*, *E. necatrix* affects the middle part of the small intestine and the lesions seen are haemorrhagic, white opaque foci with swelling (Soulsby, 1968).

In general, lesions seen in a typical clinical coccidiosis caused by *E. tenella* can include petechial haemorrhages on the wall of the caeca, ballooning of the intestines, thickening of the caecal wall and bloody contents, distension of the intestines, mucus present in the intestines, putrid odour of intestinal contents, intestinal intussusceptions, white spots (Molla and Ali, 2015), reddening and coagulation necrosis can be seen most often in the lower part of the intestine (Conway and McKenzie, 2007) (Table 1.2).

Under the microscope, pathology includes a decrease in the height of the villi and microvilli of the intestine and the depths of the crypts and this can indicate the extent of infection (Giannenas *et al*., 2014). In a study to assess intestinal morphometry in broilers infected with *Eimeria* under different anticoccidial treatments, it was reported that coccidiosis causes morphologic alterations in intestinal epithelia resulting in reduction of the absorptive surface (Nabian *et al*., 2018). The study also reported that microscopically, in the presence of infection, anticoccidial administration significantly increased the villus length (Nabian *et al*., 2018).

Table 1:2: Summary of the pathogenic and pathological characteristics of *Eimeria* species

Species	**Site of development**	**Pathogenicity**	**Gross lesions**
E. tenella	Caeca	++++	Thickening of the caecal wall and bloody contents at the proximal end, caecal distension and villi destruction causing extensive haemorrhaging and death (Chapman, 2014) (Duffy *et al*., 2005)

E. necatrix	Jejunum, Ileum, caeca	++++	Ballooning of intestine and thickening of mucosa and lumen filled with fluid, blood and tissue debris, black and white plaques, lesions at post-mortem; salt and pepper appearance (Chapman, 2014)
E. brunetti	Caeca and rectum	++++	Inflammation of the intestinal wall with patechial haemorrhages and sloughing of epithelia (Chapman, 2014)
E. maxima	Jejunum and ileum	+++	Inflammation of the intestinal wall with petechial haemorrhages and sloughing of epithelia (Chapman, 2014)
E. mitis	Ileum	++	Fluid loss as a result of limited enteritis, nutrient malabsorption (Chapman, 2014)
E. acervulina	Duodenum, ileum	+++	Fluid loss as a result of limited enteritis, nutrient malabsorption, gross white lesions (Chapman, 2014, Williams *et al*., 2009)
E. praecox	Duodenum and jejunum	++	Mucus and mucoid casts, watery intestinal contents (McDougald and Fitz-Coy, 2008)

++ denotes less pathogenic +++ denotes moderate pathogenic and ++++ denotes highly pathogenic

1.7 Clinical manifestation of infections with *Eimeria*

Coccidiosis in chickens is largely categorized into two forms based on the pathology of infections; mild sub-clinical infections also known as coccidiasis and clinical coccidiosis (Conway and McKenzie, 2007). When an infection is slight and infected chickens don't show any apparent clinical signs it is referred to as coccidiasis (Conway and McKenzie, 2007) and represents a mild interaction between parasite and host (Williams, 2002, Vermeulen *et al*., 2001). Subclinical infections can manifest as low feed efficiency, energy loss, decreased body weight gain and decreased egg production in layers (Price, 2012, Haug *et al*., 2008a, Teeter *et al*., 2008).

When a susceptible chicken has clinical coccidiosis, common signs include bloody diarrhoea, dehydration and loss of skin pigmentation and listlessness (Swayne *et al*., 2013, Ola-Fadunsin and Ademola, 2014, Singh and Mukherjee, 2018). Other signs that could be seen include a drop in egg production in layers and impaired growth rate in broilers; anaemia is also often seen in caecal coccidiosis (Mohammed and Sunday, 2015). The effects of infection are as a result of malabsorption of nutrients which is a consequence of impairment of the intestinal epithelia. Infection also results in an increased susceptibility to other disease agents especially necrotic enteritis due

to *Clostridium perfringens* and infections with *Salmonella typhimurium* species (Mcdougald, 1991), this can lead to a high mortality especially in young chicks (Tewari and Maharana, 2011). The severity of the disease also depends on the number of oocysts ingested, with mild infections occurring when a small number of oocysts are ingested and a moderate to severe infection occurring when a large number of oocysts are ingested (Swayne *et al*., 2013, Peek, 2010).

1.8 Epidemiology of *Eimeria*

Eimeria have been reported to be found everywhere chickens are raised and since they are host specific, wild birds are not a source of infection (Swayne *et al*., 2013). The most common means of spread of infection is mechanically through personnel spreading infection between poultry houses and farms (Swayne *et al*., 2013). There has been a report of coccidiosis being less frequent when the number of poultry house attendants and their movement from one poultry house to another is limited, reducing the possibility of spread of infection through fomites (Rasheed and Shameer, 2016). The spread of coccidiosis is also aided by poor cleaning of equipment, and the presence of mechanical vectors in the farm such as rodents and insects. In addition, the occurrence of other infectious agents such as enteric bacteria and viruses worsens the severity of the infection (Peek, 2010).

An important factor in the spread of coccidiosis is oocyst survival and sporulation. Because infection occurs only when chickens ingest sporulated oocysts and infected chickens shed oocysts in their faeces, factors that affect the sporulation of oocysts should be monitored and controlled to avoid spread of infection (Molan *et al*., 2009, Conway and McKenzie, 2007). There are reports that low oxygen levels and higher content of ammonia in litter reduce the chances of oocyst survival thereby reducing their numbers. Certain management strategies such as housing poultry on raised wired floors can disrupt the faecal-oral lifecycle of *Eimeria* species thereby reducing occurrence and outbreaks of coccidiosis as compared to those managed in deep litter system because of the tendency of rapid oocysts accumulation in the litter (Lunden *et al*., 2000, Dakpogan and Salifou, 2013). Similarly, good management practices like good choice and correct use of disinfection and biosecurity measures significantly reduce the chances of infection (Gharekhani *et al*., 2014)**.** Age of slaughter of chickens and breed of chicken are also factors that have been reported to influence *Eimeria* occurrence (Bumstead and Millard, 1992, Shirzad *et al*., 2011)**.** Climatic conditions affect the occurrence of coccidiosis as elevated parasite levels and disease have been recorded during warmer and wetter weather conditions (Luu

et al., 2013, Awais *et al*., 2012, Jatau *et al*., 2012). Epidemiological studies of coccidiosis have revealed that each of the seven species of *Eimeria* that cause disease in chickens have a global distribution and at least six species of *Eimeria* have been identified on many individual farms (Kučera, 1990, Williams *et al*., 1996, Thebo *et al*., 1998, Mattiello *et al*., 2000, Morris *et al*., 2007b, Prakashbabu *et al*., 2017, Fornace *et al*., 2013). (Clark *et al*., 2016) in their survey of *Eimeria* across 6 continents and 20 countries found all seven species of *Eimeria* in every country and region sampled.

Some of these epidemiological surveys have been limited by the diagnostics employed; the use of size and shape of *Eimeria* oocysts, predilection site in the intestine, and pre-patent period of the parasite when passaged in experimentally infected chickens. These approaches may not be very efficient in distinguishing infecting *Eimeria* species as there is an overlap of such characteristics amongst some species (Long and Joyner, 1984, Morris *et al*., 2007b).

The economic effects of coccidiosis are more severe in broiler production largely due to the effects of weight gain and feed conversion (Williams, 1999). Many epidemiological studies have revealed that *E. acervulina* has been the most commonly isolated species in broilers in several studies often recording a prevalence rate of up to 90% (McDougald *et al*., 1997, Györke *et al*., 2013). *Eimeria tenella, E. maxima,* and *E. praecox* have also been commonly found in broiler chickens, and more rarely, *E. mitis* and *E. necatrix* (Haug *et al*., 2008b, Sun *et al*., 2009, Hamidinejat *et al*., 2010, Ogedengbe *et al*., 2011, McDougald and Reid, 1997). Morris *et al* (2007b) in their study of seven Australian broiler flocks confirmed that all seven recognized *Eimeria* species of poultry were found but reported a higher prevalence rate for *E. acervulina, E. maxima and E. mitis* and *E. praecox.* The prevalence of *E. brunetti* and *E. tenella* were relatively lower while *E. necatrix* was the least detected.

As mentioned earlier, *Eimeria* has been discovered to have OTU genotypes which were described for the first time outside Australia by (Fornace *et al*., 2013) who described two out of the three OTUs; OTUx and OTUz in three African countries: Ghana, Tanzania and Zambia, and found that they are most related to *E. maxima, E. brunetti* and *E. mitis,* respectively. (Clark *et al*., 2016) in their survey of *Eimeria* across 6 continents, found that the OTUs were not present in the northern hemisphere despite large-scale sampling but had a wide distribution in the southern hemisphere. They found at least one OTU variant in every southern hemisphere country and it is not certain what the drivers are for the polarised distribution of these OTUs but the presence of genetically variant *Eimeria* which may spread across the hemispheres poses a threat to effective treatment and food security. They found the OTUz was

detected in all 8 countries, OTUx was detected in 6 out of 8 countries and OTUy was detected only in Nigeria (Clark *et al*., 2016).

1.9 Immunity and host responses to poultry coccidiosis

Immune responses to an infection are broadly classified into adaptive or specific immunity and innate or non-specific immunity. Adaptive immunity involves two different classes of lymphocytes and is classified into cell mediated (T cells) and humoral (B-cells). The different aspects of the immune system play important roles in the elimination of invading substances and microorganisms foreign to the host. The innate immune response is the first line of defence against any pathogen and comprises of physical barriers such as skin, innate cells such as natural killer cells, the complement system and the inflammatory response. Invading pathogens are recognised by the innate immune system by the detection of molecular structures absent in host tissue but present in pathogens (Swinkels, 2008). The adaptive immune response is mounted after the innate response, it is specific to the pathogen and this clears the pathogens rapidly in the event of a secondary exposure (Bremner, 2018). Chickens have a well-developed immune system much like mammals (Lillehoj *et al.,* 2007).

1.9.1 Immune response to *Eimeria*

The life cycle of the *Eimeria* parasite is complex comprising of both intracellular and extracellular stages as well as sexual and asexual stages hence host immune responses are diverse and complex involving different facets of innate and adaptive immunity (Bremner, 2018, Lillehoj *et al*., 2004, Yun *et al.*, 2000, Rose and Long, 1962). Immune responses to *Eimeria* in poultry depends on the prior exposure of the host to parasites, stage of the parasite development, nutritional status of the infected chickens, pathogen characteristics and genetic makeup of the host (Yun *et al*., 2000, Swinkels, 2008). (Li *et al*., 2002) in their study of interleukin-2 production in two lines of chickens, SC and TK infected with *E. tenella* found out that the chicken lines displayed differential immunity to coccidiosis, one being more resistant while the other was more susceptible to disease as shown by clinical signs, oocyst production and lesion score. The quantity of oocysts infecting a host is of importance in host response; immune response to *Eimeria* infections in poultry is usually generated after a large number of oocysts are ingested, but some exceptions have been noted such

as *E. maxima* which is highly immunogenic and only requires a small number of oocysts (Yun *et al*., 2000, Blake *et al*., 2005).

It was concluded from early studies that cell-mediated immune responses are most important in protection against *Eimeria*, with humoral immunity playing a minor role (Rose and Hesketh, 1979, Song *et al*., 2015). However, other studies have revealed that antibodies have the ability to hinder parasite invasion, development and transmission as well as providing passive and maternal immunity against infection (Wallach, 2010).

It has been established that infection with one species of *Eimeria* induces protective immunity in the host that is life-long and specific to that species, sometimes even strain specific (Basak *et al*., 2006, Blake *et al*., 2006, Yun *et al*., 2000). This elicits little protection against challenge with other species (Rose and Long, 1962, Yun *et al*., 2000, Swinkels, 2008).

1.9.1.1 Innate immune responses to Eimeria

This is a major defence mechanism and it involves a non-specific immune response activated following exposure to the *Eimeria* antigens. Upon exposure to *Eimeria* pathogens, an inflammatory immune response characterised by increased production of proinflammatory mediators is elicited and infiltration and activation of innate cells such as macrophages, granulocytes and heterophils which phagocytise invading pathogens (Min *et al*., 2013, Bremner, 2018, Lillehoj *et al*., 2004). Antigen presenting cells (APC) undertake multiple roles during *Eimeria* infection which includes production of proinflammatory cytokines, phagocytosis, and antigen presentation for the development of cell mediated immune responses.

Macrophages play an important role as one of the first line of defence against *Eimeria* and are mainly responsible for elimination of the parasites (Vervelde *et al.,* 1996). However, macrophage-like cells also have a role in the transport of sporozoites to the crypt regions where large numbers of *E. tenella* and *E. acervulina* sporozoites were reported to have been seen within this cell type (Challey and Burns, 1959). The role of heterophils as one of the first lines of defence against *Eimeria* infections is documented. Heterophils display phagocytosis and oxidative burst and degranulation (Bremner, 2018). (Petrone *et al*., 2002) reported an increase in infiltration of the mucosa with heterophils after *E. tenella* infection. Natural killer cells (NK) present in the epithelium of the intestine are also likely to play an important role as a first line of defence against *Eimeria* infections because they are one of the first cells to come in contact with the parasite (Bremner, 2018).

1.9.1.2 Cell mediated immune responses to Eimeria

Eimeria being highly immunogenic, elicits a high degree of protection to homologous challenge and there is little or no protection following heterologous challenge with different species of *Eimeria* (Rose and Long, 1962). Chicken $CD4^+$ cells play a role in coordinating protective immunity while $CD8^+$ induces protective immunity. Both T cell populations are known to increase following *Eimeria* infections (Rose and Hesketh, 1979) and T helper 1 type cytokines have been reported to play a role in limiting parasite in the early stages of infection (Choi and Lillehoj, 2000)

1.9.1.3 Humoral response to Eimeria

Humoral immunity is involved in *Eimeria* infections even though cell-mediated immunity plays a major role. At approximately 9 - 20 days after infection, high titres of *Eimeria*-specific IgY, IgA and IgM are detectable in intestinal secretions and serum. The role of B cells in *Eimeria* infections were studied using chemically and hormonally bursectomised chickens and they were seen to exhibit an increase in clinical disease and oocyst shedding in response to *E. acervulina and E. maxima* challenge compared to control chickens who had their bursars intact (Rose and Hesketh, 1979). The role of passive immunity in protection against *Eimeria* has been documented, antibodies present in chicken sera after *Eimeria* infection provided passive immunity to chickens challenged with the same species (Rose, 1974).

1.10 Diagnosis of poultry coccidiosis

Accurate diagnosis of *Eimeria* is important to the poultry industry (Barkway *et al*., 2015). The diagnosis of poultry coccidiosis is complicated because of the multiple *Eimeria* species infecting chickens and infections with mixed species of *Eimeria* are very common (Godwin and Morgan, 2015, Jenkins *et al.,* 2008). There is a continuous need for improved diagnostics to coccidiosis in poultry as it forms the basis of the development of effective control measures (Fatoba and Adeleke, 2018). Effective diagnostics are useful when monitoring vaccine efficacy, coccidiosis control programs as well as determining the species present in a particular flock. This consequently enables the assessment of disease risk, minimization of vaccine costs and prevention of introduction of species that were not previously present in the flock (Singla and Gupta, 2012, Morgan *et al*., 2009).

Diagnosis at species level have traditionally been based on developmental biology and morphological characteristics which have not been very efficient especially in

mixed species infections, such approaches also require expert parasitological skills (Morgan *et al*., 2009).

Improved coccidiosis diagnostic assays would allow better management of the disease leading to more profitable and efficient broiler and egg production, improved animal welfare owing to better disease control as well as reduced risk to the environment owing to less use of anticoccidial compounds (Singla and Gupta, 2012, Kadykalo *et al.,* 2018). An ideal diagnostic assay should have several key characteristics such as high sensitivity to detect even low levels of infection, high specificity to minimize false positives and to detect the species of *Eimeria*, minimal flock handling time to reduce stress, minimal sample processing steps to lessen the risk of cross contamination and low intra-species diversity (Singla and Gupta, 2012). Over the last two decades, monitoring and diagnosing coccidiosis have evolved from labour intensive methods, which include lesion scoring of the GI tract and microscopic examination of oocysts passed out in faeces or found in intestinal scrapings to more advanced techniques such as molecular based diagnostics (Frölich *et al.,* 2013). Several conventional polymerase chain reaction (PCR), real time-PCR (RT-PCR) and more recently Loop-mediated isothermal amplification (LAMP) assays have been developed for the species-specific diagnosis of *Eimeria* in poultry (Vrba *et al.,* 2010, Kumar *et al.,* 2014, Barkway *et al.,* 2015).

1.10.1 Current diagnostics for *Eimeria* in poultry

Traditionally, *Eimeria* infection is diagnosed using flock history, and microscopic examination of litter and faecal samples for the presence of any oocyst stage and counting of oocysts (Conway and McKenzie, 2007, Barkway *et al.,* 2015). Then post-mortem examination of lesions within the GI tract are carried out including location of lesions because different species have different predilection sites and scores. However, *Eimeria* species can rarely be differentiated based on a single criterion and as such, a balance of several criteria are used in diagnosing *Eimeria* infections and these criteria may differ in significance for particular species (Wan *et al*., 2017). Overall, *Eimeria* species have been differentiated based on clinical signs, characteristic gross lesions present at certain sites of infection of the intestine, morphology and size of oocysts, pre-patent period, morphology of the intracellular stages and minimum sporulation time (Conway and McKenzie, 2007).

History and clinical signs are employed as a first line and a basic diagnostic approach, history describes the onset and progression of the disease and clinical signs that are characteristic to coccidiosis are observed (Das *et al*., 2004, Haftu *et al*., 2014).

The comparison of morphometric parasite characteristics and host responses which involves measurement of oocysts sizes to compare them with reference sizes for that species as well as clinical signs or lesions characteristic to the *Eimeria* species have been used to differentiate between infecting species (Long *et al*., 1976, Long and Reid, 1982). Oocysts of *E. maxima* can be distinguished with certainty because of its large size and distinctive yellowish appearance (Joyner and Long, 1974). (McDougald *et al*., 1997) in their survey of coccidia on 43 poultry farms in Argentina were able to recognise *E. praecox* definitively using morphometric analysis but had to rely on a combination of morphometric analysis and characteristic gross lesions to recognise *E. tenella* and *E. acervulina*. This procedure is time-consuming and somewhat expensive and since there is the tendency for overlapping of characteristics by the different species, it is difficult to rely on them for definitive diagnosis of all *Eimeria* species of poultry (Table 1.3) (Joyner and Long, 1974, Long and Joyner, 1984, Thebo *et al*., 1998).

Oocysts of at least six of the seven species are reported to be seen in the litter samples during the first 6 weeks; *E. maxima, E. acervulina, E. mitis, E. tenella, E. brunetti,* and *E. praecox* (Williams 1995) but it has been reported that counting of oocysts per gram of faeces (OPG) or litter does not have a very accurate relationship with the impact of this disease on the performance of the flock (De Gussem, 2007). The presence of oocysts alone is not of much importance and is not a reliable method for diagnosis of coccidiosis because mortality starts well before oocysts are shed in faeces in acute cases. Therefore, definitive diagnosis of *Eimeria* is not often based on the detection of *Eimeria* oocysts in chicken faecal samples (Luu *et al*., 2013).

Scoring of lesions, which is an interpretation based on visible macroscopic lesions caused by infection with *Eimeria,* usually following a scoring system from zero to four is also typically used for coccidiosis diagnosis (Johnson and Reid, 1970, Conway and McKenzie, 2007). Some species are more easily identified at post-mortem than others based on the characteristic gross lesions they produce. Identification of *E. brunetti, E. necatrix, E. acervulina, E. maxima* and *E. tenella,* based on the gross lesions they produce is relatively easier than the other species (Singla and Gupta, 2012). However, the method is hugely labour intensive as the individual scores for all the species present for the individual predilection sites are usually compiled for a certain number of birds per flock under investigation and a Total Mean Lesion Score (TMLS) is determined (Johnson and Reid, 1970). This method is very likely to be subjective

and if not performed by skilled personnel it may not be reliable (De Gussem, 2007). However, it is reported that there is a better correlation between lesion scores and flock performance than with OPG, but this is still not always accurate (De Gussem, 2007). It is especially so in the diagnosis of sub-clinical infections (Woods *et al*., 2000).

Though the traditional approaches to diagnosis of *Eimeria* that have been discussed above can be effective, they are laborious, time consuming, highly subjective as well as requiring technical expertise particularly when species level identification of the parasite is required (Haug *et al*., 2007).

Aside from these limitations, they are also not able to effectively diagnose subclinical infections and as a result, the true prevalence of *Eimeria* species in much of the world may not be known (Haug *et al*., 2008a, Barkway *et al*., 2015). Diagnosis of coccidiosis have evolved with the development of molecular diagnostics. Some of the assays that have been developed have reduced the flock handling time and have proven to be sensitive and some assays have even been found to be species-specific. Broadly, some of these advanced techniques that have been developed are either serological based or molecular based.

Serological assays that have been employed for the diagnosis of coccidiosis include indirect ELISA and species-specific immunoblotting. Researchers in Japan developed *a* species-specific ELISA for *E. necatrix* which uses a recombinant merozoite antigen (Morgan *et al*., 2009). This assay can detect infections with either *E. necatrix or E. tenella* but is not able to differentiate between the two species. This assay detects circulating antibodies to the merozoite antigen and can detect if the chickens have developed immunity to the species even when the parasites are not there anymore. This assay can also be used as a valuable tool to assess immune response after vaccination. Species specific immunoblotting tests that can distinguish chickens infected with *E. necatrix* or *E. tenella* from chickens infected with the other species have been developed with antigen made from gut scrappings. Sera from *E. tenella* immune chickens recognised a low and high molecular weight antigen while sera from *E. necatrix* immune chickens recognised only the low molecular weight antigen. Sera from the other species failed to recognised any antigen on the immunoblots (Singla and Gupta, 2012, Morgan *et al.,* 2009).

Some of the molecular based diagnostic tools for the *Eimeria* infections include enzyme variation analysis, southern blot analysis and randomly amplified polymorphic DNA assay (Shirley, 1975, Southern, 1975, Procunier *et al*., 1993). (Shirley, 1975) in the investigation of genetic variation among and within species of *Eimeria* infecting chickens first described enzyme variation as a means of

differentiating *Eimeria* species in poultry. Previously, it had been applied to identify and classify *Plasmodium*, *Leishmania* and *Trypanosoma* (Shirley, 1975). Using oocyst lysates, the isoenzyme electrophoresis profile of four distinct enzymes present on the wall of the *Eimeria* oocysts are used to identify the species. The enzymes were lactate dehydrogenase (LDH), glucose phosphate isomerase (GPI), 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD). Horizontal starch gel separation of non-denatured proteins was carried out and their migration through a gel under the influence of an electric field allowed 11 strains of 6 species of chicken *Eimeria* to be identified. Over the years, various laboratories have used enzyme variation to enable differentiation among *Eimeria* species from samples taken from around the world; USA and Canada (Johnston and Fernando, 1997), Australia (Andrews *et al*., 1990), and Japan (Nakamura *et al.,* 1986, Shirley *et al.,* 1989, Morris and Gasser, 2006). This technique is however, not without some significant limitations; the process is time consuming and tedious often requiring the passaging of oocysts through chickens to amplify the number of oocysts which adds expense to the method and consumes more time (Chapman, 1982). Another limitation is that in cases where there is an underlying variation in the DNA sequence, it may not translate to a variation in amino acids and even if it does, there may be no variation in the motilities during electrophoresis (Shirley, 1994). (Thebo *et al*., 1998) who used the isoenzyme electrophoresis profile of glucose phosphate isomerase to identify the seven *Eimeria* species of chickens in Sweden for the first time found similar migration patterns having used PAGE gel to analyse the isoenzyme electrophoresis profile of GPI, two species had to be differentiated by the size of their oocysts and characteristic gross lesions produced. These limitations have hindered the widespread replacement of traditional methods of diagnosis with the enzyme variation analysis.

(Ellis and Bumstead, 1990) used the Southern blot technique to demonstrate that rDNA and rRNA probes could be used to identify individual *Eimeria* species through characteristic restriction fragment patterns and they successfully probed and differentiated the genomic DNA of *E. tenella*, *E. acervulina* and *E. necatrix* with an RNA probe after digesting the DNA with EcoRI endonuclease. (Shirley, 1994) using a panel of endonucleases and four DNA probes that were derived from reiterating sequences within the genome of *E. tenella* examined seven strains of *E. tenella* and derived restriction fragment length polymorphism patterns that are characteristic to them. It was recommended in this study that a panel of species-specific probes could be isolated that will aid differentiation of species of *Eimeria*.

(Procunier *et al*., 1993) employed a randomly amplified polymorphic DNA assay (RAPD) to differentiate *E. tenella* and *E. acervulina* which relies on the amplification of genomic DNA fragments using single primers of random sequence followed by electrophoretic separation of amplicons to reveal unique amplicon patterns for different species.

Towards the end of the 1990's, new DNA based diagnostic techniques for the detection, identification and differentiation of *Eimeria* in chickens were developed, specifically using PCR (Schnitzler *et al*., 1998). Afterwards, the PCR technique has undergone improvements and then in the 2000s real-time PCR assays were optimised (Kawahara *et al*., 2008). Real-time PCR improved the sensitivity of assays, reduced analysis time and also reduced cross-contamination between samples. Molecular assays including genus-specific and species-specific quantitative PCR (qPCR), genome number estimations were intended to replace traditional diagnostic methods such as microscopic counting of oocysts (Raj *et al*., 2013). The optimization of real-time PCR for simultaneously obtaining qualitative and quantitative results has allowed for accurate identification of *Eimeria* species present in a sample as well as quantification of the parasites (Nolan *et al*., 2015).

PCR methods have continued to be refined over the years but the presence of faecal inhibitors have been reported as one of the limitations inherent to this method. This may often lead to underestimation of the target genome copy numbers being determined (Morgan *et al*., 2009, Raj *et al*., 2013). False negative results due to reaction inhibition have now largely been reduced as a result of the development of optimized methods of obtaining and purifying DNA (Gerhold *et al*., 2015) and the addition of internal controls in each reaction well (Raj *et al*., 2013).

The identification of circulating *Eimeria* species has important implications for epidemiology as well as control. It can aid in disease control by informing the selection of appropriate anticoccidial drugs and vaccines (Sun *et al*., 2009). Improvements cannot be made without properly diagnosing the species that infect both backyard and commercial poultry flocks (Godwin and Morgan, 2015). High specificity, increased speed, objectivity, high output, robustness, automation potential and a reduced need for specialist knowledge of parasitology are some of the advantages of qPCR. However, the high cost of running qPCR, the need to use standardised protocols, calibrated instruments and trained personnel, in addition to the presence of PCR inhibitors are disadvantages.

Loop mediated isothermal amplification (LAMP) assays were developed by (Barkway *et al*., 2015) as uncomplicated, relatively inexpensive and quick assays. These provide a panel of species-specific LAMP assays targeting the seven *Eimeria*

species. They have been found to be species-specific and able to detect between 1 and 10 *Eimeria* genomes. This technique however requires a post-mortem examination and intestinal scrappings from predilection sites of each *Eimeria* species. The method also included extraction of the DNA for use as a template for this assay and so required a laboratory setting and expert personnel (Barkway *et al*., 2015).

Recently, a method measuring volatile organic compounds in air samples has been developed to diagnose coccidiosis in poultry farms; a prototype that is able to determine an 'air fingerprint' was developed and tested. Even though this assay diagnosed *Eimeria* infections, it has not been applied to diagnose coccidiosis at a species level (Table 1.3) (Grilli *et al*., 2018). Table 1.3 summarises the diagnostic techniques that have been used for diagnosing *Eimeria* infections in chickens, the advantages and disadvantages of each technique.

Table 1:3: Summary of advantages and disadvantages of techniques that have been used for the diagnosis of *Eimeria* infections in chickens

Diagnostic techniques	**Advantages**	**Disadvantages**
Faecal microscopy and lesion scoring	Does not requires expensive equipment, some species are easily identified based on lesions e.g. *E. tenella* or oocyst morphology e.g. *E maxima*	Subjective, time consuming, requires expert parasitological skills, labour intensive
Serological tests	High throughput, objective	Not developed for all species of *Eimeria*
Enzyme variation analysis, southern blot analysis and randomly amplified polymorphic DNA assay	Genetic based techniques that can be far more reliable than traditional techniques	Time consuming and low sensitivity
PCR based techniques	High sensitivity and specificity, objective	Need for specialist equipment, difficulty in obtaining template DNA
LAMP assays	Technically simple, relatively inexpensive, fast and objective	Requires extensive sample preparation to obtain template DNA
Air fingerprinting	Quick, cheap, convenient	Cannot differentiate between infecting species

1.10.2 Limitations and challenges of current diagnostics available

Despite the availability of molecular tools for species level diagnosis of *Eimeria* infections in poultry, conventional methods are still being relied on in the field even though they are time consuming, laborious, and require expertise (Barkway *et al*., 2015, Long and Joyner, 1984).

As mentioned, mixed and overlapping infections in affected zones leading to variation in extent and nature of lesions are common and makes accurate species level diagnosis using conventional methods difficult (Godwin and Morgan, 2015, Jenkins *et al*., 2008, Long and Joyner, 1984).

It is proposed that the application of molecular based diagnostic assays is limited by the need for specialist equipment, their expense as well as access to template DNA (Barkway *et al*., 2015). Species specific diagnosis using such methods has been argued to be an 'academic exercise', costly and not practical in the field by some representatives of the poultry industry (Morris and Gasser, 2006). However, the accurate knowledge of circulating species and strains is required to inform the adequate control of this disease (Sun *et al*., 2009, Blake *et al*., 2015). Continual improvement of available diagnostics and research into new diagnostics that are inexpensive, quick and easily applicable in the field should therefore be ongoing.

1.11 Prevention and control of poultry coccidiosis

Within the poultry industry, especially broiler production, the incorporation of ionophores and synthetic coccidiostats into the feed during the first weeks of life, combined with standard biosecurity practices as well as the use of shuttle programs to periodically introduce attenuated live parasites is the most widely and commonly used approach for the prevention of coccidiosis (Table 1.4) (Amare *et al.,* 2012, Frölich *et al.,* 2013, Clark *et al.*, 2017). These practices have enabled farmers and poultry producers to maintain low levels of infection needed for flocks to develop protective levels of specific immunity without the occurrence of clinical coccidiosis (Morris *et al*., 2007b, Clark *et al*., 2017). These strategies are cost effective and have worked well but the presence of drug resistance and the negative views about the use of drugs in food animals as well as increased demand for organic produce have challenged these strategies in recent times (Amare *et al.,* 2012, Yadav and Gupta, 2001)**.**

When control of coccidiosis through the use of in-feed anticoccidial drugs is suboptimal, either through inadequate or ineffective application or parasite resistance, the occurrence and disease burden increases (Chapman *et al*., 2010). The negative views about the use of these drugs as a result of increasing knowledge in the industry and public of the emergence of possible drug resistance and the presence of drug residues in food, led the European Union (EU) Commission to propose a phasing out of the use of prophylactic anticoccidial drugs. As at present, there is no official ban yet because it is dependent on the poultry industry establishing

alternative control strategies for rearing chickens without compromising the health and welfare of animals as well as commercial production performance.

Alternatively, vaccination with live vaccines of attenuated or non-attenuated strains of coccidia in controlled doses have been employed to induce a low level of infection in commercial flocks leading to immune protection against coccidiosis (Blake and Tomley, 2014, Shirley *et al.,* 2007). Following ingestion and recycling of controlled doses of vaccine oocysts, robust immune protection is often achieved (Blake *et al.,* 2017). These vaccines are produced by the passage of coccidia oocysts through chickens thereby making their production both expensive and laborious. Also, due to the species-specificity of *Eimeria* species and high genetic diversity between different *Eimeria* species, successful vaccines must be composed of all seven species or the most important species of *Eimeria* to confer adequate protection in the field (Shirley *et al.,* 2005, Lillehoj and Lillehoj, 2000, Vermeulen *et al.,* 2001). This has limited the widespread use of live vaccines especially in the broiler production sector (Shirley *et al.,* 2005). Nevertheless, anticoccidial rotation programs in the USA have incorporated the use of live non-attenuated vaccines and up to 35-40% of commercial broiler companies have been reported to use them (Chapman and Jeffers, 2014, Clark *et al.,* 2017).

Experimentally, recombinant subunit vaccines have yielded good results in inducing protective immunity against parasite infections and therefore represent an alternative control strategy (Lillehoj and Okamura, 2003). Subunit vaccines have been considered as potential alternatives for the control of coccidiosis for many years and many antigens have been proposed for use in novel anticoccidial vaccines because of their capacity to decrease parasite replication thereby reducing disease severity, improve feed conversion experimentally and increase body weight (Blake *et al.,* 2017).

Since host immunity in *Eimeria* is species-specific, an effective recombinant vaccine should have antigens from most species especially the most important ones, as well as antigens from most developmental stages of the pathogen (Jenkins and Dame, 1987). Recombinant vaccines depend on the expression of a small number of antigens, and low genetic variability in the target antigen is a requirement for a successful vaccine (Blake *et al.,* 2004, Blake *et al.,* 2011). However, *Eimeria* is a complex parasite and vaccination with a small portion of antigens from such a parasite may lead to eventual resistance (Table 1.4) (Blake *et al., 2015,* Clark *et al.,* 2017).

Other strategies used by farmers for the control of coccidiosis on poultry farms are management related which includes the elimination of the source of *Eimeria* oocysts, strict biosecurity measures, routine disinfection using anti-oocysts preparations and

cleaning of the poultry house between consecutive batches. These practices are useful in minimising the introduction and spread of *Eimeria* but they do not prevent coccidiosis outbreaks because some infective oocysts will survive any practical cleaning procedure (Vermeulen *et al.*, 2001). Outbreaks of coccidiosis are a result of drug resistance, environmental persistence and high prevalence of the parasite and in such instances subclinical infections are common with attendant consequences on productivity and food security (Blake and Tomley, 2014).

These limitations for existing vaccines, increased regulation and bans on the use of anticoccidial drugs and the high costs of developing vaccines and new drugs have all increased the need for the development of new and improved approaches and alternative control strategies for coccidiosis. (Dalloul and Lillehoj, 2006, Godwin and Morgan, 2015, Quiroz-Castañeda and Dantán-González, 2015). A summary of the types of commercially available and experimental vaccines used for the prevention of *Eimeria* infections in chickens as well as their advantages and disadvantages are listed in Table 1.4 below.

Table 1:4: Types of commercially available and experimental vaccines for *Eimeria* infections in chickens

Vaccine category	**Advantages**	**Disadvantages**
Live attenuated	Elicits strong and lasting immunity quickly	Difficulty in obtaining uniformity of oocyst intake per bird, escape of immunity as a result of differences in variants/strains within the same species, live non-attenuated vaccines of different *Eimeria* species being introduced to a farm may revert to virulence thereby causing outbreaks
Live non-attenuated	Safer than non-attenuated vaccines Elicits strong immunity	Difficulty in obtaining uniformity of oocyst intake per bird and possible escape of immunity as a result of differences in variants/strains within the same species
Subunit and recombinant	Safe technology which targets the induction of immunity in the host to surface proteins of the *Eimeria* parasite in different stages of lifecycle including merozoites, sporozoites, and gametocytes	They are administered as injections which is logistically difficult and labour costs are higher. Also, development of these vaccine types is slow and challenging

DNA based	These vaccines avoid maternal immunity which could neutralize vaccine antigens by the use of vectored genes which are delivered to the chickens. They undergo a translation process in the host cells generating immunogenic proteins of the *Eimeria* species	The protein translated from the delivered genes might be inadequate in quantity to induce a protective level of immunity against coccidiosis. Also, the vaccines can induce immunity which might not protect against the many species of *Eimeria*,

1.12 Non-conventional methods used for the control of coccidiosis

The search for alternative methods of controlling coccidiosis is encouraged by drug resistance issues associated with the use of in-feed anticoccidials and the tightening of legislations around the use of such compounds (Innes and Vermeulen, 2006). These have also been compounded by negative consumer views on the use of drugs in food producing animals as well as the increased demand for organic products. This has led to the investigation of the possible role of natural/herbal compounds (Tan, 2005, Lillehoj *et al*., 2007). Alternative compounds for the control coccidiosis include fats (containing high concentrations of docosahexaenoic acid, eicosapentaenoic acid and linoleic acid), antioxidants, essential oils, herbal extracts/medicinal plants, apple cider vinegar (Hayajneh *et al*., 2018) and immune response modulators. They have all been reported to have anticoccidial activity (Quiroz-Castañeda and Dantán-González, 2015). At present, there are up to four plant products that can be used as anticoccidial feed additives commercially available in the market (Muthamilselvan *et al*., 2016). Researchers have argued that herbal extracts do not have the risk of contaminating meat with harmful substances and it's also been reported that resistance to herbal products among *Eimeria* species is yet to be documented (Nogareda *et al*., 2016, Naidoo *et al*., 2008). The results obtained from the use of natural/herbal compounds in the form of pre- and pro-biotics, phytotherapy and aromatherapy have so far been confusing and inconsistent, therefore they have not been applied at large scale in the field (Peek and Landman, 2011).

1.13 Phage display technology

1.13.1 Introduction to phage display

A key methodology in the current study is antibody phage display. Phage display technology, originally introduced by George Smith in 1985 (Smith, 1985) is a robust and versatile method that allows the display of proteins and peptides as fusion proteins on the surface of bacteriophages by splicing the genes encoding the

protein/peptide into one of the genes that encodes a phage coat protein (Petty *et al*., 2007, Paschke, 2006). Phage display directly links the genotype of the displayed ligand with its binding properties (phenotype). In 1985, (Smith, 1985) successfully incorporated foreign DNA into the genome of the M13 phage such that the displayed foreign peptides were tethered to the pIII coat protein (Smith, 1985) and since then other coat proteins have been used for display of proteins/peptides (Hufton *et al*., 1999, Fagerlund *et al*., 2008, Løset and Sandlie, 2012) with gene VIII protein (pVIII) and gene III protein (pIII) the most commonly used (Clackson and Lowman, 2004).

In 1990, (McCafferty *et al*., 1990) were one of the first to describe antibody phage display and were able to fuse genes encoding the antibody binding domain in the form of single chain variable fragments (scFv). This technology utilized the domain architecture of antibodies which, because of the modular design of antibodies, allows the display of the minimum binding domain fragment in the selection of antigen recognising phage fusions (McCafferty *et al*., 1990).

Vectors used to construct phage display libraries are of two types. Firstly, phage vectors derived from the entire genome of filamentous bacteriophage having the natural Ff phage sequence which codes for all proteins required for the replication and assembly of phage particles (Marvin, 1998). Secondly, phagemids, which are a hybrid of phage and plasmid vectors designed with the origin of replication (ori) from the Ff phage, the plasmid origin of replication from *E. coli*, multiple cloning site, and an antibiotic resistant gene. Phagemids however lack the phage genes required to produce a functional phage and therefore require a helper phage. They are grown as plasmids in *E. coli* and they replicate in *E. coli* cells as double stranded DNA plasmid and packaged as single stranded (ss) phage DNA when host cells are superinfected with helper phage. The latter providing all the necessary other genes for phage assembly (Mullen *et al*., 2006, Foster *et al*., 2015).

1.13.2 The structure and biology of filamentous phage

Bacteriophage are a group of viruses that infect prokaryotic cells and are capable of infecting and replicating in a bacterial cell (Bratkovič, 2010). While some phages such as T4 or T7 are lytic and kill their host, filamentous phages such as M13 phage are lysogenic and do not kill the host bacterial cells they infect (Loh *et al.,* 2019). The most important and best characterised filamentous phage strains are the M13, fd and f1 phage together referred to as the Ff bacteriophage due to their mode of infection through the F pilus of male *E. coli* (Sidhu and Geyer, 2015). Since their discovery in the early 20th century, they have been used widely in scientific applications such as

studies and analysis of protein-protein interactions and epitope mapping (Pennazio, 2006, Pande *et al.,* 2010). They have a circular single-stranded DNA genome which is enveloped by a long cylinder of coat proteins ~7 nm wide by 900–2000 nm in length (Hill and Petersen, 1982). The genome encodes 11 genes of which five are responsible for expression of structural coat proteins. The coat is predominantly made up of 2700 molecules of pVIII being the major coat protein which extends the whole length of the phage particle as well as an additional four minor capsid proteins at the tips (Mullen *et al*., 2006) (Figure 1.2).

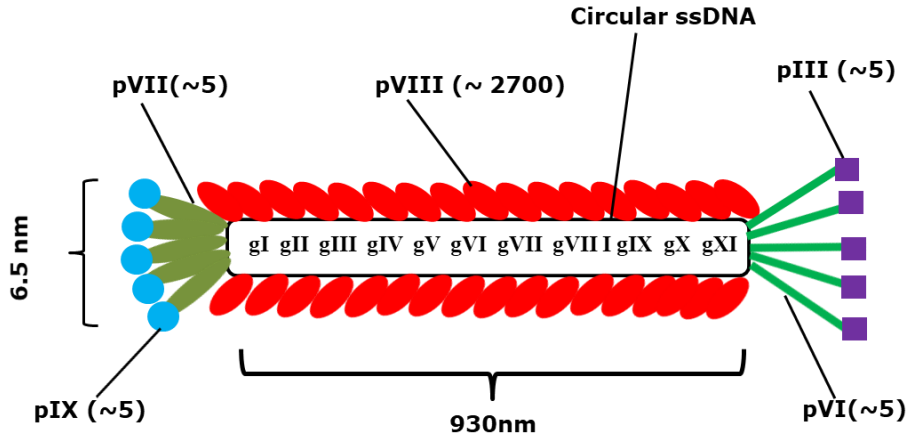


Figure 1:2: Schematic representation of the dimensions and structure of the M13 filamentous bacteriophage. Cylindrically shaped M13 comprising of a single stranded DNA, 6400 base genome that codes for eleven genes gI-gXI, five structural proteins, major coat protein pVIII and other minor coat proteins (PIII, PVI, pIX and pVII). Modified from (Mullen *et al*., 2006, Lebani, 2015). Coat proteins gII, gV, gX and gXI are involved in ssDNA replication while coat proteins gI and gIV are proteins for phage assembly and extrusion.

The display of proteins and peptides on the surface of filamentous bacteriophage is a powerful methodology for selection of proteins with specific binding properties, including antibodies and peptides. Since phage display was introduced by George Smith in 1985, various very high diversity libraries expressing over 10^9 distinct peptides or antibodies have been produced (Scott and Smith, 1990, Hoogenboom *et al.,* 1991, Barbas *et al.,* 1991, Bass *et al.,* 1990, Holliger and Hudson, 2005).

As mentioned, phage display links the phenotype and genotype physically because the displayed ligand and its encoding DNA fragment exist in the same phage particle. The construction of a library that contains a very large diversity of ligand sequences is key in the success of *in vitro* selection strategies; typically the library should contain

between 10^9 and 10^{11} distinct ligands (Hust and Dübel, 2004). This ensures that a wide range of structural diversity is displayed. When a phage library is screened against a target protein *in vitro*, the process is known as panning. Phages are introduced to the target (usually immobilised on a solid support such as an ELISA plate) and non-binding phage are washed away, bound phage are then eluted and re-amplified by growing in bacteria. Panning is repeated several times to enrich the population of phage-ligands that binds to the target structure. The proteins that are displayed are thereafter identified by DNA sequencing of the ligand gene in the phage clones (Figure 1.3) (Ravn et al., 2010a).

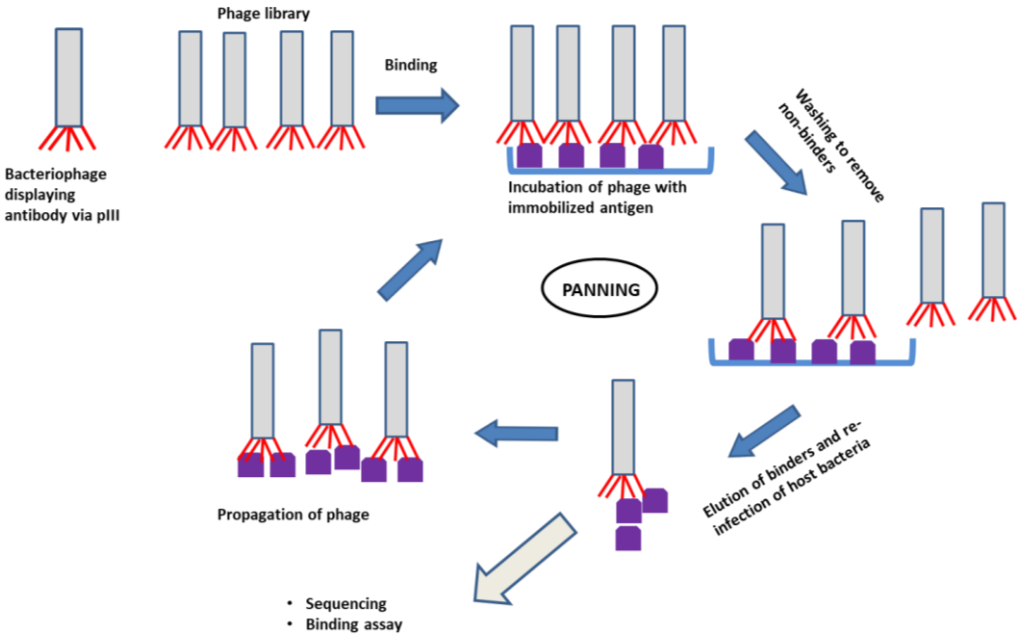


Figure 1:3: Schematic representation of phage panning procedure. The phage are incubated with an immobilised target and non-binders are washed away after incubation. Bound phage are eluted (by direct addition of TG1 or by a pH shift) and used to re-infect a bacterial host and bacterial glycerol stocks of phage sub-libraries are created. The sub-libraries are then used for future panning rounds or deep sequenced to identify binders.

Filamentous bacteriophages are lysogenic not lytic; they are assembled in the cytoplasm of the host and secreted from the bacteria without lysing the cell (Clackson and Lowman, 2004). The reproductive life cycle begins with interaction of pIII with the tip of the F pilus of the *E. coli* host and there is an adsorption of the phage DNA into the *E. coli* cell. The F pilus retracts as a result of de-polymerization of pilin subunits and this process pulls the bacteriophage towards the cell (Maneewannakul *et al*., 1993), the phage disassembles and the coat protein is inserted into the bacterial membrane as illustrated in Figure 1.4 (Nakamura *et al*., 2003). The coat proteins integrate into the host cytoplasm and there is a translocation of the ssDNA into the host's cytoplasm. The penetration of *E. coli* membrane and the uptake of the phage

ssDNA into the cytoplasm is dependent on the integrity of the N-terminal of the pIII protein and the products of certain bacterial genes especially tolA which is essential for infection (Nakamura *et al*., 2003, Click and Webster, 1998, Ploss and Kuhn, 2010). Once inside the cell, the host enzymes synthesize the complementary DNA strand and then the ssDNA is converted into the supercoiled double-stranded (ds) DNA known as the replicative form (RF). The RF creates a ssDNA by undergoing rolling circle replication which serves as a template for phage gene expression (Azzazy and Highsmith Jr, 2002, Ploss and Kuhn, 2010). Newly synthesized phage particles are assembled by packaging of ssDNA into phage protein coats and these are expelled through the bacterial envelope into the medium (Ploss and Kuhn, 2010, Azzazy and Highsmith Jr, 2002). Phage assembly occurs in a non-lytic manner and gVIII, the major coat protein is incorporated into the bacteriophage coat as the phage particle is being expelled from the cell (Qureshi, 2016).

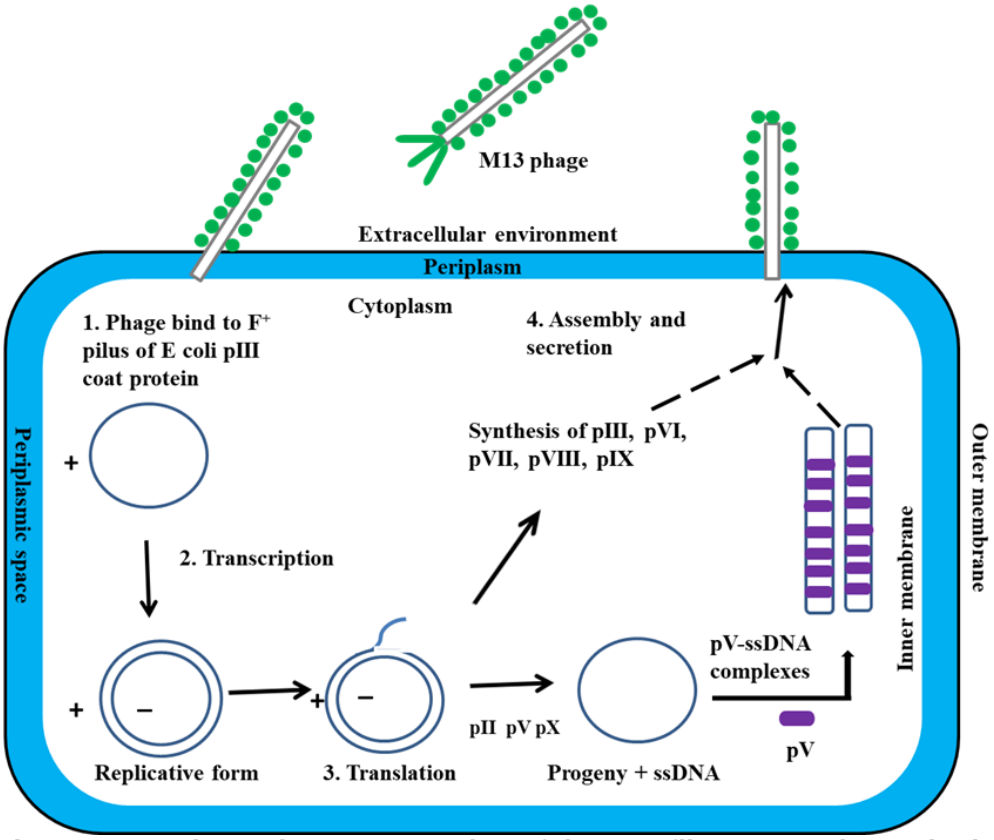


Figure 1.4: Schematic representation of the M13 filamentous bacteriophage life cycle in *E. coli*. Phage binds to the tip of the F^+ pilus of *E. coli* through the pIII coat protein. The single stranded (+) genome of the virus is injected into the bacterial cell and a complementary strand (-) is synthesized forming a double-stranded phage genome. Phage encoded proteins are then produced by host mediated protein synthesis; phage coat proteins, proteins for replication and proteins for assembly and export. Virions are then assembled and exported across the bacterial membranes (Adapted from (Mullen *et al*., 2006).

1.13.3 Types of Antibody libraries

There are different types of antibody libraries based on how they are derived; immune naïve and synthetic/semi-synthetic antibody libraries (Ledsgaard *et al.,* 2018, Carmen and Jermutus, 2002). Immune libraries are directed against the immunogen. Naïve and synthetic/semi-synthetic libraries are known as single pot libraries as they can be used to produce binders to any target.

1.13.3.1 Single pot libraries

Naïve libraries are a collection of antibody genes that are derived from healthy individuals. They are obtained from v-gene repertoires from B cells (IgM) of non-immunized donors (Qi *et al*., 2012) and they are not biased towards any particular antigen. Naïve libraries are designed to isolate antibodies binding to any antigen (Willats, 2002, Bahara *et al*., 2013). The advantage of naïve libraries is that they can be used to detect antibodies against a wide range of targets including toxic targets (Wang *et al*., 2012, Ledsgaard *et al*., 2018), non-immunogenic targets and self-antigens. However, a concern when dealing with naive libraries is the true unbiased nature of the repertoire. This is because most individuals must have encountered one form of infection or another in their lifetime. Also, antibodies isolated from naïve libraries may not have as high an affinity as those from immune libraries because of a lack of affinity maturation *in vivo* (Richard *et al*., 2013, Bahara *et al*., 2013, Stewart *et al*., 2007). In order to generate high affinity antibodies from naïve libraries, library size must have at least 10^9 independent clones (Vaughan *et al*., 1996). In addition, different *in vitro* affinity maturation techniques have been employed successfully. The sequences of the specific antibody fragments are first diversified and phage display selections are carried out to select antibodies with increased affinity to the target antigen (Ledsgaard *et al.,* 2018, Bradbury *et al.,* 2011). Mutagenesis can be employed in the variable regions of antibodies to increase affinity to the target which could be random or site directed. HCDR3, having the highest natural diversity amongst the variable regions of the antibodies is usually targeted for mutagenesis, although some libraries have targeted other CDRs (Goletz *et al.,* 2002, Rothe *et al.*, 2008). Chain shuffling whereby antibody fragments obtained after selection have either their heavy chain or a light chain paired with the complementary chain from an

immune library in which the initial antibody fragment was selected can also improve affinity to the target (Marks *et al.,* 1991, Vaughan *et al.,* 1996).

When single pot libraries are produced by mutagenesis of a small diversity of natural antibody sequence, it's termed a semi-synthetic library. Synthetic library on the other hand are made up of a single or small number of antibody scaffold sequences and diversity is introduced in the CDR3 region or multiple CDRs. These libraries are constructed artificially by *in vitro* assembly of V gene segments to construct antibody variable domains that then undergo random mutagenesis of the CDRs within a single or multiple frameworks in both library types (Ch'ng *et al.,* 2016, Ponsel *et al.,* 2011, Lim *et al.,* 2014). The human scFv library (Griffin) which is a semi-synthetic library was constructed by using six diverse synthetic CDR3 regions as the framework (Ch'ng *et al*., 2016, Strachan *et al*., 2002, Griffiths *et al*., 1994). Unlike naïve libraries, in synthetic libraries, customization can be carried out on the genetic sequence, overall diversity and local variability. The needs of the user can be suited by the use of a specific codons, for instance, features such as solubility and heat stability can be improved by modifications to the framework (Sidhu and Fellouse, 2006).
Single pot libraries have been successfully used to generate antibodies against a wide range of antigens including whole cells, haptens and proteins (Lim *et al*., 2016, Zhu and Dimitrov, 2009).

1.13.3.2 Immune libraries

Immune libraries are constructed from antibody V-genes isolated from IgG secreting plasma cells of immunized donors (Hoogenboom *et al*., 1998, Clackson *et al*., 1991, Trott *et al*., 2014, Arakawa *et al*., 2007). Immune libraries are generated to obtain antibodies against a particular target antigen because the libraries are already biased towards the antigen in question (Adams *et al*., 2014, Chen and Sidhu, 2014). This limits the use of immune libraries compared to naïve and synthetic/semi-synthetic libraries. Antibody fragments in an immune antibody library have increased affinity to the antigen type used for immunization and this can be advantageous for antibody discovery because high affinity antibodies can easily be generated because the antibodies have gone through an affinity maturation process *in vivo*. This also means that the immune library size does not have to be as high as single pot libraries, typical library sizes are between 10^6-10^7 (Bahara *et al.,* 2013, Gough *et al.,* 2015).
Immune libraries are also useful for studying vaccination strategies, human immunity and immune responses (Ch'ng *et al*., 2016). The drawback of such libraries is that a

new library will have to be generated to study every different disease or target antigen. (Willats, 2002, Bahara *et al*., 2013, Lim *et al*., 2014).

Immunized libraries have been prepared from rabbits (Li *et al*., 2000a), chickens (Schmitz *et al.,* 2000, Andris-Widhopf *et al.,* 2000), sheep (Li *et al.,* 2000b), llamas (Miyazaki *et al.,* 2015), sharks (Leow *et al.,* 2018), mice (Saggy *et al*., 2012a) and humans (Amersdorfer *et al.,* 2002).

It has been reported that in some cases, scFv antibodies isolated from naïve or immune libraries are difficult to express in bacteria which complicates the production of the soluble proteins at a preparative scale. This may be overcome to some extent when using synthetic libraries and if the parental framework is known to be well expressed in *E. coli* then it will be more likely to support a good expression of a library of inserted CDR3 sequences (Ch'ng *et al*., 2016).

Just like naive libraries, immune libraries have been applied to isolate a wide variety antigens and have successfully used different antibody formats such as Fab, scFv (Saggy *et al*., 2012a), V_HH (Miyazaki *et al.,* 2015) and V_{NAR} (Leow *et al.,* 2018).

1.13.4 Types of antibody formats

1.13.4.1 Antigen binding fragment (Fab)

First described by (Kang *et al*., 1991), they were the first antibody library formats displayed on the surface of bacteriophage as well as the first therapeutic antibody fragment format. They are composed of antibody light chain (VL and CL) domains linked by a disulphide bond to the antibody heavy chain (VH and CH1) domains (Figure 1.5) (Bates and Power, 2019). They are more functionally stable than scFvs due to mutual stabilization across the V_H/V_L and the C_H1/C_L interface (Röthlisberger *et al.,* 2005).

(Quintero-Hernández *et al*., 2007) found that three of four specific scFvs against a scorpion toxin that were not neutralising as scFv formats became neutralising once expressed as Fabs showing an improved stability of Fabs over scFvs. Fabs have good tissue/ tumour penetration as compared to IgG but may not be as good as other smaller antibody formats (Li *et al.,* 2016).

1.13.4.2 Single chain fragment variable (scFv)

They are heterodimers consisting of the light and heavy chain variable domains (VL and VH) of antibodies tethered by a flexible linker (Figure 1.5) (Bhat *et al.,* 1990). First described by Bird *et al.* (1988), scFvs have high specificity, sensitivity and ease of expression. The coding sequences of the VL and VH are genetically linked hence they are expressed from a single gene which makes their expression easier and

production quicker and in higher yields compared to Fabs (Skerra and Pluckthun, 1988). However, as mentioned earlier, scFvs have lower long time stability compared to Fabs and may display lower levels of affinity (Bird *et al.,* 1988).

1.13.4.3 Camelid V_HH antibodies

V_HH antibody fragments which are also known as nanobodies or single domain antibodies (sdAb) were first described by (Hamers-Casterman *et al*., 1993) and are found in the sera of animals of the Camelidae family that contain a species of antibody lacking the light chain (Hamers-Casterman *et al*., 1993). The V_HH, just like the VH domain of a typical IgG has a disulphide bond between the framework regions (FRs) that stabilises its structure (Figure 1.5). It has 3 CDR regions held by FRs with the CDR1 and CDR3 being longer. They are heat stable being able to refold after denaturation, small at approximately 13–15 kDa (Figure 1.5), soluble and readily produced in *E. coli* and can display distinct antigen-binding properties compared to traditional antibody fragments (Muyldermans, 2013, De Vlieger *et al*., 2019). This is because their small paratope with a longer CDR3 region aids their binding to hidden antigenic sites (Harmsen and De Haard, 2007). The variable domain of these antibodies can be cloned for phage display which results in one of the smallest known functional antigen-binding units (White *et al*., 2019).

1.13.4.5 V_{NAR} Antibodies

The single variable new antigen receptors (V_{NAR}) are obtained from cartilaginous fish such as sharks. The V_{NAR} are similar to the V_HH having a long CDR3 region which represents most of the diversity (Feng *et al.,* 2018). Just like the V_HHs*,* they are highly stable, have disulphide bonds that stabilise their structure and they are small at approximately 12 kDa (Figure 1.5). They have only the CDR1 and CDR3 regions as well as two mutation-prone regions; HV2 and HV4 of which HV4 has been shown to contribute to antigen binding (Zielonka *et al.,* 2015, Stanfield *et al.,* 2007). *V_{NARs}* are easily cloned being from a single gene family (Dooley *et al.,* 2003) and both naïve and immune *V_{NAR}* libraries have been produced (Nuttall *et al.,* 2002, Leow *et al.,* 2018).

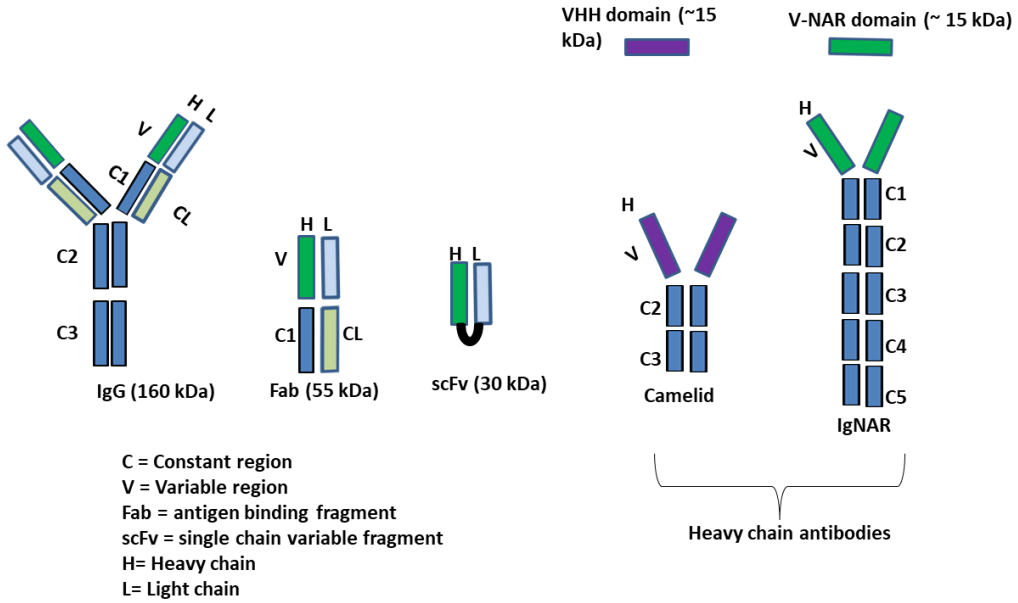


Figure 1:5: Schematic representation of different antibody formats and domains, showing V_HH, V_{NAR}, Fab and scFv formats as well as intact IgG molecule or heavy chain antibodies

1.14 Next generation sequencing in relation to phage display

In recent years, genomic research has greatly advanced because of improved capacity for DNA sequencing provided by next generation sequencing (NGS). As an advantage over traditional sanger-based sequencing methods, NGS has the ability to evaluate millions of sequences in parallel. NGS technologies have more recently been employed for analysing antibody sequences following phage panning experiments to identify binders not found by conventional ELISA screens (Barreto *et al.,* 2019). When applied to isolated populations of phage (usually through the sequencing of the HCDR3 regions), the resulting data is a very thorough interrogation of the library in question after selection against a target. Often 10,000's to millions of phage ligand sequences can be analysed. In comparison, conventional phage panning methods usually select just a few 100 phage clones for analysis by ELISA and Sanger sequencing.

The use of NGS for the analysis of antibody sequences both in phage libraries and during *in vitro* selection processes has become increasingly popular in the last few years (Ravn, Didelot *et al*. 2013). NGS has been used for phage library quality control and also to support antibody and peptide discovery (Ravn et al., 2010a, Glanville et al., 2009, AC't Hoen et al., 2012, Naqid et al., 2016b, Naqid et al., 2016a).

Conventional panning experiments are often unsuccessful because of parasitic antibodies/ peptides. Parasitic peptides/antibodies are those that are enriched during panning due to a growth advantage in bacteria compared to other clones or due to their binding to a non-target component, such as blocker or the solid support used to immobilise the target. The screening of several hundred phage by ELISA in conventional panning does not identify target-specific ligands as they can often be rare due to the fact that they can be outcompeted by phage coding for parasitic clones and/or they are swamped by non-enriched background phage. NGS screening can analyse thousands to millions of ligands to establish which ones are enriched over background or parasitic phage (Naqid *et al*., 2016a). One strategy is to use NGS screening to compare the binding of antibody phage libraries isolated after panning against a target compared to a control (Zhang *et al*., 2011, Naqid *et al*., 2016b). This screening can be used to dismiss parasitic phage that are present in equal amounts between the target and control binding experiments and identify ligands that are binding only to the target (Naqid *et al*., 2016b).

1.15 Aims and Objectives

Reliance on conventional diagnostics for *Eimeria* infections does not allow efficient identification of *Eimeria* species. Whilst molecular techniques are available for species level diagnosis of *Eimeria* infections in poultry they are not widespread due to the need for specialist equipment, high costs and the challenges of obtaining template DNA. Therefore, there is a need for improved diagnostics which are relatively inexpensive and easily applied, possibly in the field. This is especially important because the scale of poultry production has increased and there is the need to provide food security to an increasing human population (Prakashbabu *et al.,* 2017).

The aim of this research was to develop novel species-specific antibody-based diagnostics for *Eimeria* infections in chickens using next generation phage display technology (NGPD). This was to be carried out by antibody-phage panning against all seven pathogenic *Eimeria* species that infect chickens to attempt to isolate species-specific antibodies. Comparisons were also made between the efficacy of traditional phage panning and NGPD.

1.16 Research hypothesis

It was hypothesized that NGPD will allow comparative screening of antibody binding events to identify antibodies to species-specific epitopes of *Eimeria* oocysts and such ligands could then be used to develop immunoassays to differentiate infecting species of *Eimeria* in chickens.

2. Materials and Methods

2.1 Reagents and Media

I. Glucose Solution 25%

25g of glucose (Sigma Aldrich) was dissolved in 100ml of deionized water and then filter sterilized through a 0.22 µM sterile filter unit (Millipore). Glucose stock was stored at room temperature.

II. 2YT Media

2YT media was prepared by weighing out 16g of tryptone (Sigma Aldrich), 10g of yeast extract (Merck) and 5g of NaCl (Sigma Aldrich) and dissolving it in 1 litre of distilled water. The broth was then sterilized by autoclaving.

III. 2YT Agar

2YT agar was prepared by dissolving Bacto-agar (15 g) (Sigma Aldrich), tryptone (16 g), yeast extract (10 g) and NaCl (5 g) in 1 litre of deionized water. The broth was then sterilized by autoclaving (121°C for 30 minutes). The broth was then cooled to 37°C; Ampicillin 150 µg/ml (Sigma Aldrich) and 1% (w/v) of glucose solution (Fisher Scientific) were added. Plates were poured and stored at 4°C until required

IV. M9 Minimal agar glucose plates

M9 salts (5 x solution) was prepared by dissolving Na_2HPO_4 Anhydrous (15 g) (Fisher 5/4520/53), KH_2PO_4 (15 g) (Sigma P9791-500g), NH_4Cl (5 g) (Fisher A/3880/53) and NaCl (2.5 g) (Fisher 5/3160/60) in 1 litre of deionized water, the solution was then autoclaved. Minimal agar (3 g) was dissolved in 150 ml of deionized water in a 250 ml flask, and then autoclaved. The agar was then cooled to 37°C and 50 ml of 5x M9 salts, 3.2 ml of 25% (w/v) glucose solution, 200 µl of $MgCl_2$ 20% (w/v) (Sigma Aldrich), and 100 µl of 10 mg/ml thiamine solution (Sigma Aldrich) added. Plates were poured and stored at 4°C until required.

V. Polyethylene glycol (PEG) Solution

PEG-6000 (20 g) (Sigma Aldrich) and NaCl (14.61 g) were dissolved in 100 ml of deionized water and then filtered through a 0.22 µM sterile filter unit (Millipore), and stored at room temperature.

VI. Bicarbonate/carbonate coating buffer (100 mM)

Na_2CO_3 (3.03 g) and $NaHCO_3$ (6.0 g) (Sigma-aldrich, UK) were dissolved in 1 litre of deionized water and the pH adjusted to 9.6. The solution was stored at room temperature.

VII. Phosphate Buffered Saline

Phosphate buffered saline (PBS) was made by dissolving 1 tablet of pre-made PBS in 500 ml distilled water (1× PBS) (Life Technologies, UK). PBS-Tween 20 (0.1% PBS-T (v/v)) was prepared by adding 0.5 ml of Tween-20 (Thermo Scientific, UK) to 0.5 L of 1× PBS.

VIII. A stock solution of 50x TAE

Tris-acetate EDTA (TAE) electrophoresis buffer (Thermo Scientific, UK) was used in the preparation of agarose gels. A stock solution of 50x TAE was prepared by dissolving Tris base (242 g) (Sigma-Aldrich) in 750 ml deionized water. Glacial acetic acid (57.1 ml) and 0.5 M EDTA (100 ml) (pH 8.0) was added and the stock solution adjusted to a final volume of 1 litre. To prepare the 1x TAE buffer, 20 ml of 50x TAE stock was added to 980 ml of deionised water.

IX. 20% (w/v) $MgCl_2$

10 g of magnesium chloride was added to 50 ml distilled water, it was then autoclaved.

X. Antibiotics

Ampicillin and kanamycin (Sigma-Aldrich, UK) stock solutions were made with deionized water to final concentrations of 150 mg/ml and 50 mg/ml, respectively. They were sterilized by filtration through a 0.22 µm sterile filter unit (Millipore). Antibiotic stocks were aliquoted and stored at - 20°C until required.

2.2 Quantification and storage of *Eimeria* oocysts

Oocysts from seven species of *Eimeria* in chickens were supplied by MSD Animal health. The oocysts were in suspension within 50 ml of PBS. The oocysts were quantified using a haemocytometer.

The oocysts were prepared by first inverting the tubes that contain the cells 4-6 times to ensure that the cells were evenly distributed and before the cells settled, 100 µl of each suspension of the individual *Eimeria* species was taken out and put in a sterile

eppendorf tube. To each tube 900 µl of PBS was added and gently mixed. This tenfold dilution of the individual oocysts sample was analysed.

The haemocytometer was cleaned by wiping to remove dust particles. The oocysts suspension was loaded by capillary action using a pipette after placing the coverslip over the counting surface. The liquid cell suspension was just enough to cover the mirrored surface; around 10 µl. Duplicate samples were loaded on each of the two sides of the coverslip. The loaded haemocytometer was then placed on the stage of the microscope and the counting grid brought into focus at x10 magnification. The cells were counted in each of the four outer grids of the heamocytometer containing 16 squares making sure to count just one side each of the length and width of the squares to avoid duplicate counts. Once the cell count was obtained, the average count in the grids were taken as well as the average from the 2 sides of the haemocytometer, the cell concentration was thus calculated using the following formula:

Total cells/ml = average cells counted x dilution factor x 10000

[10000 was used as the volume of sample examined on the haemocytometer is:

height X width X depth = 1 X 1 X 0.1 mm3 = 0.0001 mL]

The number of oocysts in the original sample were calculated by multiplying the cell concentration by total sample volume. For all the seven *Eimeria* samples, the total volume was 50 ml.

Amphotericin B was added at 5 mg/ml to prevent fungal growth and oocysts were stored at 4°C until required.

2.3 Phage display: identification of antibodies to whole oocysts using the Griffin scFv library

Whole oocysts in solution were used together with the Griffin library of bacteriophage antibody-fusions to identify displayed scFv that bind to the different species of oocysts.

2.3.1 Bacteria and helper phage stocks preparation, titration and storage

The bacteria used for all library propagation, manipulation, phage panning experiments and phage rescue carried out in this thesis were *Escherichia coli* TG1 supE thi -1Δ (lac-proAB) Δ (mcrB-hsdSM) 5 (rK-mK-) (F' traD36 proAB lacUqZΔM15) which was stored as glycerol stocks in aliquots at -80°C before use. The glycerol stock of bacteria was prepared by prewarming 2YT media (500 ml) containing 150 µg/ml

ampicillin and 1% glucose in a 2 L flask to which 500 µl of glycerol library stock (*E. coli* + phage) was added and then grown to OD_{600nm} 0.4 to 0.6 in a shaking incubator at 180 rpm and 37°C. After the culture had reached mid log at OD_{600nm}, it was grown overnight. The next day, the culture was spun down, supernatant discarded and then re-suspended in 10 ml 2YT containing 25% glycerol and stored at -80°C until needed. Whenever required, the TG1 glycerol stock was thawed and streaked onto minimal agar glucose plates and then incubated at 37°C for 48 hours and thereafter stored at 4°C. Every two weeks, this was repeated to ensure the cells were always viable. Whenever mid-log TG1 cells were needed, colonies from the minimal agar glucose plate were picked and grown shaking overnight in 10 ml of 2YT media at 37°C, 200 rpm shaking. The next day, the overnight culture was then diluted with fresh 2YT media 1:100 and grown shaking at 37°C for approximately 2 hours until OD_{600} reached between 0.4-0.6.

Helper phage stocks used in all experiments was the Exphage (Ex12) helper phage which is a mutant M13K07 helper having two amber stop codons within the gIII. Since there is an inefficient read-through of the UAG codons in amber suppressor strains, Ex12 helper phage produces about 10% of the intracellular wild type pIII in its host cells (*E. coli*), which makes the antibody pIII fusion display far more efficient (Oh *et al.,* 2007).

Helper phage was prepared by inoculating 1 litre 2YT with 10 ml overnight grown TG1 and growing to mid log at $OD_{600nm.}$ To the mid log cells, Exphage at multiplicity of infection (MOI) of 10 was added and incubated static at 37°C for 30 minutes and then incubated shaking at 180 rpm for another 30 minutes at 37ºC. To the incubated culture, 1 litre of 2YT was added making a total of 2 litres and then split between two flasks; 1 litre per flask. Kanamycin at 50 µg/ml was added to each flask and grown overnight at 30°C shaking at 180 rpm. The following day, the culture was centrifuged at 3000 xg for 20 minutes and the supernatant collected. The supernatant was Polyethylene glycol (PEG) precipitated by adding 250 ml 20% (w/v) PEG-8000/2.5M NaCl for every litre of phage supernatant and the sample placed on ice for 1 hour. The culture was centrifuged at 8000 xg for 20 minutes and the supernatant discarded. All residual PEG/NaCl was removed by pelleting a further two times at 8000 xg for 30 seconds to 1 minute and all liquid removed after each spin. The phage pellet was re-suspended in 30 ml of PBS, 15% glycerol was added and thereafter titred and stored in 500 µl aliquots at – 80°C until required.

2.3.1.1. Titration of Exphage

Exphage was titred using the H-top agar method. Briefly, nine 2YT agar plates labelled A-I were poured and allowed to set and then kept at 37ºC. 100 ml of H-top agar was made by adding 1g tryptone, 0.8g NaCl and 0.7g agar in a 250 ml glassware, this was autoclaved and allowed to cool and then 3 ml of the agar was aliquoted into 15 ml tubes and kept molten at 42°C. TG1 was grown to an OD of 0.2. A dilution series of phage was created as shown in table 2.1 and 20 µl of each dilution was added to 180 µl TG1 suspension and thereafter added to the H-top agar before plating on the warm 2YT plates, it was allowed to set and this was incubated overnight at 37°C.

Table 2:1: Showing serial dilution regime for Exphage titration using H-top agar method

Plate	PBS+exphage (µl)	Dilution factor	Exphage + TG1 (20+180, df10)	200 µl added to H-top (µl)	Cfu/ml
A	999 + 1 exphage	$1x10^{-3}$	$1x10^{-4}$	$2x10^{-2}$	Cfu $x10^{-5}$ /2
B	990 + 10 A	$1x10^{-5}$	$1x10^{-6}$	$2x10^{-4}$	Cfu $x10^{-7}$ /2
C	990 + 10 B	$1x10^{-7}$	$1x10^{-8}$	$2x10^{-6}$	Cfu $x10^{-9}$ /2
D	990 + 10 C	$1x10^{-9}$	$1x10^{-10}$	$2x10^{-8}$	Cfu $x10^{-11}$ /2
E	990 + 10 D	$1x10^{-11}$	$1x10^{-12}$	$2x10^{-10}$	Cfu $x10^{-13}$ /2
F	990 + 10 E	$1x10^{-13}$	$1x10^{-14}$	$2x10^{-12}$	Cfu $x10^{-15}$ /2
G	990 + 10 F	$1x10^{-15}$	$1x10^{-16}$	$2x10^{-14}$	Cfu $x10^{-17}$ /2
H	990 + 10 G	$1x10^{-17}$	$1x10^{-18}$	$2x10^{-16}$	Cfu $x10^{-19}$ /2
I	990 + 10 H	$1x10^{-19}$	$1x10^{-20}$	$2x10^{-18}$	Cfu $x10^{-21}$ /2

PFU (colony forming unit) which is the number of colonies counted on the plates the next day in each of the dilution factor. This was divided by 2 to obtain the actual number of PFU/ml.

2.3.2 Growing the Griffin scFv phage library

The library that was used for the initial phage display was the Griffin library, a semi-synthetic antibody library consisting of the human V_L and V_H chain gene segments with additional HCDR3 diversity generated using synthetic oligonucleotides supplied by MRC laboratories, Cambridge, UK (Goletz *et al.,* 2002). This naive scFv library has a reported repertoire of ~$1x10^9$ (Goletz *et al.,* 2002). This was prepared by prewarming 500 ml of 2YT (Fisher scientific UK) media containing 150 µg/ml ampicillin and 1% (w/v) glucose to 37ºC. To the culture, 500 µl of glycerol stock of the Griffin library was added and allowed to grow to mid log at 37ºC with shaking at 200 rpm. The helper phage Exphage was added at a multiplicity of infection (MOI) of 10 when the optical density at 600 nm (OD) of the culture reached an absorbance value between 0.4-0.6, indicating ~$4x10^8$ cells. The helper phage was left to infect the culture for 60 minutes, static for 30 minutes then shaking for the remaining 30 minutes at 37ºC. It was then centrifuged for 10 minutes at 3000 xg. The pellet was re-suspended in 500 ml 2YT containing 150 µg/ml ampicillin and 50 µg/ml kanamycin (Sigma Aldrich, UK) and left overnight at 30ºC and shaking at 180 rpm to produce recombinant bacteriophage.

2.3.2.1 Griffin library Phage Precipitation with Hydrochloric acid

The overnight phage culture was centrifuged at 3000 xg for 20 minutes and the supernatant containing the phage was collected. The phage were acid precipitated from the supernatant by lowering the pH to 4.2 using 1:1 water and hydrochloric acid (Jończyk *et al.,* 2011). The phage supernatant was left on ice for 1 hour before being centrifuged for 30 minutes at 8000 xg. The pellet was re-suspended in 10 or 5 ml of PBS as required and the pH was returned to 7-7.4 using between 10-20 µl of sodium hydroxide, respectively (2 M). The resulting phage suspension was then transferred into sterile eppendorf tubes and then centrifuged for 2 minutes to remove bacterial debris. The supernatant was transferred to a sterile container and a small aliquot was used to titre the phage. The phage were stored at 4°C and used for NGPD experiments within two weeks of precipitation.

2.3.2.2 Titration of phage library

The phage titres were determined for libraries and sub-libraries to count and measure the infectivity level of the phage preparation.

To titre the phage, one hundred fold serial dilutions were performed where 1 µl of phage was added to 99 µl of PBS (dilution factor 1), 1 µl of this was then diluted in 99

µl of PBS (dilution factor 100) and so on until all of the dilution series was completed; dilution series went up to a dilution factor of 10^{10}. After that, 900 µl of log phase TG1 *E. coli* cells at an OD600 of 0.4 were added to each tube and then incubated for 30 min at 37ºC, this was to allow the infection to take place.

On 2YT agar +150 µg/ml ampicillin and 1% (w/v) glucose plates, 100 µl of each dilution was plated out and incubated overnight at 37ºC. Negative control was uninfected TG1 plated on 2YT agar +150 µg/ml ampicillin and 1% (w/v) glucose. The following day, the phage concentration was determined by counting plaque forming units (PFU) in each plate to titre the phage per ml using this formula:

Plaque forming units per millilitre (PFU/mL) = Colonies counted x 1000 x Dilution factor

2.3.3 Griffin phage library biopanning

2.3.3.1 Round 1 biopanning

Approximately 4 x 10^5 oocysts in solution of each *Eimeria* species were put in sterile eppendorf. The oocysts were spun down and the supernatant discarded, the pellet was resuspended in sterile 3% (w/v) Marvel in PBS (1 ml) and incubated for 1 hr at room temperature. The oocysts were spun at 2000 xg for 3 minutes and the supernatant discarded after which the pellet was resuspended in 100 µl of sterile PBS. The phage were blocked for 1 hour with 200 µl 18% (w/v) Marvel in 6x PBS per ml of phage. The phage were then incubated with oocysts overnight at 4°C; 800 µl blocked phage per tube of oocysts (at 10^{11} phage/ml). The following day, the oocysts were spun at 2000 xg for 3 minutes the supernatant discarded by pipetting out. The oocysts were then washed with 1 ml wash PBST containing 0.1% (v/v) Tween 20 and thereafter spun at 2000 xg for 3 minutes and the supernatant discarded by pipette. This was repeated 6 more times followed by washing 3 times in PBS.

The phage were eluted from the oocysts by adding mid-log TG1 cells. To the oocyst pellet in each tube, 1.2 ml TG1 was added then incubated for 30 minutes at 37ºC to allow infection. A small volume of the bacteria was titred to determine the concentration of eluted phage and the remaining bacteria plated out on bioassay plates (245x245x25mm) containing 2YT agar +150 µg/ml ampicillin and 1% (w/v) glucose. A separate bioassay dish was used for panning against each of the seven *Eimeria* species. Cells were grown at 37ºC overnight. The following day, colonies were scraped from the surface of the bioassay plates using 5 ml of 2YT media. Glycerol was added to 30% (v/v) and samples stored at -80ºC

2.3.3.1.1. Titration of eluted phage

To determine the titre of eluted phage, the eluate containing phage and bacteria was diluted. One hundred-fold serial dilutions were performed where 1 µl of eluted phage was added to 99 µl of PBS (dilution factor of 1), 1 µl of this was then diluted in 99 µl of PBS (dilution factor of 100) and so on until all of the dilution series was completed; dilution series went up to a dilution factor of 10^6. After that, 900 µl of log phase TG1 *E. coli* cells at an OD600 of 0.4 were added to each tube and then incubated for 30 min at 37 ˚C to allow the infection to take place. Bacteria were plated out and number of colonies calculated as described in *2.3.2.2.*

2.3.3.2 Round 2-5 biopanning

Sub-libraries of phage were produced from bacterial stocks obtained from the phage eluted in round 1 of panning against each *Eimeria* species. 50 µl of each of the seven bacterial stock for the seven *Eimeria* species; total of 350 µl was inoculated into 100 ml of prewarmed 2YT (with 100 µl of ampicillin stock and 4 ml glucose) in a 250 ml flask. The culture was grown to $O.D_{600}$ 0.5-1 at 37ºC, 200 rpm. 50 µl of Exphage was added and incubated for 30 minutes static and another 30 minutes shaking. The culture was then transferred to x2 50 ml centrifuge tubes and centrifuged at 3000 xg for 10 minutes. Pellets were resuspended in 50 ml of 2YT containing 150 µg/ml ampicillin and 50 µg/ml kanamycin and grown overnight at 30ºC, 180 rpm. Overnight phage culture was transferred to x2 50 ml centrifuge tubes and centrifuged for 10 minutes at 8000 xg. The phage supernatant was then titred as described in *2.3.2.2* above to determine concentration and thereafter was transferred to fresh 1.5 ml tubes and refrigerated until use (usually within 1 week).

Panning in rounds 2-5 was carried out the same way as for round one. For each round, phage that bound to each of the seven species were pooled and grown into one culture which was then panned against the seven *Eimeria* species in the next round of selection. After each round of panning, eluted phage were plated out on bioassay dishes, made into glycerol stocks, and stored as individual species.

The total number of samples generated from panning was 35 from seven individual species and 5 rounds of panning as well as 5 input phage comprising of pooled phage selected against all *Eimeria* species and then the phage library itself, totalling 41 samples as illustrated in figure 2.1.

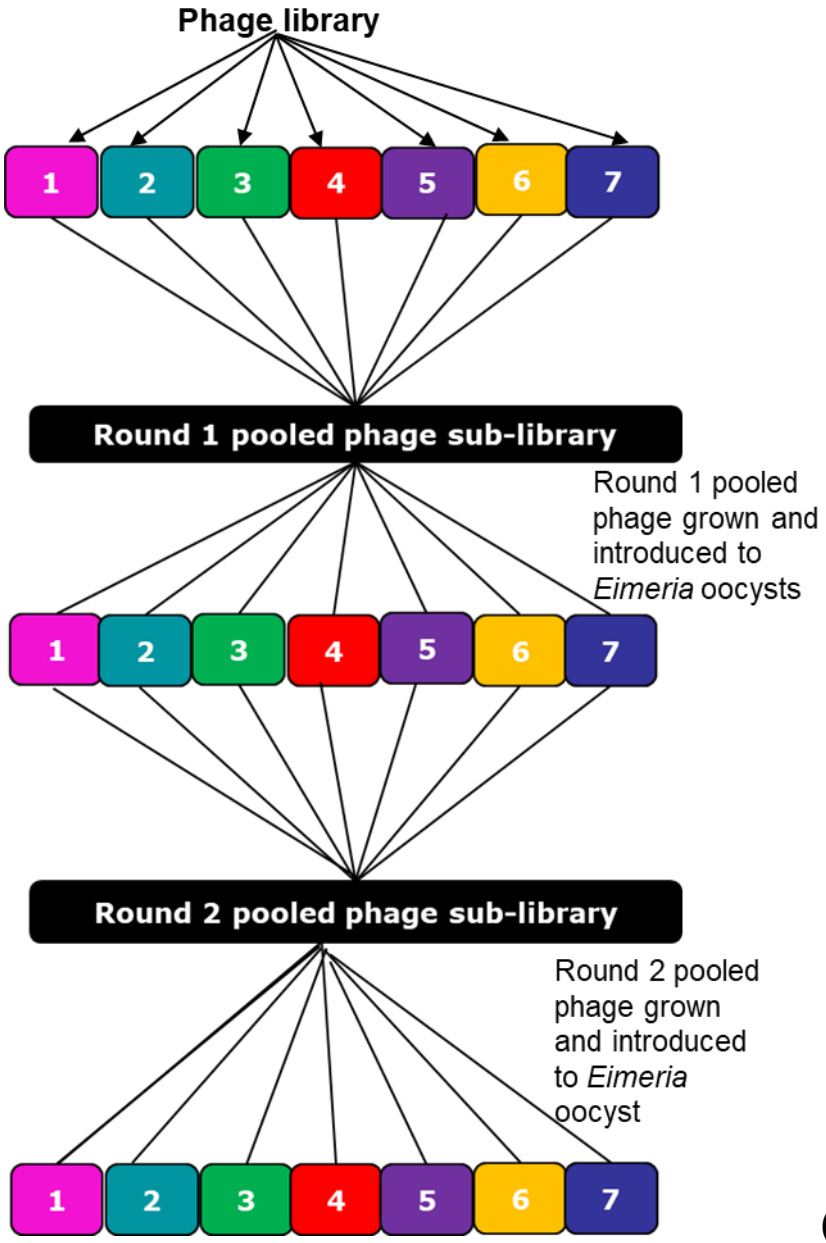


Figure 2:1: The panning strategy for whole oocysts panned with the Griffin library. 1-7 represent the seven different *Eimeria* species. They were panned against the library and glycerol stocks of output phage made, each of the sub-libraries were then pooled to make phage that was used in the next panning round (Round 1 pooled phage sub-library). A total of 5 panning rounds were carried out.

2.3.4 DNA extraction and preparation of sample for Ion Torrent sequencing

After five rounds of biopanning, phagemid DNA was isolated and purified from each sub library TG1 *E. coli* bacteria that were stored as glycerol stocks using a Qiagen Spin Mini Prep Kit (Product No. 27104) following the manufacturer's instructions. 250 µl of glycerol stock of sub libraries of all seven species from all five rounds of panning were put into separate tubes and centrifuged at 13,000 rpm for 1 minute and the cells harvested. The pellets were then resuspended in 250 µl of resuspension solution. To the resuspended cells, 250 µl of lysis solution was added to each tube and was mixed by inverting the tubes 5 times. After 4 minutes, 350 µl of neutralization solution was added and mixed by gently inverting the tubes 6 times then centrifuged for 10 minutes at 13,000 xg to pellet the cell debris. After the centrifugation, 800 µl of the supernatant was added to the QIAprep 2.0 spin column and centrifuged 1 minute and the flow through was discarded. The QIAprep 2.0 spin column was washed by adding 750 µl of the wash buffer and centrifuged for 1 minute, the flow through was discarded and the tubes were further centrifuged for another 1 minute to remove residual wash buffer. The QIAprep 2.0 column was then placed in a clean 1.5 ml microcentrifuge tube and DNA was eluted by adding 50 µl of elution buffer NE. It was allowed to sit for 2 minutes at room temperature then centrifuged for 1 minute and the DNA was collected.

Single stranded phage DNA from the five input phage for each round of panning against oocysts was isolated using a Qiagen spin M13 kit following the manufacturer's instructions. Briefly, 250 µl of the phage were added to sterile eppendorf tubes and 2.5 µl (1/100 dilution) of precipitation buffer was added, mixed and incubated at room temperature for 2 minutes. QIAprep spin columns were placed in microcentrifuge tubes and samples were added. These were then centrifuged for 15 seconds at 8000 xg and the flow through was discarded. 700 µl of the lysing and binding solution was added to the columns twice, incubating for 1 minute the second time and then centrifuged for 15 seconds after each application at 13000 xg. 700 µl of wash buffer was then added to each tube and centrifuged at 8000 xg for 15 seconds. The wash buffer was to prevent residual ethanol from being carried over into subsequent reactions. The QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added to the centre of each tube, incubated for 10 minutes and then centrifuged for 30 s at 13000 xg and stored at -20˚C.

2.3.4.1 Quantification of DNA by Qubit Fluorometer

Estimation of DNA concentration was performed using Qubit 2.0 Fluorometer (Invitrogen, UK) and Qubit reagent dsDNA BR (Broad range, Thermofischer) following the instructions of the manufacturer. Two assay tubes were set up for standards (0 µg and 1 µg of DNA) and one tube for each test sample. The master mix was prepared by diluting 200 µl of buffer and 1 µl of fluorophore dye per tube. To 2 µl of test samples, 198 µl of master mix was added making a total of 200 µl in each tube. The standards; 0 ng/µl and 100 ng/µL respectively were prepared by adding 10 µl of each standard into 190 µL of master mix. The samples were mixed by pipetting and measured using a Qubit Fluorometer (Invitrogen, UK)

2.3.4.2 DNA quantification by Spectrophotometer

Phage DNA (single stranded) were quantified using NanoDropTM ND-8000 spectrophotometer (ThermoScientific, UK) according to the manufacturer's instructions. The software program NanoDrop ND-8000 version 2.2.0 was used. A 2 µl blank was read initially. After that, 2 µl of DNA samples were dispensed onto each lower measurement pedestal and read at 260 nm and recorded.

2.3.4.3 PCR amplification of scFv HCDR3 gene sequences from the Griffin scFv library

The eluates from DNA extractions were quantified either using Qubit fluorometer or using Nanodrop. Working solutions of 10 ng/µl were prepared diluted in DNase/RNase-free deionised free water. The DNA was used as a template for PCR reactions. The PCR reaction master mix with the final concentrations of the PCR components within the 50 µl reactions were prepared as shown in table 2.1.

Two rounds of PCR were conducted for the amplification of the HCDR3 region of the scFv and the generated amplicons had linker and barcode sequences compatible with the Ion Torrent NGS platform as represented in figure 2.2.

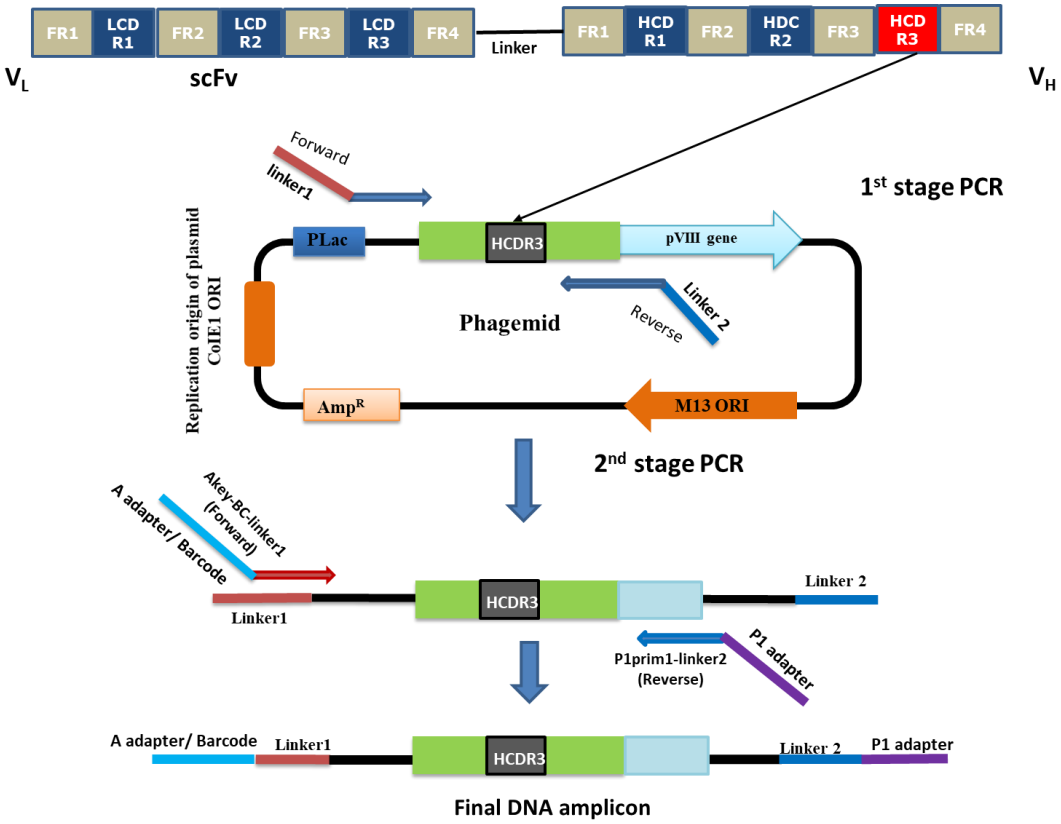


Figure 2:2: A Schematic representation of round 1 and 2 PCR. Prior to the NGS, two rounds of PCR were conducted on all 41 samples generated after extraction of DNA in order to amplify the HCD3 domain and add linker and barcode sequences for each sample. Barcode sequences are required to identify each sample during data analysis.

Primers used for the first round of PCR were VHFR2UniFNEW and JHFR4RevNew while the second round of PCR was conducted using a second set of primers intended to provide unique barcode sequences for the purpose of sample identification and specific sequences, p1 and Akey as shown in table 2.3.

Table 2:2: Reaction master mix for PCR reactions

Reagent	Concentration	Volume (µl)
Nuclease free H_2O	N/A	39.8
Platinum Taq polymerase	5 U/µl	0.2
dNTPs	10mM	1
$MgSO_4$	50mM	2
Forward primer	10 µM	1
Reverse primer	10 µM	1
Platimum Hi Fi buffer	10X	5
Template	10 ng/µl	1

Table 2:3: Primers used for the PCRs

Primer name	**Sequence (5' → 3')**
VHFR2UniFNEW	CTAGAACATTTCACTTACGGVMARGGNCTKGASTGG
JHFR4RevNEW	GTAATCCTTGTGGTATCGTGARGAGACRGTGACCRKKGT
Akey-BC1-lnk1* (Forward)	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAAC-GTAATCCTTGTGGTATCG
P1prim-linker2 (Reverse)	CCTCTCTATGGGCAGTCGGTGATCTAGAACATTTCACTTAC

* The underlined bases are where the barcode region is. Up to 96 barcodes were used to differentiate each sample before being pooled together and sent off for Ion Torrent sequencing.

In the first round PCR, VHFR2UniFNEW and JHFR4RevNew primers as well as a high fidelity Platinum Taq Polymerase (Product No. 11304-011) were used to amplify the HCDR3 domain. The primers were phage-library specific and incorporate linkers to attach barcode adapters in the Round 2 amplification. The primers were complementary to the flanking regions of the Griffin library HCDR3 domain which was the region that was amplified.

The first round of PCR started with an initial denaturing step at 95°C for 3 minutes. Followed by a single PCR cycle of a denaturing step conducted at 95°C for 30 seconds, an annealing step of 55°C for 30 seconds and an extension step of 30 seconds at 68°C. A further 28 cycles were then conducted using a denaturing step at 95°C for 30 seconds, an annealing temperature of 60°C held for 30 seconds and an extension time of 30 seconds at 68°C. After the first round of PCR, the size of the PCR products were determined to be 250-300bp by running 10 µl on a 2% (w/v) agarose/TAE gel at 100 volts for one hour.

PCR products were purified using a Machery-Nagel Nucleospin Gel and PCR purification kit (fisher scientific, Product No. 740609) before carrying out the second round of PCR. The second round of PCR was conducted using a second set of primers intended to provide unique barcode sequences for the purpose of sample identification and specific sequences, p1 and Akey sequences, that are compatible with the Ion Torrent sequencing platform. High fidelity Platinum Taq Polymerase (Product No. 11304-011) was used. The Round 2 PCR products had total volumes of 50 µl and the same reagents and concentrations were used as round 1. The template DNA for round 2 PCR was 5 µl of the purified round 1 PCR products. For round 2

PCR, an initial denaturing step was conducted at 95°C for 3 minutes. Afterwards, 25 cycles involving a denaturing step at 95°C for 30 seconds, an annealing time of 30 seconds at 63°C and an extension time of 30 seconds at 68°C were conducted. The size of the amplified round 2 products were determined by running on a 2% (w/v) agarose/TAE gel at 100 volts for one hour and the amplicon size was expected to be ~350-400bp.

Individual round 2 PCR products were quantified using Qubit florescence and then pooled, ensuring an equal contribution from each sample towards a total of 3000 ng. The pooled DNA sample was then purified using Agencourt AMPure XP kit and re-quantified. When it was re-quantified 68% was recovered after purification. Samples were sent for sequencing using the commercial service offered by the University of Pennsylvania using the high-throughput Ion Torrent platform with a 318 chip.

2.3.4.4 DNA visualization using agarose gel electrophoresis

DNA was visualized using 2% (w/v) agarose gel electrophoresis to confirm the amplicon of interest had the expected size. Preparation of a 2% (w/v) agarose gel was carried out by measuring 2 grams of agarose (Fisher Scientific, UK) and adding to 100 ml of 1x TAE Buffer and was then microwaved for 3 minutes until it reached boiling temperature. After heating, 3 µl per 100 ml of Nancy–520 (Sigma-Aldrich, UK) was added and mixed to ensure that it is uniformly distributed in the matrix and the agarose gel solution was then poured into casting trays with an appropriate comb and allowed to set for 30 min at RT. After solidification, the comb was removed and the gel was put into a running tank containing 1× TAE buffer, the gel was completely immersed in the buffer. The DNA samples were mixed with tracking dye (Thermo Fisher scientific) and were loaded alongside a molecular marker, normally Low Range DNA Ladder (Thermo Scientific, UK). The agarose gel was run at a constant voltage of 100 V/cm for 50 min. The bands were subsequently visualized using a Biorad ChemiDoc ^{TM} (SV00843) and the gel photographed.

Alternatively, to prepare a 3% MetaPhor Agarose gel; high resolution agarose, 3 grams of MetaPhor agarose were added to 100 ml of 1x TAE Buffer and microwaved until it boiled and completely dissolved. After that, 3 µg/ml of Nancy–520 was added and mixed and the agarose gel solution was then poured into casting trays allowed to set for an hour at RT. The gel was then placed at 4°C for approximately 2 hours to obtain optimal resolution and gel handling characteristics. Samples were loaded and electrophoresed as described above. The general method used for NGPD is represented in figure 2.3 below.

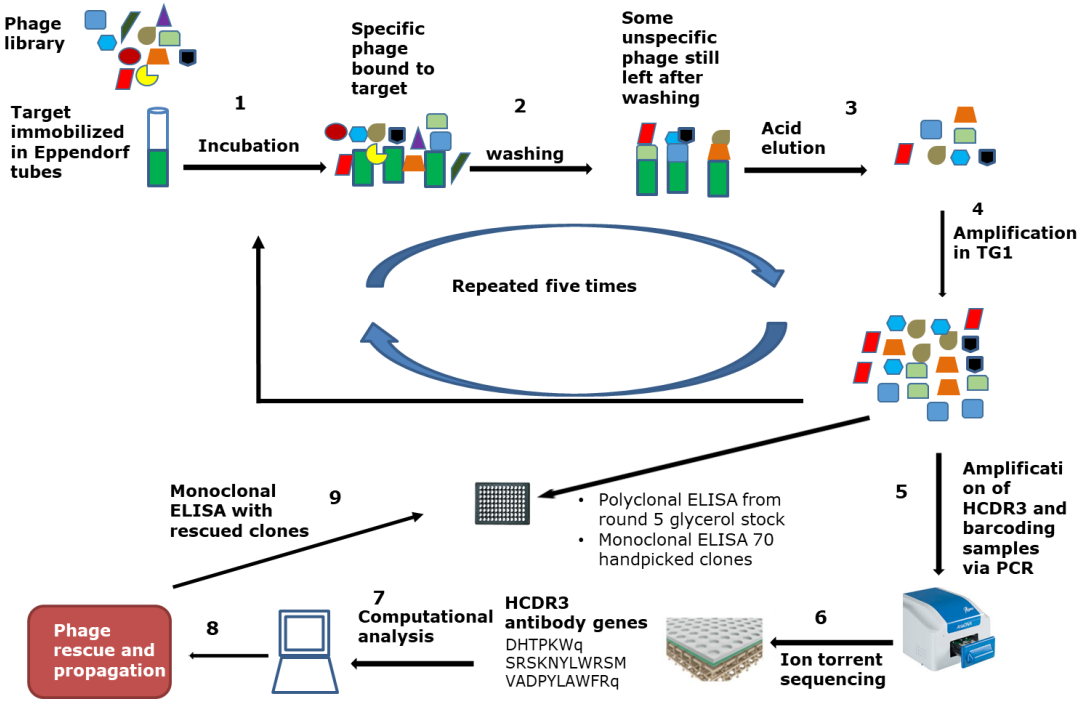


Figure 2:3: The method used for Next generation phage display 1) The phage library containing billions of different phage was incubated with the target immobilized in eppendorf tubes. 2) Non binders were removed by washing. 3) Bound phage are eluted from the antigen using midlog TG1 cells. 4) They were then amplified in bacteria which were used for further rounds of panning; steps were repeated 5 times to increase enrichment to the target. 5) The HCDR3 genes are amplified by PCR. 6) The HCDR3 region was deep sequenced. 7) Data was analysed computationally. 8) Enriched clones are identified, rescued and propagated. 9) ELISA was carried out to determine if they are true binders (polyclonal and monoclonal ELISAs)

2.3.5 Conventional screening for scFv phage binders

2.3.5.1. Screening against immobilised oocyst lysate

2.3.5.1.1 Antigen preparation

Lysing of oocysts

Glass beads (Stratech) of 1 mm diameter were added to pre-weighed 2 mL centrifuge tubes up to 1 mL mark. To the pre-weighed 2ml tubes containing glass beads 500 µl of each of the seven oocyst species in solution were added. Oocysts homogenisation was carried out at 2400 oscillations/minute for 1 minute using a mini bead beater (Stratech). The oocyst homogenates were then separated into aliquots for storage at -20°C.

Bradford assay for protein quantification

The concentration of protein in the lysed oocysts was measured according to the Bradford assay (Bradford 1976). The Bradford method is based on the ability of a Bradford reagent solution to bind protein and when bound to absorb light at 595 nm. The linear calibration curve was made with Bovine Serum Albumin (BSA) (Sigma-Aldrich, UK, Cat.No.23200). A 2 mg/ml BSA solution was prepared in PBS and this was used to make a calibration standard curve of serial dilutions such as 0, 0.25, 0.5, 1.0, 1.4, and 2.0 mg/ml. 50 µl of each of the standard dilutions was mixed in a cuvette with 1.5 ml of Bradford reagent (Sigma-Aldrich, UK, Cat. No B6916). These were incubated for 5 minutes at RT, then the optical density at 595 wavelength was measured using a Biochrom Libra S22; 190-1100nm spectrophotometer (Scientific Laboratory supplies, UK) and protein concentration was assessed by comparing the absorbance value of unknown samples to the calibration BSA standard curve.

2.3.5.1.2 Polyclonal phage ELISA

Nunc Maxisorb® plates were coated with of 20 µg/ml (by protein) of pooled oocyst lysate in carbonate buffer (100 mM pH 9.6) overnight. No antigen control plates were coated with only carbonate buffer overnight. The following day, the coating solution was discarded and the wells were washed three times with 400 µl of 3X PBS. The wells were thereafter blocked for one hour with 3% (w/v) marvel in PBS; 400 µl was used per well. The phage supernatant was also blocked for 1 hour with 200 µl of 18% (w/v) marvel in 6X PBS per ml of phage supernatant per ml.

The plate blocking solution was discarded and the plate washed once with 400 µl 1X PBS per well. Blocked phage supernatant; 100 µl from the phage library and all five rounds of panning were incubated per well for 1 hour. Anti-M13-HRP (1:5000) ratio

of antibody to PBS was blocked with 3% (w/v) marvel for 1 hour. The plates were washed 5 times with 400 µl 3X PBST containing 0.1% (v/v) Tween 20. The plates were thereafter washed 5 times with 400 µl 3X PBS. 100 µl of anti-phage primary antibody (Anti M13 conjugate; GE healthcare) was added per well and incubated for 1 hour. This bound to the bacteriophage displaying the scFv, provided its scFv was bound to the immobilised target on the plate. The plates were washed 3 times with 1x PBST containing 0.1% (v/v) Tween 20 and 3 times with PBS. 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) was added per well and incubated for ~15-30 minutes or until the desired colour change was reached. The absorbance was read with a Microplate reader (Labtech LT-4000) at 630nm when blue or the reaction was stopped using 2 M Sulphuric Acid 100 µl and absorbance read at 450nm.

2.3.5.1.3 screening colony picks

From round 5 glycerol stock of phagemid isolated against each of the seven *Eimeria* species, 2YT agar plates were streaked and incubated overnight at 37°C. The following day, the plates were stored at 4°C until required for monoclonal ELISA.

2.3.5.1.4 colony picks ELISA

Phage were grown and 70 single clones picked (10 from panning against each *Eimeria* species). Briefly 300 µl of prewarmed 2YT media (Sigma Aldrich, UK) containing 150 µg/ml ampicillin and 1% (w/v) glucose was added to microtitre 96 deep well plates (Alpha laboratories LW9445). To the culture single colonies were added. This was incubated and allowed to grow to mid log at 37°C with shaking at 200 rpm. After 2 hours, exphage was added and then the culture was incubated for 30 minutes static at 37°C and another 30 minutes shaking at 37°C. The plate was thereafter spun down at 3000 xg for 10 minutes and the pellet resuspended in 300 µl of 2YT media containing only Ampicillin 150 µg/ml and Kanamycin 50 µg/ml shaking at 180 rpm. The next day, the plate was spun down at 3000 xg for 10 minutes and phage supernatant collected.

Nunc MaxiSorp ® plates were coated with 20 µg/ml of lysed oocyst antigen of each species overnight. Monoclonal phage ELISA was carried out by incubating each phage grown from panning against a particular species with the corresponding lysate using the same protocol described in the polyclonal phage ELISA (section 2.3.5.1.2).

2.3.5.1.5 Assessment of clonal diversity of colony picks by BstNI digestion patterns

Phage DNA was isolated and purified from each of the 70 hand-picked clones using Qiagen Spin Mini Prep Kit (Product No. 27104) as described in section 2.3.4. The

PCR reaction master mix was prepared and the final concentrations of the PCR components within the 25 µl reactions were as shown in table 2.4.

Table 2:4: PCR master mix for BstNI digestion patterns

Reagent	Concentration	Volume (µl)
Sterile water	N/A	38.5
Taq DNA polymerase	5 U/µl	0.5
dNTPs	10mM	1
$Mgcl_2$	50mM	2
Fdtetseq forward primer	10 µM	1
pU19 reverse primer	10 µM	1
Taq polymerase buffer	10X	5
Template	10 ng/µl	1

The reactions were placed in the thermocycler for 30 cycles of 94°C for 1 min initial denaturation, 55°C 1 min annealing, 72°C for 2 min extension, followed by a final extension for 10 min at 72°C. The products (3-5 µl) were analysed by electrophoresis on 2% (w/v) agarose gel. The presence of an amplicon of 1kb was determined. The remaining PCR reactions were made up to 1 x BstNI buffer, 100 µg/ml BSA and 2-5 units of BstNI. This was incubated at 60°C for 2 hours. Digestion products were resolved on a 3% (w/v) agarose gel.

2.3.5.1.5 Sanger sequencing of single clones

Twenty-three (1/3rd) of the single clones were randomly selected and sent off for Sanger sequencing carried out by Source Bioscience. 5 µl of 90-100 ng/µl of plasmid DNA was sent for sequencing with the primer VHFR2UniFNEW. This was carried out to assess clonal dominance which is characteristically seen in the last rounds of most panning experiments that carry out high numbers of rounds.

2.3.6 NGS Data Analysis: for identifying scFv binders to oocysts

NGS data was downloaded as fastq.tgz archive file and was decompressed to fastq file before using perl scripts to process NGS data files. It was demultiplexed in order to sort the scFv sequences according to their barcode identifiers generating an individual fastq file for each barcoded sample. Then each barcode fastq file was converted into a fasta file. The Griffin library was sequenced in the reverse direction hence it was reverse complemented before translation. The DNA sequences were

then translated in each of the 3 reading frames into the amino acid sequence. The three reading frames were concatenated into one file (translate.pl, Appendix II) and using a script that searches through the fasta files (iterate.pl, Appendix III), the reads were sifted to only include those that contained the VYYCAR and WGQGT phagemid motifs, which are the left and right HCDR3 flanking regions respectively. The HCDR3 amino acid sequence was between the flanking regions was then used to identify individual scFv clones. Parameters used to analyse the data were as follows; not more than 1 degeneracy was allowed in each of the left and right flanking region amino acid sequence, and a minimum of 4 amino acids were required in the HCDR3 sequence. All amber stop codons were replaced with amino acid Q for subsequent analysis. Z scores were generated for each scFv based on the frequency and ratio of binding as described previously by (Faver *et al.,* 2019, Naqid *et al.,* 2016b, Zhang *et al.,* 2011). The Z scores define relative enrichment for each scFv and in this case the comparison was carried out through progressive rounds of panning, i.e. scFv enrichment against each individual *Eimeria* species (when analysing phagemid sequences from bacterial glycerol stocks after each round of panning) from round 1 to round 2, round 2 to round 3, round 3 to round 4, and round 4 to round 5 were calculated. The scFvs were then selected based on those that had more than 5 reads, and a Z score of >5 in at least two panning rounds such that those scFvs which didn't meet these criteria were discarded. A script was written to pool together barcodes of the phagemid sequences in bacterial stocks for each of the seven species, hence 5 output phagemid were pooled across species for each of the five rounds of panning. Input phage for each round of panning were pooled before panning hence there was only one sample for each species input phage. A comparison was then made between grown pooled input phage for each round of panning and the pooled bacterial stock (output) which served to compare growth only phases (comparing the output glycerol stock of one round to the subsequent phage input of the next round) and selection+growth phases (comparing the input phage to the subsequent bacterial glycerol stock) characteristics in each round of panning. The pools were from panning against all seven *Eimeria* species. This was aimed at identifying the scFv's that were enriched during selection in each round of panning and not just those that had growth advantage. This is because a major problem encountered in library screening is the enrichment of false positives that have no actual affinity to the target but possess growth advantages over other clones, amplification of the library which is carried out between rounds of biopanning may select such clones (Bakhshinejad *et al.,* 2016). It was based on these criteria that the list of scFv's for further characterisation were generated.

2.3.7 scFv clone rescue

The primers that were used to rescue specific scFv were designed based on a universal framework 4 sequence and the unique heavy chain CDR3 sequence of enriched scFv (Figure 2.4) based on the method by (Spiliotopoulos *et al.,* 2015). The framework 4 region and HCDR3 sequences were amplified in an inverse PCR reaction.

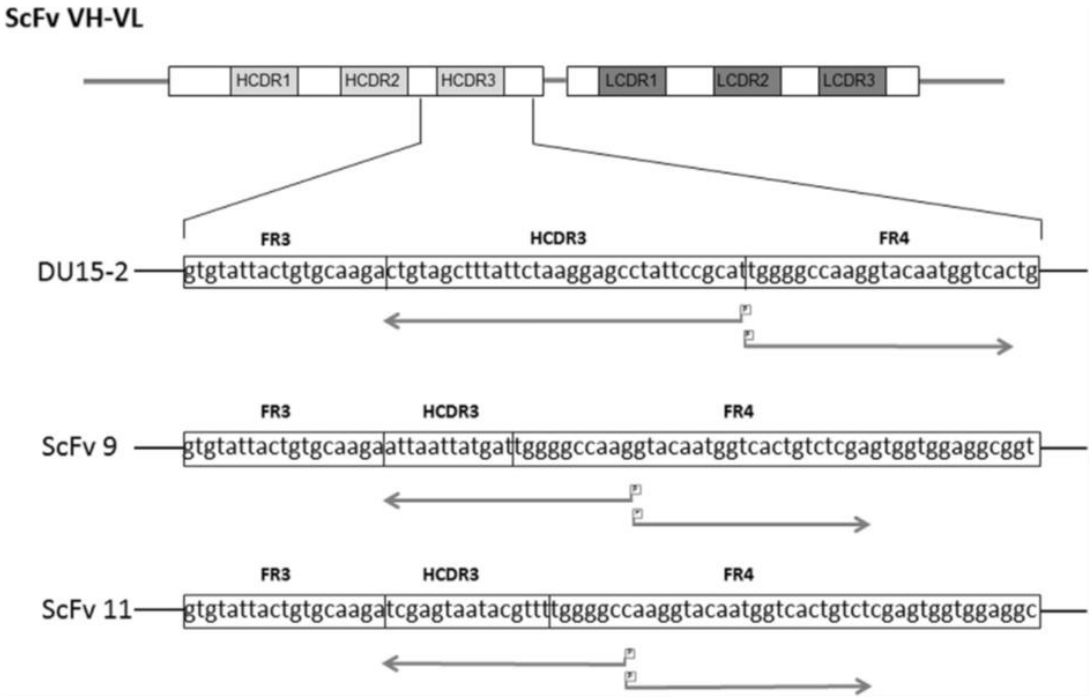


Figure 2:4: ScFv rescue using inverse PCR. Primers were designed to anneal to the heavy chain HCDR3 region and framework 4 regions of the clone of interest and amplify the entire phagemid using inverse PCR. The reverse primer spans the entire HCDR3 region and its forward primer pair targets the framework 4 region. In instances in which the HCDR3 were less than 6 amino acids long, the reverse primers encompass the whole HCDR3 and extended into the framework 4 region and the forward primer redesigned to anneal further downstream in the FR4 region (Spiliotopoulos *et al.*, 2015).

2.3.7.1 Optimisation of Inverse PCR

Inverse PCR for the rescue of Griffin NGS clones was optimised using different conditions. Annealing temperatures, primer concentrations and template concentration were all optimised. This included gradient PCR in which different annealing temperatures were tested to find the best annealing temperatures for the primers.

Rescues were performed using the 10 ng/µl DNA from bacterial glycerol stocks of the samples with the highest enrichment (assessed by Z score as described in 2.3.6) for

the individual scFv to be rescued. Inverse PCR was conducted using; 0.5 µl Q5 DNA Polymerase (NEB), 2 µl dNTP mix, 10 µl GC enhancer buffer, 10 µl 5x Q5 reaction buffer, 2.5 µl of Forward primer at 10 µM, 2.5 µl of Reverse primer at 10 µM, 3 µl of DNA template and 19.5 µl molecular biology grade water to make up a final volume of 50 µl. N.B. primers were designed for each clone to be rescued according to the specific HCDR3 sequence and framework 4 sequence and are detailed in the results section.

The inverse PCR started with an initial denaturing reaction conducted at 95°C for 2 minutes followed by 15 cycles of 95°C for 30 seconds, 50°C for 30 seconds and an extension time of 5 minutes at 72°C. The following 25 cycles involved a denaturing time of 30 seconds at 95°C, 30 seconds annealing time at 50°C and an extension time of 5 minutes at 72°C.

Alternatively, asymmetric PCR was also carried out in which specific HCDR3 primers were added initially and after 10 cycles, universal framework 4 primers was added. Primer details are provided in the results section.

Another alternative method was also carried out, PCR was carried out with each scFv to be rescued having an annealing temperature 10°C lower than the Tm of the HCDR3 primer in the first 10 cycles and then the temperature was increased to 5°C lower than each scFv HCDR3 primer Tm in the last 18 cycles. This was carried out to look at various annealing temperatures and find the optimum annealing temperature.

The primers for the framework 4 and the HCDR3 region had been phosphorylated at the 5' end to allow a ligation reaction using T4 Ligase (M0202S) to produce circular DNA (Table 2.5). The ligation reaction was conducted at 12°C overnight. The following day, the T4 ligase was deactivated by heating the samples to 65°C for 20 minutes. The ligation reactions were then microdialysed by placing on 0.025 µm filters (Product No. VSWP02500) and left on the surface of pure water (Product No. 10011342) for 15 minutes before pipetting into a sterile eppendorf tube. From the ligation reaction of each scFv rescue, 2 µl was then used to transform 50 µl of electro-competent TG1 cells (Product No. 200123, Agilent) using electroporation cuvettes with a 0.1 cm gap (Product No. 10573273, Invitrogen). Electroporation was conducted using a BioRad GeneP Pulsar Xcell Electroporator using a pre-set exponential decay protocol for bacteria. After electroporation, cuvettes were placed on ice immediately until 950 µl of pre-warmed SOC recovery medium (B9020S) was added to each sample. The samples were then transferred into 15 ml falcon tubes and were then allowed to recover for 45 minutes to 1 hour at 37°C, shaking at 200 rpm. Following incubation, 100 µl was then spread on 2YT plates with 1% (w/v) glucose and 150 µg/ml ampicillin and were allowed to grow static overnight at 37°C. A positive control

using 2 µl of the plasmid pUC19 (400 Units) (N3041S) and a no-ligase negative control were also used to transform cells.

The following day 3 colonies from each plate were grown overnight in 10ml 2YT, 150µg/ml ampicillin and 1% (w/v) glucose before being stored in 30% (w/v) glycerol stocks at -80. From the transformed cultures grown overnight, plasmid DNA was isolated for each sample using a Qiagen Spin Mini Prep Kit (Product No. 27104) following the manufacturer's instructions. The DNA extracts were quantified using the Qubit fluorometry and 5 µl of each sample at a concentration of 100 ng/µl, were sent for Sanger Sequencing to confirm the HCDR3 sequence.

Table 2:5: Inverse PCR primer pairs designed for phagemid rescues for selected scFvs

scFv	**Specific primers 5' 3'**	**Framework 4 primers 5' 3'**
AWTVNQTTNY	5' PHO- ATAATTCGTAGTCTGATTAACAGTCCACGC-3'	5' PHO- TGGGGCCAAGGTACCCCGGTCACCGTCTCT-3'
DGKPPSG	5' PHO- CCCACTAGGCGGCTTCCCATC-3'	5' PHO- TGGGGCCAAGGTACCCCGGTC-3'
DKSSqGT	5' PHO- CGTACCCTACGACGACTTATC-3'	5' PHO- TGGGGCCAAGGTACCCCGGTC-3'
DRSYTACDTSK	5' PHO- CTTAGAAGTATCACACGCCGTATAACTCCTATC-3'	5' PHO- TGGGGCCAAGGTACAATTGGTCACCCGTCTCTT-3'
EDRqCKHG	5' PHO- ACCATGCTTACACTACCGATCCTC-3'	5' PHO- TGGGGCCAAGGTACCCTGGTCACT-3'
ESSqMNS	5' PHO- CGAATTCATCTAAGAAGACTC-3'	5' PHO- TGGGGCCAAGGTACCCCGGTC-3'
VAADPFDY	5' PHO- ATAGTCAAAAGGATCAGCAGCAAC-3'	5' PHO- TGGGGCCAAGGTACACCGGTCACT-3'
YIRYTSPV	5' PHO-AACCGGCGAAGTATAACGAATATA-3'	5' PHO-TGGGGCCAAGGTACCCCGGTCACC-3'
STQHP	5' PHO-CCACGGATGCTGAGTCGA-3'	5' PHO-GGCCAAGGTACAACGGTC-3'
SSDAP	5' PHO- CCACGGAGCATCCGAAGA-3'	5' PHO- GGCCAAGGTACCCCGGTC-3'
SPRLPFDY	5' PHO-ATAGTCAAACGGCAGCCTCGGACT-3'	5' PHO-TGGGGCCAAGGTACCACGGTCACC-3'
QTGMN	5' PHO-CCAATTCATCCCAGTCTG-3'	5' PHO-GGCCAAGGTACCCCGGTC-3'
LMVYKH	5' PHO-ATGCTTATACACCATAAG-3'	5' PHO-TGGGGCCAAGGTACCCCG-3'
KDVGVVPK	5' PHO- CTTAGGCACCACACCAACATCCTT-3'	5' PHO- TGGGGCCAAGGTACACCGGTCACC-3'
IHRMVYL	5' PHO-CAAATAAACCATCCGATGAAT-3'	5' PHO-TGGGGCCAAGGTACCCCGGTC-3'
HPMLGGLQGK	5' PHO- CTTACCCTGCAGCCCCCCAAGCATAGGATG-3'	5' PHO- TGGGGCCAAGGTACCCCGGTCACCGTCTCC-3'
SRSKNYLWRSM	5' PHO- CATAGAACGCCAAAGATAATTCTTACTACGACT-3'	5' PHO- TGGGGCCAAGGTACCCCGGTCACCGTCTCCTCA-3'
RDLTqDMWR	5' PHO-CCGCCACATATCCTAAGTCAAATCCCT-3'	5' PHO-TGGGGCCAAGGTACACCGGTCACCGTC-3'
TTALP	5' PHO-CCAAGGCAGCGCCGTAGT-3'	5' PHO-GGCCAAGGTACCACGGTC-3'
SAqHP	5' PHO- CCAGGGATGCTACGCCGA-3'	5' PHO- GGCCAAGGTACACTGGTC-3'

2.4 An alternative NGS analysis to enhance recovery of species-specific clones

The NGS data for scFv panning was reanalysed using different scripts in an attempt to prioritise the selection of species-specific scFvs to whole oocysts.

A script was written to select for each scFv that was enriched in a particular species but not enriched in the other six, hence each species of *Eimeria* oocyst was analysed versus the rest and Z scores were calculated for this comparison (find_species1_vs_the_rest.pl-find_species7_vs_the_rest.pl, Appendix IV). The Z scores were ranked in a descending order from those that had the highest to the lowest Z scores. The scFvs that had been isolated previously from the colony picks and the initial NGS analysis that compared selection and growth were removed. Also, scFvs that showed enrichment to one or two other species were removed leaving only those that were enriched in only one species compared to the others. Motifs with sequences that had one or two amino acid changes were identified which could be represented in multiple data sets (for the different *Eimeria* species) but likely bind the same epitope. Once these were identified in datasets for multiple species, they were removed. A list was drawn up, according to the scFvs that had the highest Z score which is a measure of enrichment and were only found in datasets for panning against a single species. The top 10 sequences in the list were targeted for each species, totalling 70 scFvs for rescue. Primers for scFv rescue by inverse PCR were designed as previously (Table 2.6).

Table 2:6: Primers used to rescue species-specific clones

Species	Sequence	Framework 4 universal primers 5'3	HCDR3 Specific primers 5' 3
E. mitis	SEYCEKTPGG	Universal	5'PHO-CCCACCAGGAGTCTTCTCACAATACTCCGA
E. mitis	ANACID	Universal	5'PHO-ATCAATACACGCATTAGC
E. mitis	RWRAMR	Universal	5'PHO-CCGCATAGCACGCCAACG
E. mitis	CGNVN	GGCCAAGGTACCCTGGTC	5'PHO-CCAATTAACATTACCACA
E. mitis	TCTTLPIGLQA	Universal	5'PHO-AGCCTGAAGCCCAATCGGCAACGTCGTACAAGT
E. mitis	DDTINVEV	Universal	5'PHO-CACCTCAACATTAATCGTATCATC
E. mitis	LWRNYYSS	Universal	5'PHO-ACTACTATAATAATTCCTCCAAAG
E. mitis	KHICRFDY	Universal	5'PHO-ATAGTCAAACCTACAAATATGCTT
E. mitis	PHYVLM	Universal	5'PHO-CATCAACACATAATGCGG
E. mitis	VWSTESI	Universal	5'PHO-AATACTCTCCGTACTCCACAC
E. necatrix	AFEWKTRVLF	Universal	5'PHO-AAACAAAACACGAGTCTTCCACTCAAAAGC
E. necatrix	GYRFCHNSRD	Universal	5'PHO-ATCCCGCGAATTATGACAAAACCTATAACC
E. necatrix	RQTKYWYDCSD	Universal	5'PHO-ATCCGAACAATCATACCAATACTTCGTCTGCCG
E. necatrix	RRPDLLM	Universal	5'PHO-CATAAGCAAATCAGGCCGCCG
E. necatrix	ELPPTPITS	Universal	5'PHO-CGAAGTAATCGGAGTCGGCGGAAGCTC
E. necatrix	FEWRPPTSCQG	Universal	5'PHO-ACCCTGACACGAAGTAGGAGGCCGCCACTCAAA
E. necatrix	LESTSV	Universal	5'PHO-CACACTCGTACTCTCAAG
E. necatrix	CWKGQG	Universal	5'PHO-CCCCTGCCCCTTCCAACA
E. necatrix	VNPTKFDY	Universal	5'PHO-ATAGTCAAACTTCGTAGGATTAAC
E. necatrix	WVPAGWY	Universal	5'PHO-ATACCAACCAGCCGGCACCCA
E. acervulina	ARWRAEK	Universal	5'PHO-CTTCTCAGCACGCCAACG
E. acervulina	FCVSHFDY	Universal	5'PHO-ATAGTCAAAATGAGAAACACAAAA
E. acervulina	PSYWRFDY	Universal	5'PHO-ATAGTCAAAACGCCAATAAGAAGG
E. acervulina	ICSPRATRRQ	Universal	5'PHO-CTGCCGCCGCGTAGCCCGCGGACTACAAAT
E. acervulina	DQHWQPLAMS	Universal	5'PHO-AGACATAGCCAAAGGCTGCCAATGCTGATC
E. acervulina	EKTWDFDY	Universal	5'PHO-ATAGTCAAAATCCCAAGTCTTCTC
E. acervulina	ASNCERE	Universal	5'PHO-CTCCCGCTCACAATTCGACGC

E. acervulina	CAPEE	GGCCAAGGTACCCTGGTC	5'PHO-CCACTCCTCAGGAGCGCA
E. acervulina	QQNENSD	Universal	5'PHO-ATCAGAATTCTCATTCTGCTG
E. acervulina	RDQSHKQEMS	Universal	5'PHO-AGACATCTCCTGCTTATGACTCTGATCCCT
E. brunetti	NHMRTFDY	Universal	5'PHO-ATAGTCAAACGTCCGCATATGATT
E. brunetti	KWNSGRDAD	Universal	5'PHO-ATCAGCATCACGACCAGAATTCCACTT
E. brunetti	APRWDI	Universal	5'PHO-AATATCCCACCGCGGAGC
E. brunetti	FAAMCDDV	Universal	5'PHO-CACATCATCACACATAGCCGCAAA
E. brunetti	TGVENWTL	Universal	5'PHO-CAAAGTCCAATTCTCCACCCCGGT
E. brunetti	MAEPPLTTHT	Universal	5'PHO-CGTATGAGTAGTCAAAGGAGGCTCCGCCAT
E. brunetti	HSQVPAVAPqF	Universal	5'PHO-AAACTAAGGAGCCACCGCCGGCACCTGCGAATG
E. brunetti	IWGPQFDY	Universal	5'PHO-ATAGTCAAACTGAGGACCCCAAAT
E. brunetti	RADRASF	Universal	5'PHO-AAAACTAGCCCTATCAGCCCT
E. brunetti	RAILSRNGLHC	Universal	5'PHO-ACAATGAAGCCCATTCCTAGACAAAATAGCCCT
E. maxima	SPRMF	GGCCAAGGTACCCCGGTC	5'PHO-CCAAAACATACGCGGAGA
E. maxima	NRYSKT	Universal	5'PHO-AGTCTTACTATACCTATT
E. maxima	RLFQVE	Universal	5'PHO-CTCCACCTGAAACAACCT
E. maxima	WRMPTP	Universal	5'PHO-AGGCGTAGGCATACGCCA
E. maxima	ENTMRGFGL	Universal	5'PHO-AAGCCCAAAACCACGCATAGTATTCTC
E. maxima	FMVGDG	Universal	5'PHO-CCCATCACCAACCATAAA
E. maxima	VEGPRFDY	Universal	5'PHO-ATAGTCAAACCGCGGCCCCTCCAC
E. maxima	ASRDPKMNQVL	Universal	5'PHO-CAGCACCTGATTCATCTTCGGATCCCGACTAGC
E. maxima	MTTVFR	Universal	5'PHO-CCTAAACACAGTCGTCAT
E. maxima	SFTLVMDHPS	Universal	5'PHO-CGACGGATGATCCATAACCAGAGTAAAAGA
E. praecox	KELIQPTV	Universal	5'PHO-AACAGTCGGCTGAATAAGCTCCTT
E. praecox	YTRKRFRRPER	Universal	5'PHO-CCTCTCAGGACGCCGAAACCGCTTACGCGTATA
E. praecox	PWPTPNQ	Universal	5'PHO-CTGATTAGGAGTAGGCCACGG
E. praecox	SGSPPFDY	Universal	5'PHO-ATAGTCAAACGGAGGAGACCCAGA
E. praecox	LRNGKSHMMRTV	Universal	5'PHO-AACAGTCCTCATCATATGAGACTTACCATTCCGAAG
E. praecox	PAGSRTL	Universal	5'PHO-CAACGTCCGAGACCCAGCCGG
E. praecox	HRPKQGKA	Universal	5'PHO-AGCCTTACCCTGCTTAGGCCTATG
E. praecox	RFIMSHS	Universal	5'PHO-ACTATGAGACATAATAAACCT

E. praecox	TRLRPTNLKV	Universal	5'PHO-AACCTTCAAATTAGTAGGCCTCAAACGAGT
E. praecox	LNTNDV	Universal	5'PHO-CACATCATTCGTATTCAG
E. tenella	KRAHGRNEPA	Universal	5'PHO-CGCAGGCTCATTACGACCATGAGCCCGCTT
E. tenella	LVNRSSNEQG	Universal	5'PHO-ACCCTGCTCATTAGAAGAACGATTAACAAG
E. tenella	LAPSLSERPV	Universal	5'PHO-CACAGGCCGCTCAGACAAAGAAGGAGCCAG
E. tenella	IGPSA	GGCCAAGGTACCCCGGTC	5'PHO-CCAAGCAGAAGGCCCAAT
E. tenella	SHQTqWQSSIAE	Universal	5'PHO-CTCAGCAATCGAAGACTGCCACTAAGTCTGATGCGA
E. tenella	RILDLDT	Universal	5'PHO-CGTATCAAGATCCAGAATCCT
E. tenella	ASYRGH	Universal	5'PHO-ATGACCCCTATAAGACGC
E. tenella	TIAIGFDY	Universal	5'PHO-ATAGTCAAAACCAATCGCAATAGT
E. tenella	GGFSMTGLF	Universal	5'PHO-AAACAAACCAGTCATCGAAAAACCACC
Framework 4 Universal primer 5'3			5'PHO-TGGGGCCAAGGTACMMYGGTCACYGTCTC

2.5 Sub cloning Griffin scFv genes into the PMAL-p5x expression vector

From the traditional picking of clones, and the initial analysis of the NGS data and subsequent rescue of clones, five scFvs showed binding to all or some of the oocyst species. These were cloned from the Griffin vector into pMAL-p5X vector. This was carried out to produce scFv as a maltose binding protein (MBP) tag-scFv fusion by using the NdeI or NcoI site along with a downstream NotI site in the multiple cloning site to transfer the scFv to the pMAL-p5X vector.

Briefly, 500 ng of vectors (Griffin and pMAL) were digested using 1 µl (10 units) of restriction enzymes NcoI (R0193S), 0.5 µl of restriction enzyme NotI HF (R3189S) adding CutSmart® Buffer to make up to 20 µl. The reaction was incubated for 1 hour at 37°C. The enzymes were then heat inactivated by incubating at 80°C for 20 minutes. Complete digestion of the vectors was checked by running the entire reaction on a 1.2% agarose gel at 90 volts for 1 hour. Uncut versions of the vectors were analysed alongside to compare for both P5x and Griffin vectors. The vector (p5X) and scfv gene (Griffin vector) were gel purified by cutting the correct bands from the agarose gel. The digests were gel purified using Machery-Nagel kit and quantified using Qubit fluorometry. Following quantification of digests, 40ng of gel purified P5X vector was mixed with 20ng of gel purified scfv gene and molecular water was added to make up to 10µl. To that, 2X quick ligation buffer (M2200S) was added and Quick T4 DNA ligase (M2200S) and the sample incubated for 10 minutes at room temperature. 2 µl of the ligation reaction was mixed with 50 µl of electro-competent ER2523 then pulsed using a pre-set exponential decay protocol for bacteria; 25 uF, 200ohm, 1800 volts, 0.1 cm gap. After electroporation, cuvettes were placed on ice immediately until 950 µl of pre-warmed SOC recovery medium (B9020S) was added to each sample. The samples were then transferred into 15 ml falcon tubes and were then allowed to recover for 45 minutes to 1 hour at 37°C, shaking at 200 rpm. Following incubation, 100 µl was then spread on 2YT plates with 1% (w/v) glucose and 150 µg/ml ampicillin and were allowed to grow static overnight at 37°C. A positive control using 2 µl of the plasmid pUC19 (400 Units) (N3041S) and a no-ligase negative control were also used to transform cells. The following morning, 3 colonies were picked from each plate and grown overnight. The overnight cultures were then miniprepped and the DNA extracts were Sanger sequenced to confirm the clone. Sub cloning into pMAL-p5X may produce higher level expression than from the Griffin library and also allows purification of the scFv-MBP fusion by amylose affinity chromatography (Simabuco *et al.,* 2007). The aim was to produce soluble scFv that will then be used to test the individual specificities of the scFv.

2.5.1 Affinity Chromatography of expressed scFv-MBP fusion protein

2.5.1.1 Periplasmic extraction of expressed protein

Cells containing the fusion plasmid were grown overnight in 10 ml Lysogeny broth (LB) media (5g tryptone, 2.5g yeast, 2.5g NaCl per 500 ml) containing 0.5% (v/v) glucose and 150 µg/ml ampicillin at 37°C shaking at 180 rpm. The next morning, one litre LB media containing 0.5% (v/v) glucose and 150 µg/ml ampicillin were inoculated with the overnight culture. This was grown to an optical density of 0.5 (2-4 X 10^{8} cells/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration 0.3 mM. The cells were then incubated at 37°C for 2 hours. The cells were harvested by centrifugation at 4000 g for 20 minutes and the supernatant was discarded. The cells were resuspended in 400 ml 30 mM Tris-HCL containing 20% sucrose at pH of 8.0; the pellet was weighed and resuspended in 80 ml per pellet. Ethylene diamine acetic acid (EDTA) was added to 1 mM and incubated for 10 minutes at room temperature shaking. The sample was centrifuged at 8000 X g for 20 minutes at 4°C and the supernatant removed. The pellet was resuspended in ice cold 5 mM $MgSO_4$ for 10 minutes while stirring in an ice bath. The sample centrifuged at 8000g for 20 minutes at 4°C and supernatant, which is the cold osmotic shock fluid, collected.

Affinity purification of scFv MBP fusion proteins utilizes the natural affinity of MBP to maltodextrin. Briefly, to the osmotic shock fluid, 8 ml of 1 M Tris-HCl pH 7.4 was added. For each of the five different clones of scFv-MBP fusions, different 2.5 x 10 cm columns were obtained. Amylose resin was poured in each of the columns and the columns were washed with 5 column volumes of column buffer (50 mM Tris-HCL pH8, 200 mM NaCl, 1 mM EDTA).

Crude extract was loaded to the column at a flow rate of 1 ml per minute. The columns were thereafter washed with 12 column volumes of column buffer at a rate of 2 ml per minute. The fusion protein was eluted with column buffer + 10 mM Maltose using gravity. The eluate was collected in 6 fractions of 1 ml each and it was then quantified using Bradford protein assay.

2.5.2 Analysis of scFv-MBP Expression

Osmotic shock fluid containing scFv-MBP samples was mixed 1:1 with 2X LDS/5% β-mercaptoethanol and boiled in a heat block for 3-5 minutes and 15 µl analysed on a 12% (w/v) Bis Tris SDS-PAGE gel using Magic Marker ladder, an scFv-MBP positive control and negative MBP-only control. Proteins from the gel were transferred to polyvinylidene fluoride (PVDF) membrane using the NuPAGE blotting apparatus.

After this was run for 45 minutes at 25 volts, the PVDF paper was rinsed in water and blocked 5% Marvel in PBS or Tris Buffer then left in the fridge overnight at 4°C. Anti-MBP antibody; 1: 10000 was diluted in blocking buffer, 3% (w/v) Marvel and then incubated for 1 hour with the membrane and left on a rocking platform. The blot was thereafter washed with 3% Marvel in 3x Tris buffer. Goat anti-mouse antibody; 1:2000 was then incubated with the blot for at least 1 hour and then washed with Tris-Marvel buffer changing it every 10 minutes, 3 times with increasing rocking speed. Final wash buffer was removed and the blot was rinsed in RO water. The enhanced chemiluminescent (ECL) substrate was added (3 ml per blot of the ECL substrate for HRP) before recording the chemiluminescence on a Biorad ChemiDoc ™ (SV00843).

2.6 Optimisation of oocyst immobilisation and detection

Initial ELISA screening of scFv clones used an oocyst lysate produced by a bead beater method (section *2.3.5.1.1)*. Alternative oocyst immobilisation and detection methods were required.

2.6.1 Using buffers of different pH conditions and different temperatures to immobilise whole oocysts

Different methods were used to whole oocysts on Nunc MaxiSorp® plates. Buffers of different pH conditions were used (acetic acid, pH of 3.0 and pH of 5.0, PBS pH of 7.0 and carbonate buffer pH 9.0). To these buffers, 10^4 oocysts were added and then coated on Nunc MaxiSorp® plates overnight. Coating was carried out at room temperature and at 37°C. Phage was prepared from a clone that had been confirmed to be a positive clone from spin down ELISA assays. This phage was used to carry out a monoclonal phage ELISA to assess oocyst immobilisation using the general phage ELISA protocol described in subsection *2.3.5.1.2* with the exception of the phage preparation being from a single clone and not polyclonal phage.

2.6.2 Immobilizing whole oocysts on distinct assay plate types

Four different plates were obtained; Polysorp® plate (Thermo Scientific™ 442404), Maleic Anhydride plate (Thermo Scientific™ 15110), Nunc™ Edge 96 well cell culture plates (Thermo Scientific™ 152640) and Nunc MaxiSorp®. There were two different conditions for each plate; oocysts were incubated in room temperature in different pH buffers (as above), and then in 37°C. Oocysts were viewed under an inverted

microscope as being in all the plates before washing and also following washing steps (as used in the ELISA in subsection *2.3.5.1.2*).

2.6.3 Immobilization of whole oocysts using polyclonal sera

Anti-*Eimeria tenella* antibody was obtained from chickens that had been infected with *E. tenella*, sacrificed and their serum collected (provided by MSD-Animal Health). This antibody had been tested in 96 well plate ELISA against oocyst lysates of *E. maxima, Eimeria necatrix* and *Eimeria tenella* alongside a normal chicken sera negative control and was found to be a positive antibody. A sandwich ELISA incubating anti-*Eimeria tenella* polyclonal antibody (1:500) on Nunc MaxiSorp® plate overnight. The next day the plate was washed and blocked with Marvel as described in subsection *2.3.5.1.2. Eimeria tenella* oocysts (10^4) were added and the plate incubated for an hour. Plate washing and bound phage detection was carried out as described in subsection *2.3.5.1.2.*

2.6.4 Immobilization of whole oocysts using Nitrocellulose membrane/ Dot blots

Grids of 1 cm were drawn with a pencil to indicate regions on Nitrocellulose membrane (0.45 µm A10195922 GE Healthcare life science). 2.5 µl of whole oocysts (10^4 cells on average) or 2.5 µl of 20 µg/ml protein derived from oocyst lysates as well as BSA negative control were spotted onto the nitrocellulose membrane at the centre of the grid. The membrane was left to dry and was blocked by immersing the entire membrane in 3% (w/v) Marvel in TBS at 4°C overnight. The next morning, the membrane was left to warm to room temperature and it was thereafter rinsed with PBS. The primary antibody which was a pool of anti-*Eimeria* species chicken sera diluted at 1:1000 dissolved in TBST and blocked with 0.5% Marvel for 1 hour at room temperature was incubated with the membrane for 1 hour and thereafter washed three times with TBST. Each wash lasted for 10 minutes with slow rotating. The secondary antibody which was anti-chicken IgG AP (Product No. A9171, Sigma-Aldrich) 1:2000 was incubated with the membrane for 1 hour and was thereafter washed with TBST three times at RT for 10 minutes. BCIP®NBT substrate (Product No. 35655, Sigmafast) was added and it was observed for colour change which was imaged using Biorad ChemiDoc ™ (SV00843).

2.6.4.1 Phage dot blots

Grids were drawn on Nitrocellulose membrane and 2.5 µl of whole oocysts as well as 2.5 µl of BSA negative control were dotted on the membrane exactly as described in subsection 2.6.4. Drying, blocking and rinsing of membrane were carried out exactly as described in subsection 2.6.4. Phage was produced for the clone designated SRSKNYLWRSM as well as anti-BSA control phage and they were used to screen all seven species of oocysts on colorimetric dot blots (5 mls per blot) using nitrocellulose membrane. The phage supernatant (~10^{11} PFU/ml) was pre-blocked with 3% (w/v) Marvel milk for 30 minutes before incubating with the membrane for 1 hour and thereafter washing the membrane. Washing of the membrane was carried out exactly as described in subsection 2.6.4. Rabbit anti-fd (1:2000) was pre-blocked for 30 minutes before incubating for 1 hour with the membrane and then washing it as described in subsection 2.6.4. Goat anti-rabbit AP (1:2000) was pre-blocked with 3% (w/v) Marvel and then incubated with the membrane for 1 hour and washes were carried out the same way as with previous incubations.

2.6.4.2 Optimisations of phage dot blot

With chemiluminiscent detection system

A chemiluminescent detection system was used to determine if the phage dot blot assays will produce better results. Phage dot blot assay was carried out exactly as described in subsection 2.6.4.1 with the exception of the detection system. An anti-M13-HRP (1:5000) substrate was used to detect phage and substrate used was the enhanced chemiluminescent (ECL) substrate (3 ml per blot of the ECL substrate for HRP) before recording the chemiluminescence on a Biorad ChemiDoc ™ (SV00843).

Validation of phage dot blots using lysozyme

Phage dot blots were validated before optimising phage dot blot conditions. Briefly, 2.5 µl concentrations of 200 µg/ml and 20 µg/ml of lysozyme as well as the same concentration of BSA were dotted on Nitrocellulose membrane. Drying, blocking and incubation conditions were carried out exactly as described in 2.6.4 above. Detection was carried out as described in subsection 2.6.4.1 above.

Optimisation of dot blots with control proteins, different phage concentrations, membrane and wash conditions

Using PVDF membrane, BSA and lysozyme were spotted as before and phage prepared from anti-BSA was used to detect the proteins. The phage was used in three

different concentrations; 10^{11}, 10^{10} and 10^{9} /ml (5 mls of phage per blot). Two different wash conditions were used, the first one was TBST containing 0.1% (v/v) Tween 20 while the second wash condition included 1 M NaCl to the TBST containing 0.1% (v/v) Tween 20 to enhance the wash stringency. Six different conditions were tested: A (10^{11} phage concentration + TBST), B (10^{10} phage concentration + TBST), C (10^{9} phage concentration + TBST), D (10^{11} phage concentration + TBST + 1 M NaCl), E (10^{10} phage concentration + TBST + 1 M NaCl) and F (10^{9} phage concentration + TBST + 1 M NaCl). All other conditions of the phage dot blot remained the same and were carried out exactly as described in subsection 2.6.4.1.

*Optimisation using acid and PEG precipitated phag*e

Phage dot blot was conducted exactly as described in subsection 2.6.4.1 except that Both phage were used in three different concentrations; 10^{11}, 10^{10} and 10^{9} /ml (5 mls of phage per blot). The wash, buffer and incubation conditions remained the same as described in 2.6.4.1.

Optimisation using HRP Detection system

Phage dot blot assay was conducted using an HRP detection system. The SRSKNYLWRM and anti-BSA control phage (~10^{10} PFU/ml) were screened against the seven species of *Eimeria*. The assay was performed exactly as described in subsection 2.6.4.1 with the exception of the phage being detected with an anti-M13-HRP antibody and a membrane adapted TMB substrate (Serva, 160837).

Optimisation of phage dot blots by improving blocking conditions and phage concentrations

Phage was grown from five scFv clones (SRSKNYLWRSM, DHTPKWq, SAqHP, STQLP and STqHP) alongside anti-BSA phage. They were used at a concentration of 10^{10} PFU/ml to probe blots dotted with whole oocysts of the seven species of *Eimeria* as well as a BSA negative control. The assay was performed exactly as described in subsection 2.6.4.1 with the exception of the phage being pre-blocked with 10% (w/v) Marvel and detected with an anti-M13-HRP antibody and a membrane adapted TMB substrate. This assay was repeated using a PVDF membrane applying the same conditions.

2.6.5 Using nonylphenylpolyethylene glycol Nonidet®40 (RB10355) and boiling to release membrane components for immobilisation

Duplicate pooled oocysts (10^4) were put in two different eppendorfs containing 10% (v/v) and 5% (v/v) respectively of nonylphenylpolyethylene glycol Nonidet® 40 RB 10355 (Nonidet P-40) in PBS and were subjected to two different treatments. Treatment one was heating the oocysts at 90°C for 15 minutes and treatment 2 was at 50°C for 10 minutes. This was aimed at lysing the oocysts by disrupting membranes and extracting oocyst proteins and other macromolecules. Both treatments successfully lysed oocysts and oocyst proteins were quantified using Bradford's assay. The detergent treated lysates were then added to Nunc MaxiSorp® and Polysorp® plates and phage ELISA was carried out as previously described in subsection *2.3.5.1.2.*

2.6.6. Sonication of oocysts to generate homogenates

The sonicator was placed inside a sterile hood. Three millilitres (each species having different concentrations refer to **chapter 3**, Table 3.1) of each oocyst species were put in different 15 ml centrifuge tubes and then incubated in ice for 30 minutes. The sonicator was set to pulse eight times (1800000 Joules) at 10s intervals (10 seconds sonication and 10 seconds pause). The sonicator probe (1/4 inch diameter) was cleaned after sonication of each species with 70% ethanol. After sonication, samples were incubated in ice for another 5 minutes then solubilized with 0.5% (v/v) Nonidet P-40 in PBS. Samples were spun down at 10000 xg for 5 minutes to remove cell debris and then protein concentration in the supernatant was quantified using Bradford protein assay. Samples were transferred to microcentrifuge tubes and these stock solutions of samples were stored at -20°C. The homogenates were then used at 50 µg/ml for phage ELISAs as previously described in subsection *2.3.5.1.2.*

2.6.8 Development of a spin down immunoassay to detect whole oocysts

2.6.8.1 Phage spin down ELISA

Monoclonal phage from the most abundant (in round 5 of panning) scFv clone, designated DHTPKWq, was grown using the protocol for growing phage as has been described previously in subsection 2.3.2. Here, the initial starting culture of this is 50 ml and not 500 ml as described in subsection 2.3.2.

Pooled oocysts (10^5 of each of the seven species) or individual oocyst species (10^5) were suspended in 1ml of sterile PBS in 3% (w/v) Marvel solution in sterile eppendorf tubes and incubated for 1 hour at room temperature. They were then spun at 3000 g

for 3 minutes and the supernatant was discarded by pipetting and the pellet resuspended in 100 µl of sterile PBS. Phage, 400 µl per oocyst preparation were pre-blocked for 1 hour with 200 µl of 18% (w/v) Marvel in 6 x PBS. The blocked phage were incubated with oocyst overnight at 4°C gently rotating. The following day, the tubes were spun at 3000 g for 3 minutes and the supernatant was discarded by pipetting. Each tube was washed with 1 ml PBST containing 0.1% (v/v) Tween 20. This was spun at 3000 g for 3 minutes and discarded and repeated twice. The oocysts were washed once more with PBS. Anti-M13-HRP (1:5000) in PBS was pre-blocked with 3% (w/v) Marvel for 1 hour before incubating 100 µl with the oocyst preparation for 1 hour. This was then spun and supernatant discarded and the oocysts washed 3 times with PBST containing 0.1 %(v/v) Tween 20 and once with PBS only. The oocyst pellets were then resuspended in 150 µl of PBS and then 100 µl of the preparation was transferred into a well on Nunc Maxisorb® plates. To the wells, 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) was added and colour was allowed to develop and then absorbance was read at 650nm or the reaction was stopped using 2M Sulphuric Acid in which case plates were read at 450nm.

2.6.8.2 Phage fluorescent spin down ELISA

Phage and oocysts were prepared and interacted with phage as described in 2.6.8.1. An anti-BSA negative control scFv phage was included. Rabbit Anti-fd (1:2000) in PBS was pre-blocked with 3% (w/v) Marvel for 1 hour before incubating 100 µl with the oocyst preparation for 1 hour. This was then spun and supernatant discarded then washed 3 times with PBST containing 0.1 %(v/v) Tween 20 and once with PBS. Anti-rabbit Alexafluor 488 (1:2000) was pre-blocked for 1 hour with 3% (w/v) Marvel and then incubated with oocysts. This was washed 3 times with PBST containing 0.1 % (v/v) Tween 20 and 3 times with PBS. The oocyst pellets were then resuspended in 150 µl of PBS and then read with Corning® 96 well black polystyrene microplate (Product No. CLS3603) using a fluorescent plate reader (BMG, Labtech) at wavelength of 485 nm.

2.6.9 Development of an oocyst filtration immunoassay

A filtration ELISA was carried out to improve wash conditions and reduce background signals with the spin down ELISA, as it was difficult to remove void volumes of buffer during washing steps and retain oocysts. Filtration tubes were used that had pore sizes smaller than the oocysts to allow thorough washes, the sizes of oocysts species range between ≤18.8 µm and ≥23.9 µm long (Reid and Long, 1979, Haug *et al.,*

2008b). Two tube types were assessed, the initial filter tube had 0.45 µm filter pore size which retained oocysts but was easily clogged. A spin column with 10 µm filter (Mobitec M1002S) was then used to aid in the wash steps. The protocol was the same the described above for the spin down ELISA (subsection *2.6.8.1*) except oocysts were transferred into the filter columns for every wash step.

2.6.10 Fluorescent Microscopy analysis of scFv phage binding to oocysts

Oocysts in solution of each *Eimeria* species (10^4) were quantified and put in sterile eppendorf tubes. They were spun down and resuspended in 500 µl 3% (w/v) Marvel for 1 hour. After one hour, they were spun down and washed once with PBS. Anti-oocyst and Anti-BSA control phage scFv (10^{11} PFU/ ml) were pre-blocked in 3% (w/v) Marvel for 1 hour and thereafter incubated with the oocysts for 1 hour rotating. The oocysts were then washed three times with PBST containing 0.05% (v/v) tween 20 and once with PBS. Rabbit anti-fd 1:2000 was pre-blocked in 3% (w/v) Marvel in PBS for 1 hour and was then incubated with the oocysts for 1 hour at room temperature. It was washed as before and then incubated with Anti-rabbit Alexafluor 1:2000 which had been pre-blocked for 1 hour with 3% (w/v) Marvel. It was thereafter washed as described above. Then resuspended in 100 µl of PBS and viewed under the microscope at X40 magnification.

2.6.10.1 Optimisation of fluorescent microscopy

Wash conditions were optimised as well as blocking conditions. Wash conditions were optimised to 3 times with PBST containing 0.05 %(v/v) Tween 20 and once with PBS after each antibody incubation. Exposure, gain and saturation settings of the microscope were optimised to reduce background noise.

2.7 V_HH library panning against oocyst lysate

In order to select for antibodies that were easily rescued after identification in NGS datasets a V_HH library which had diversity only in the HCDR3 was used. This allows synthesis of clones of interest directly into the phagemid or an alternative expression vector without needing to rescue clones of low abundance from polyclonal phage pools as was carried out with the scFv library. In addition, a readily immobilised *Eimeria* preparation was used as the target antigen; this was so that any final assay could easily be carried out in a 96 well format, as opposed ot using whole oocysts that are difficult to immobilise.

2.7.1 Growing of V_HH camelid library

Camelid V_HH library was used for the second NGPD approach. The library was propagated as a 12mer, 16 mer and 21 mer individually and grown exactly as the Griffin scFv phage library was grown as described in subsection 2.3.2, after which they were combined before phage precipitation.

2.7.2 V_HH Phage Precipitation with Polyethylene glycol (PEG)

The phage from the three different cultures were combined in one flask and the phage were PEG precipitated. 250 ml 20% (w/v) PEG-8000/2.5M NaCl was added for every litre of phage supernatant and placed on ice. After 1 hour, the culture was centrifuged at 8000 xg for 30 minutes. The supernatant was discarded and all residual PEG/NaCl were removed by centrifuging a further two times at 8000 g for 30 seconds. The pellet was then resuspended by adding 10 ml of PBS. The pellet was allowed to soak in PBS for a few minutes before gently pipetting to re-suspend the purified phage pellet. The phage was then titred as previously described in subsection *2.3.2.2*.

2.7.3 Round 1 biopanning

For round 1 panning, 100 µl of individual species of oocyst lysate at 50 µg/ml protein was coated on 6 wells per species of a 96 well Nunc MaxiSorp® plate and was allowed to bind overnight, static at room temperature. The next day, plates were washed once with 400 µl PBS and subsequently blocked with 3% (w/v) marvel in PBS for one hour while the phage suspension from 2.7.2 was blocked for one hour with 200 µl of 18% Marvel and 6X PBS per ml of phage. The plates were then washed with 400 µl PBS and 300 µl of blocked phage suspension was added to each of the Nunc MaxiSorp® wells and allowed to bind for one hour. The plate was then washed fifteen times with PBST (PBS containing 0.05% Tween 20) and then five times using dH2O.

Phage were eluted from the plates by direct addition of 300 µl of mid-log TG1 at an O.D of 0.4-0.6 and then incubated for 30 minutes at 37°C static. The eluted wells for each species were then pooled together; 300 µl in 6 wells, 1800 µl eluted phage in total. Of each elution pool, 600 µl was transferred to sterile eppendorf tubes out of which 1 µl was used to titre the enriched phage and the rest stored at -20ºC. The remaining 1.2 ml solutions were then spread on bioassay plates (containing 2YT agar and 150 µg/ml ampicillin, 1% (w/v) glucose), and grown at 37ºC overnight. The

bacterial colonies were scraped from the bioassay plates and suspended in 2 ml 2YT 40% glycerol and stored at -80°C.

2.7.4 Round 2 biopanning

For round 2 panning, a pre-panning subtractive step was carried out. The sub-libraries from each of the seven species produced from round 1 panning were re-grown individually shaking at 37°C, using 100 µl of round 1 glycerol stocks to inoculate 50 ml 2YT containing 150 µg/ml ampicillin and 1% (w/v) glucose. At an O.D of 0.4-0.6, exphage was added to each library and allowed to incubate for 30 minutes static and 30 minutes shaking. Cultures were there spun for 10 minutes at 3000 xg and the pellets were re- suspended in 30 ml 2YT containing 150 µg/ml ampicillin and 50 µg/ml kanamycin. They were then allowed to grow overnight at 30°C and shaking at 180 rpm. The following day, all seven cultures were centrifuged at 8000 xg and the supernatants were transferred to sterile containers and titred.

Seven 96 Nunc MaxiSorp® plates were used for the second round of panning and on each plate, 6 reps of 100 µl, 50 µg/ml of oocyst lysate for each of the seven species were coated overnight at room temperature static. The next day, the plates were washed once with 400 µl, 1 XPBS and subsequently blocked with 3% (w/v) Marvel in PBS for one hour while the seven phage suspension from 2.7.3 were blocked for one hour with 200 µl of 18% Marvel and 6X PBS per ml of phage. Subtraction was carried out by incubating phage from one of the seven species with oocyst lysates from the other six species sequentially for 15 minutes each before finally incubating the unbound phage with the species that the phage was panned against in round 1. This was carried out for targeting all 7 species. These subtraction steps should ideally remove non-specific binders from the phage pool before they were incubated with the lysate from the *Eimeria* species that they were panned against in round 1, the aim being to maximise the chances of isolating phage that were specific to the target species. The plate was then washed fifteen times with PBST (PBS containing 0.05% Tween 20) and then five times using dH_2O.

The phage were eluted from the lysate by adding mid-log TG1 cells followed by incubation, pooling of eluates, spreading in bioassay dishes, titration of eluted phage, incubation overnight, scraping from the bioassay dishes the next day, adding of glycerol stock and storage at -80°C as has been described in 2.7.3 above.

2.7.5 Round 3 panning

A pre-panning subtractive step and post-panning subtraction were carried out for round 3 panning. Input phage was grown exactly as for round 2 panning. A total of 14 Nunc MaxiSorp® plates were coated with six reps of 100 µl, 50 µg/ml of individual species of oocyst lysates overnight at room temperature static. The pre-panning subtractive step was carried out exactly as was described in round 2 panning. Elution was carried out by a pH shift using glycine (Tu *et al.,* 2012) given that a post-panning subtraction of phage was going to be carried out; using TG1 cells was not feasible. 100 µl of 0.1M glycine at a pH of 4.2 was added to individual wells and incubated for 10 minutes and then immediately neutralised with an equal volume of 0.1M Tris buffer. The eluate from the six reps of individual species was then subtracted against the other six species as for pre-panning subtractive step, this again aimed at increasing the specificity of the phage pool to the target species. Phage were then collected and pooled together. Half of each of the eluate pool were transferred to a sterile eppendorf and the remaining half were incubated with 1 ml of TG1 and left for 30 minutes static at 37°C and then another 30 minutes shaking at 37°C. Eluted phage titration was performed as has been described in subsection *2.3.3.1.1* and eluted phage incubated with TG1 were spread on bioassay dishes containing 2YT agar and 150 µg/ml ampicillin, 1% (w/v) glucose, then grown at 37°C overnight. Scraping and storage of clones were carried out exactly as described in 2.7.4 above.

2.7.6 Screening of round 3 panning output phage

Round 3 panning output phage from panning against all seven species was grown using the same protocol as described in 2.7.4 above. The phage was then screened against oocyst lysates from all seven species as illustrated in figure 2.5. Nunc MaxiSorp® plates were used for the screens. For the binding of each polyclonal phage, six reps of 100 µl, 50 µg/ml oocyst lysate for each species were coated overnight at room temperature static. The next day, plates were washed once with 400 µl 1 XPBS and then blocked with 3% (w/v) Marvel in PBS for one hour while the phage suspension was blocked for one hour with 200 µl of 18% Marvel and 6X PBS per ml of phage. The plates were then washed with 400 µl PBS and 300 µl of blocked phage suspension was added to each of the corresponding wells and allowed to bind for one hour. The plate was then washed fifteen times with PBST (PBS containing 0.05% Tween 20) and then five times using dH_2O. Elution of phage was carried out by pH shift using 100 µl of 0.1M glycine at pH 4.2 and thereafter neutralised after 10 minutes by adding the same volume of 0.1M Tris buffer. Phage was transferred to

sterile eppendorfs and 40% glycerol was added and then stored at -80°C until needed. Eluted phage was titred as has been described in subsection 2.3.3.1.1.

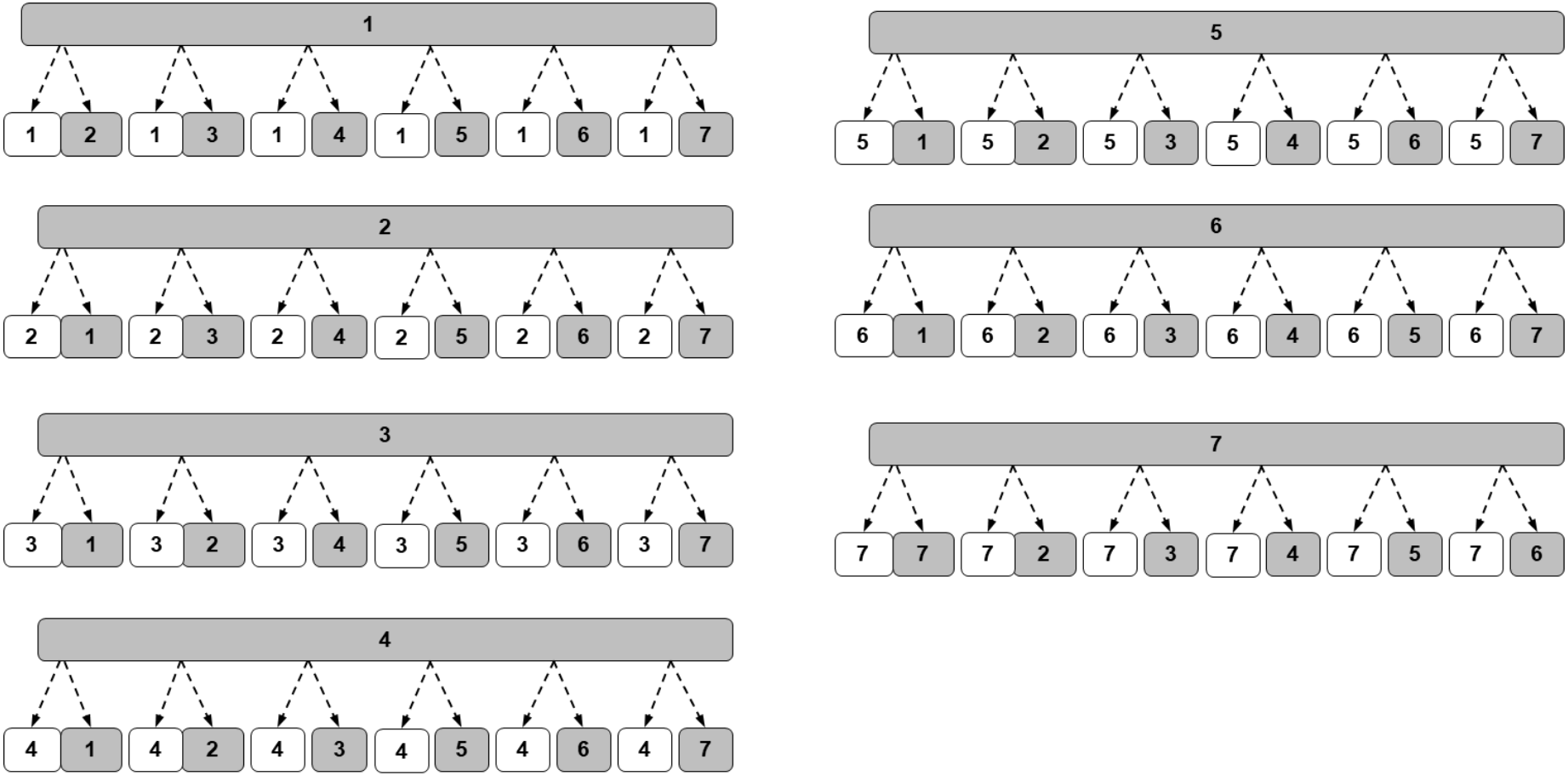


Figure 2:5: Schematic representation of V_HH-phage screening against *Eimeria* lysates from 7 different species. Each phage pool isolated after 3 rounds of panning against a single *Eimeria* species (1-7, grey rectangles) was screened in 6 replicates against the target species it was panned against (positive samples, white boxes) and single replicates of the other six other species (negative samples, grey boxes).

2.7.7 Conventional screening of V_HH clones

2.7.7.1 V_HH polyclonal phage ELISA

Phage from the V_HH library and the sub-libraries of each species from the three rounds of panning were grown and titred in a similar protocol as described in 2.7.4. Nunc Maxisorb® plates were coated with 100 µL of oocyst lysate at 50 µg/ml in PBS overnight at room temperature static. Washing and blocking of wells as well as blocking of phage and incubation were carried out as previously described in subsection *2.3.5.1.2.* Rabbit Anti-fd (1:2000) in PBS was pre-blocked with 3% Marvel for 1 hour before adding 100 µl to the wells and incubating for 1 hour. The plates were then washed 3 times with PBST containing 0.05 %(v/v) Tween 20 and then 3 times PBS. Goat anti-rabbit AP (1:1000) was pre-blocked for 1 hour, 100 µl added to each well and incubated for 1 hour. It was then washed 3 times with PBST containing 0.05 %(v/v) Tween 20 and then 3 times PBS. The substrate p-Nitrophenyl phosphate disodium Salt (PNPP) was added, 100 µl per well and incubated for 30 minutes until colour change and absorbance was read at 405 nm using the Microplate reader (Labtech LT-4000).

2.7.7.2 V_HH monoclonal phage ELISA; initial approach

From the round 3 panning glycerol stocks for each species, 2YT plates were streaked and then ten single colonies were picked for each species; totalling 70 and phage was grown for each clone using the same protocol as described in subsection *2.3.5.1.4*. Nunc MaxiSorp ® plates were coated with 50 µg/ml of oocyst lysates antigen of each species overnight. Monoclonal phage ELISA was carried out by incubating each phage grown from panning against a particular species with the corresponding lysate using the same protocol described in the polyclonal phage ELISA (sub-section *2.3.5.1.2*).

2.7.7.3 V_HH monoclonal phage ELISA; second approach

From the round 3 panning glycerol stocks for each species, 2YT plates were streaked and 96 single colonies were picked for each species; totalling 672 clones for seven species of *Eimeria*. Phage was grown for each clone using the same protocol as described in subsection *2.3.5.1.4*. and phage ELISA was carried out following similar protocol as described in 2.7.7.1.

2.7.8 NGS analysis of V_HH-phage panning against oocyst homogenate

After receiving the NGS data, it was converted to fasta file, demultiplexed, reverse complemented, translated and the three reading frames concatenated as previously described in subsection 2.3.6. The reads were also sifted to only include those that contained the IYYCAA and WGQGT phagemid motifs and the unique V_HH CDR3 motifs were identified. Because the V_HH library is only randomised in the HCDR3 region, no degeneracy was allowed in the sequences of the flanking regions.

In the initial analysis, for each target that was screened, the negative control barcodes were pooled together while the positive barcodes were not pooled. Using a script (compare_collapsed_sequences_2a.Zscore.pl, Appendix V), comparison was made between each positive barcode (n=6) versus the pool of negative barcodes and on that basis, Z scores were generated as previously described by (Naqid *et al*., 2016b). Subsequently, the files generated from the comparison of positive versus negative barcodes were subjected to another analysis in order to improve the chances of selecting truly enriched clones. A script (Find_Z_scores_above3_sort_count.sh) was written for the seven species to select CDR3s that had a Z score of above 3, and they were sorted and counted according to the number of times they appeared. Those that were seen in at least 4 barcodes out of 6 were sorted and a list of HCDR3s were selected for each species. Within the list for each species, five HCDR3s that had the top Z scores were selected, totalling 35 selected HCDR3s for the seven *Eimeria* species. As a final check, each HCDR3s selected was then searched for in the files to see if it appeared enriched in any other barcodes having Z score of 3 and above (for panning against a distinct *Eimeria* species) and if it appeared as being enriched against other species, it was not selected.

2.7.9 Primer design for synthesis of V_HH clones

Amino acid sequences for HCDR3 selected from the NGS data were converted to their optimal nucleotide codons for expression in prokaryotic *E. coli* systems. This was performed using the online converter and the conversion removed stop codons, replacing them with a codon for Q (JCat, http://www.jcat.de/ assessed 31/08/2019). The inverse PCR primers were then designed to synthesise the optimal V_HH sequence by each covering half of the V_HH CDR3 sequence and 18 nucleotides into the adjacent framework region of the scaffold V_HH using to construct the original library. The forward primer thus covered half the CDR3 and the framework region 4, and the reverse primer covered the other half of the CDR3 and the framework region 3, amplifying in the other direction (Figure 2.6). The CDR3 part of the primer would

initially not have had any complementary sequence to anneal to in the template; only the framework region part of the primer would anneal to the template. The template in the synthesis method is the anti-beta lactamase clone in the PSD3 vector. This was the scaffold used to produce the V_HH library. The CDR3 of the primer which initially would not have anything to bind to will then be synthesized by the polymerase. The entire vector will then be amplified. This method is superior to the inverse PCR/ rescue that was carried out with the Griffin library in that clones of interest can be directly synthesized into the vector or any alternative expression vector like P5X provided it already had a V_HH clone within it. This bypasses the need to rescue the clones from a panning sub library where the target clone can be present at very low levels and other non-target clones can interfere with amplification. This is possible because of the constancy of the V_HH framework and HCDR1 and HCDR2 regions which is not so with the scFv library. Primers for rescue of V_HH clones in for V_HH-oocyst homogenate panning and V_HH-peptides are represented in Table 2.7 and Table 2.10 respectively.

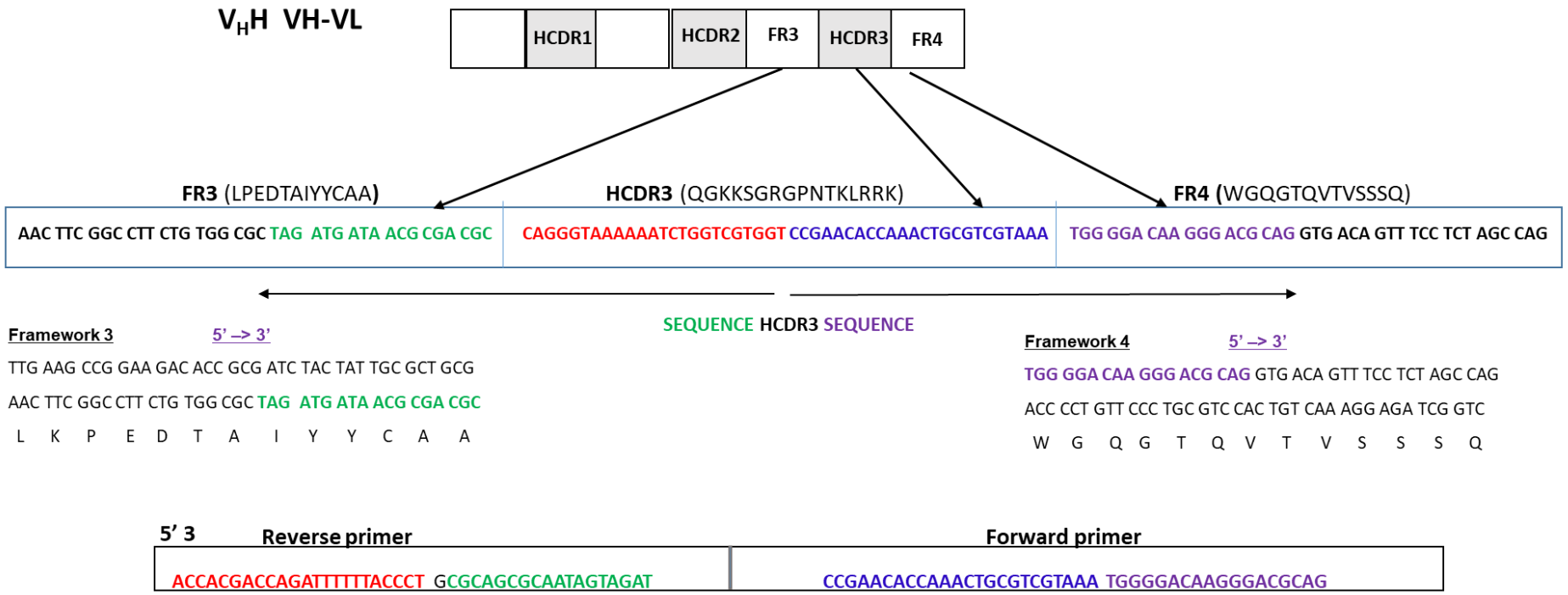


Figure 2:6: Schematic design showing primer design and inverse PCR strategy for V_HH clones of interest. Primers were designed to cover half of the CDR3 sequence each. One forward, one reverse with 5' phosphorylated ends to allow ligation. The primers extended 18bp into the framework 3 or 4 region to allow annealing to any V_HH template. These primers were compatible with any vector that already contained a V_HH sequence.

Table 2:7: Inverse PCR primers for phagemid synthesis of V_HH selected against oocyst homogenate

Sequence	**Optimised sequence**	**Forward primer 5'3**	**Reverse primer 5'3**
DqRSPNSLCAqC	DQRSPNSLCAQC	TCTCTGTGCGCTCAGTGCTGGGGACAAGGGACGCAG	GTTCGGAGAACGCTGGTCCGCAGCGCAATAGTAGAT
TSTGTLQSSRPPKHSH	TSTGTLQSSRPPKHSH	TCTCGTCCGCCGAAACACTCTCACTGGGGACAAGGGACGCAG	AGACTGCAGGGTACCGGTAGAGGTCGCAGCGCAATAGTAGAT
YqLTFAHTPAAFVYVFRSVAS	YQLTFAHTPAAFVYVFRSVAS	CTTTCGTTTACGTTTTCCGTTCTGTTGCTTCTTGGGGACAAGGGACGCAG	CAGCCGGGGTGTGAGCGAAGGTCAGCTGGTACGCAGCGCAATAGTAGAT
AKSSPQPMTVSLACqP	AKSSPQPMTVSLACQP	ACCGTTTCTCTGGCTTGCCAGCCGTGGGGACAAGGGACGCAG	CATCGGCTGCGGAGAAGATTTAGCCGCAGCGCAATAGTAGAT
AqSHAqCRLSNKELKR	AQSHAQCRLSNKELKR	CTGTCTAACAAAGAACTGAAACGTTGGGGACAAGGGACGCAG	ACGGCACTGAGCGTGAGACTGAGCCGCAGCGCAATAGTAGAT
WaqYSKPPSTYVYYLGKRYLI	WAQYSKPPSTYVYYLGKRYLI	ACGTTTACTACCTGGGTAAACGTTACCTGATCTGGGGACAAGGGACGCAG	AGGTAGACGGCGGTTTAGAGTACTGAGCCCACGCAGCGCAATAGTAGAT
YLRSqCEqHNFLLDALNVEDA	YLRSQCEQHNFLLDALNVEDA	TCCTGCTGGACGCTCTGAACGTTGAAGACGCTTGGGGACAAGGGACGCAG	AGTTGTGCTGTTCGCACTGAGAACGCAGGTACGCAGCGCAATAGTAGAT
KAALqMqHWHSQKREL	KAALQMQHWHSQKREL	TGGCACTCTCAGAAACGTGAACTGTGGGGACAAGGGACGCAG	GTGCTGCATCTGCAGAGCAGCTTTCGCAGCGCAATAGTAGAT
RqPVERQRMTVATAPRKTqRV	RQPVERQRMTVATAPRKTQRV	TTGCTACCGCTCCGCGTAAAACCCAGCGTGTTTGGGGACAAGGGACGCAG	CGGTCATACGCTGACGTTCAACCGGCTGACGCGCAGCGCAATAGTAGAT
CFGQCEHSqYTRGMTGRGRHS	CFGQCEHSQYTRGMTGRGRHS	CCCGTGGTATGACCGGTCGTGGTCGTCACTCTTGGGGACAAGGGACGCAG	TGTACTGAGAGTGTTCGCACTGACCGAAGCACGCAGCGCAATAGTAGAT
YqLTFCSYACCVLFMFFRSVAS	YQLTFCSYACCVLFMFFRSVAS	GTTCTGTTCATGTTCTTCCGTTCTGTTGCTTCTTGGGGACAAGGGACGCAG	GCAGCAAGCGTAAGAGCAGAAGGTCAGCTGGTACGCAGCGCAATAGTAGAT
PSASYAGMqRRWKQNL	PSASYAGMQRRWKQNL	CAGCGTCGTTGGAAACAGAACCTGTGGGGACAAGGGACGCAG	CATACCAGCGTAAGAAGCAGACGGCGCAGCGCAATAGTAGAT
lHWqREKqLTTqSRTVPAHNM	IHWQREKQLTTQSRTVPAHNM	CCCAGTCTCGTACCGTTCCGGCTCACAACATGTGGGGACAAGGGACGCAG	TGGTCAGCTGTTTTTCACGCTGCCAGTGGATCGCAGCGCAATAGTAGAT
MVVWKqHSSLAKTRCY	MVVWKQHSSLAKTRCY	TCTCTGGCTAAAACCCGTTGCTACTGGGGACAAGGGACGCAG	AGAGTGCTGTTTCCAAACAACCATCGCAGCGCAATAGTAGAT

PHLKFSCFpCFCqHHGS	PHLKFSCFPCFCQHHGS	CGTGCTTCTGCCAGCACCACGGTTCTTGGGGACAAGGGACGCAG	GGAAGCAAGAGAATTTCAGGTGCGGCGCAGCGCAATAGTAGAT
YNRLqRTGNATT	YNRLQRTGNATT	ACCGGTAACGCTACCACCTGGGGACAAGGGACGCAG	ACGCTGCAGACGGTTGTACGCAGCGCAATAGTAGAT
HsqLCKVPCVTRPRNY	HSQLCKVPCVTRPRNY	TGCGTTACCCGTCCGCGTAACTACTGGGGACAAGGGACGCAG	CGGAACTTTGCACAGCTGAGAGTGCGCAGCGCAATAGTAGAT
IcqLLLHPLCAL	ICQLLLHPLCAL	CACCCGCTGTGCGCTCTGTGGGGACAAGGGACGCAG	CAGCAGCAGCTGGCAGATCGCAGCGCAATAGTAGAT
NyqPNWNPqKTS	NYQPNWNPQKTS	AACCCGCAGAAAACCTCTTGGGGACAAGGGACGCAG	CCAGTTCGGCTGGTAGTTCGCAGCGCAATAGTAGAT
IMGPMSTANKMNTMPQ	IMGPMSTANKMNTMPQ	AACAAAATGAACACCATGCCGCAGTGGGGACAAGGGACGCAG	AGCGGTAGACATCGGACCCATGATCGCAGCGCAATAGTAGAT
YHYNRLYNSqSSAVEV	YHYNRLYNSQSSAVEV	TCTCAGTCTTCTGCTGTTGAAGTTTGGGGACAAGGGACGCAG	GTTGTACAGACGGTTGTAGTGGTACGCAGCGCAATAGTAGAT
VPYLIRHTPPDSTqYV	VPYLIRHTPPDSTQYV	CCGCCGGACTCTACCCAGTACGTTTGGGGACAAGGGACGCAG	GGTGTGACGGATCAGGTACGGAACCGCAGCGCAATAGTAGAT
YADqYASKSFKL	YADQYASKSFKL	TCTAAATCTTTCAAACTGTGGGGACAAGGGACGCAG	AGCGTACTGGTCAGCGTACGCAGCGCAATAGTAGAT
LCLNRSqLTVNQ	LCLNRSQLTVNQ	CAGCTGACCGTTAACCAGTGGGGACAAGGGACGCAG	AGAACGGTTCAGGCACAGCGCAGCGCAATAGTAGAT
DLNYPRTqHRRKQKNE	DLNYPRTQHRRKQKNE	CACCGTCGTAAACAGAAAAACGAATGGGGACAAGGGACGCAG	CTGGGTACGCGGGTAGTTCAGGTCCGCAGCGCAATAGTAGAT
KVTMPGKNQAqLSIAR	KVTMPGKNQAQLSIAR	CAGGCTCAGCTGTCTATCGCTCGTTGGGGACAAGGGACGCAG	GTTTTTACCCGGCATGGTAACTTTCGCAGCGCAATAGTAGAT
TRWPTNYLHSFDLGPq	TRWPTNYLHSFDLGPQ	CACTCTTTCGACCTGGGTCCGCAGTGGGGACAAGGGACGCAG	CAGGTAGTTGGTCGGCCAACGGGTCGCAGCGCAATAGTAGAT
qWLVESRRAKRRPMPG	QWLVESRRAKRRPMPG	GCTAAACGTCGTCCGATGCCGGGTTGGGGACAAGGGACGCAG	ACGACGAGATTCAACCAGCCACTGCGCAGCGCAATAGTAGAT
RqPVERQRMTVATATRKTqRV	RQPVERQRMTVATATRKTQRV	TTGCTACCGCTACCCGTAAAACCCAGCGTGTTTGGGGACAAGGGACGCAG	CGGTCATACGCTGACGTTCAACCGGCTGACGCGCAGCGCAATAGTAGAT
WHSWRqLQYAPAHIKG	WHSWRQLQYAPAHIKG	TACGCTCCGGCTCACATCAAAGGTTGGGGACAAGGGACGCAG	CTGCAGCTGACGCCAAGAGTGCCACGCAGCGCAATAGTAGAT

VnqMHYTIRDLV	VNQMHYTIRDLV	ACCATCCGTGACCTGGTTTGGGGACAAGGGACGCAG	GTAGTGCATCTGGTTAACCGCAGCGCAATAGTAGAT
qLqqDNPLSVPF	QLQQDNPLSVPF	CCGCTGTCTGTTCCGTTCTGGGGACAAGGGACGCAG	GTTGTCCTGCTGCAGCTGCGCAGCGCAATAGTAGAT
TSHFTQLYRYLF	TSHFTQLYRYLF	CTGTACCGTTACCTGTTCTGGGGACAAGGGACGCAG	CTGGGTGAAGTGAGAGGTCGCAGCGCAATAGTAGAT
TRCqAPNFNKPLQRAR	TRCQAPNFNKPLQRAR	AACAAACCGCTGCAGCGTGCTCGTTGGGGACAAGGGACGCAG	GAAGTTCGGAGCCTGGCAACGGGTCGCAGCGCAATAGTAGAT
CPPNRMPRHDATVDSD	CPPNRMPRHDATVDSD	CACGACGCTACCGTTGACTCTGACTGGGGACAAGGGACGCAG	ACGCGGCATACGGTTCGGCGGGCACGCAGCGCAATAGTAGAT

2.7.10 Synthesis of V_HH clones

Synthesis of V_HH clones was carried out using the anti-Beta-Lactamase V_HH clone in pSD3 as the template. PCR reaction master mix is shown in table 2.8 below.

Table 2:8: PCR reaction master mix for V_HH synthesis

Reagent	Concentration	Volume (µl)
Nuclease free H_2O	N/A	20
Q5 DNA Polymerase (NEB)	5 U/µl	0.5
dNTPs	10mM	2
GC enhancer buffer	1 X	10
Forward primer	10 µM	2.5
Reverse primer	10 µM	2.5
5x Q5 reaction buffer	5 X	10
Template	10 ng/µl	2.5

The block was pre-heated to 95 degrees and initial denaturation was set at 95 °C for 5 minutes, 20 cycles at 95°C for 30s, 55°C for 1 minute and 72°C for 5.5 minutes for denaturation, annealing and extension respectively. A further 15 cycles of 95 °C for 30s, 60°C for 1 minute and 72°C for 5.5 minutes respectively for denaturation, annealing and extension, 5 minutes extension at 72 °C and held at 4 °C. The entire PCR product was loaded onto a 1.5% agarose gel using a wide comb.

The bands were gel purified using Machery-Nagel kit and samples were elute twice with 20 µl of elution buffer that was allowed to sit on the column for 5 minutes (40 µl total elute). Ligations were carried out using 17 µl of the gel purified product, 2 µl reaction buffer (10X) and 1 µl T4 ligase. Ligations were carried out at 12°C for 64 h. After ligation, the samples were incubated at 65°C for 10 minutes for de-activation of the ligase. The samples were micro-dialysed against molecular grade water (Product No. 10011342, Thermo-scientific) and then transferred to sterile eppendorf tubes. From the ligation reaction, 2 µl was then used to transform 50 µl of electro-competent TG1 cells (Product No. 200123, Agilent) using cuvettes with a 0.1 cm gap. Electroporation, recovery, incubation, growing and rescue of clones, confirmation of CDR3 sequences via Sanger sequencing were conducted exactly as previously described in subsection 2.3.7.1.

2.7.11 screening of V_HH synthesized clones

Phage were grown for all the synthesized clones following similar protocol described in 2.7.7.2 and Phage ELISA was carried out for all the clones following the protocol as that described in 2.7.7.1.

2.8 V_HH library biopanning against *Eimeria* peptides

A further alternative approach was also carried out in an attempt to isolate V_HH antibodies that differentiated *Eimeria* species. This approach targeted a constrained outer wall protein of oocysts which is one of the most abundant surface proteins of oocysts and that has been the most widely described, the 56 kDa gametocyte antigen (Belli *et al.,* 2009). Peptides that are largely unique to each of the *Eimeria* species oocyst were identified and synthesized. The synthesized peptides were used to pan the V_HH library for binders to each specific peptide that are therefore likely to be species specific. The advantages of this approach is that these peptides are far more simple and well defined compared to whole oocysts or oocyst lysates and the final assay format could possibly target oocyst homogenates on ELISAs, denaturing western or dot blots.

2.8.1 Comparative genomics of *Eimeria* species for peptide design

Amino acid sequences of the 56 kDa gametocyte antigen of each of the seven *Eimeria* species were aligned and compared to each other and areas of at least four amino acid changes were noted. Peptides were then synthesized from these areas of amino acid differences between the species and in addition, two species had only one amino acid change in the region and so another region of 4 amino acid change was located and peptides designed from the area making nine peptides in total (details in chapter 6).

2.8.2 Peptide Preparation for Panning

Areas of 4-5 amino acid changes on the 56 kDa gametocyte antigen on oocyst wall from the seven *Eimeria* species were selected and synthesized Biomatik Corporation. They were biotinylated. All synthesized peptides were analysed by high-performance liquid chromatography (HPLC) and mass spectrometry by the manufacturers and the purity levels was >55 %. Synthesized peptides were dissolved according to the charge of the peptide as outlined in Figure 2.7. Five of the peptides were dissolved in sterile distilled water, 3 peptides were dissolved in methanol + DMSO while 1 peptide

was dissolved in methanol only (table 2.9). They were then diluted to a stock concentration of 1 mg/ml according to the manufacturer's instructions. Whenever required, stock solutions were diluted to obtain the desired concentration for ELISA assay using the plate coating buffer.

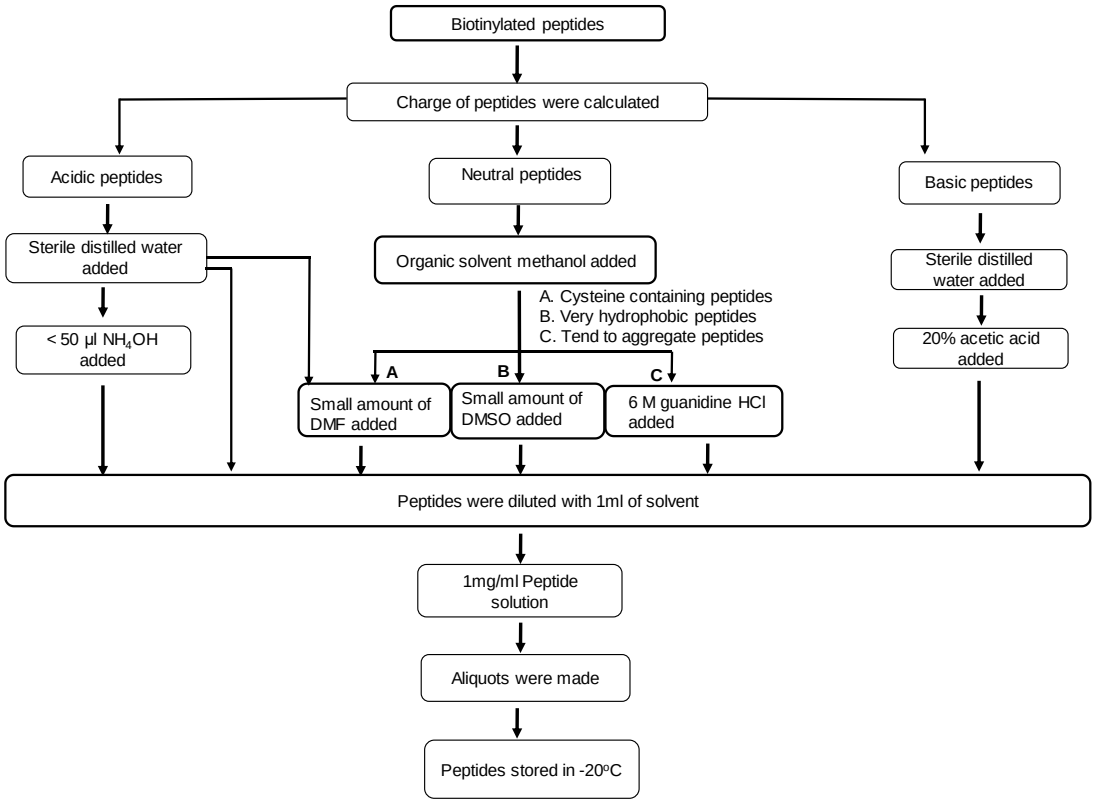


Figure 2:7: Flowchart of the strategies employed for dissolving biotinylated peptides. The number of basic peptides residues such as Arg (R), Lys (K), His (H) and the N- terminal free amine (-NH2) were counted and assigned a value of +1. The number of acidic residues such as Asp (D), Glu (E) and the C- terminal free acid (-COOH) were also counted and assigned a value of -1. The overall charge of each peptide was calculated. If the overall value was positive, then peptide was considered basic. If the overall value was negative, then the peptide was considered acidic. If the overall value was zero, the peptide was considered neutral.

2.8.2.1 Peptide preparation

The charge for individual synthetic peptides were calculated and the peptides were dissolved according to the charge of the peptide as outlined (Table 2.9). They were further diluted to a stock concentration of 10 mg/ml.

Table 2:9: Calculated peptide charge and solvents used to dissolve individual peptides

Species	Peptide	Calculated charge	Solvent
Eimeria mitis	QSFSLPIPIDRPL	Basic	Sterile distilled water
Eimeria necatix [1]	QSMAVPTTVENTV	Neutral/ hydrophobic	Methanol +DMSO
Eimeria necatrix[2]	MDSTPDFPAFNEPM	Acidic	Sterile distilled water
Eimeria acervulina	PAMAVPSTTSVESTVL	Neutral/ hydrophobic	Methanol + DMSO
Eimeria brunetti	PSLALPSSSAAEPAAVH	Basic	Sterile distilled water
Eimeria maxima	PSMAVPSTTPVENQVH	Basic	Sterile distilled water
Eimeria praecox	QSLALPTPIDRAF	Neutral	Methanol
Eimeria tenella[1]	QSLAVPTTVENTV	Neutral/hydrophobic	Methanol + DMSO
Eimeria tenella[2]	MDTMPEFPPFAEGM	Acidic/hydrophobic	Sterile distilled water + DMSO

2.8.3 Growing of V_HH Camelid phage for panning

The library was grown for panning exactly as described in 2.7.1 above

2.8.4 Round 1 panning

For round 1 panning, 100 µl of 10 µg/ml of all nine peptide types in PBS; including the two peptide types for *E. tenella* and *E. necatrix* were coated, 6 reps per peptide on wells of Pierce™ Streptavidin Coated plates Clear, 96-well ® and was allowed to bind overnight, static at room temperature. The plates were beforehand rinsed three times with PBS as per manufacturer's instructions. The next day, plate washing, blocking, phage blocking and incubation were carried out as previously described in subsection 2.7.3. Phage elution, spread on bioassay dishes, scraping of colonies and storage were also carried out as described in subsection 2.7.3.

2.8.5 Round 2 biopanning

For round 2 panning, a pre-panning subtractive step was carried out whereby the peptides were subtracted from the other peptides of the same type. The sub-libraries from each of the nine peptides produced from round 1 panning were re-grown and phage prepared as previously described in subsection 2.7.4.

Six reps of 100 µl, 50 µg/ml of individual peptides were coated on 96 well Pierce™ Streptavidin coated plates and were allowed to bind overnight, static at room temperature. The next day, the plates were washed once with 400 µl 1 XPBS and subsequently blocked with 3% Marvel and PBS (w/v) for one hour while the seven phage suspension were blocked for one hour with 200 µl of 18% Marvel and 6X PBS (w/v) per ml of phage. Subtraction was carried out by incubating phage from each of the seven related bound peptides with the other six species sequentially for 15 minutes each before finally incubating the subtracted phage with the peptide that it was panned against in round 1. The other two related peptides were subtracted one against the other (using 6 x 15min sequential incubations with the non-target immobilised peptide). These subtraction steps should ideally remove non-specific binders from the phage pool to maximise the chances of isolating phage V_HH specific to the target peptide. The plate was then washed fifteen times with PBST (PBS containing 0.5% Tween 20) and then five times using dH2O. The phage were eluted from the peptides by adding mid-log TG1 cells followed by incubation, pooling of eluates, spreading in bioassay dishes, titration of eluted phage, incubation overnight, scraping from the bioassay dishes the next day, adding of glycerol stock and storage at -80°C as has been previously described in subsection 2.7.3.

2.8.6 Round 3 biopanning

A pre-panning subtractive step and post-panning subtraction were carried out for round 3 panning. Input phage was grown exactly as was grown for round 2 panning. 96 well Pierce™ Streptavidin coated plates were coated with six reps of 100 µl, 50 µg/ml all nine peptides in duplicate plates overnight at room temperature static. A pre-panning subtractive step was carried out exactly as was described in round 2 panning. Elution was carried out by a pH shift using glycine since a post-panning subtraction was going to be carried out using the eluted phage; elution using TG1 cells was not feasible. Glycine, 100 µl of 0.1M at a pH of 4.2 was added to individual wells for not more than 10 minutes and then immediately neutralised with an equal volume of 0.1M Tris buffer. The eluate from the six reps of peptides were again subtracted against the other six non-target peptides for the seven related peptides; 15 minutes of

subtraction from each of the non-target peptide before pooling the reps of eluates for each peptide. The eluates of the other two related peptides were subtracted from one another for 15 minutes each before being collecting and pooling the 6 reps of each peptide together. Half of each of the eluate pool were transferred to a sterile eppendorf and the remaining half were incubated with 1 ml of TG1 and left for 30 minutes static at 37 ^{o}C and then another 30 minutes shaking at 37°C. Eluted phage titration was carried out as has been described and eluted phage incubated with TG1 were spread on bioassay dishes containing 2YT agar and 150 µg/ml ampicillin, 1% (w/v) glucose, and grown at 37°C overnight. Scraping, and storage were carried out exactly as described in 2.8.5 above.

2.8.7 Screening of round 3 panning output phage

Round 3 panning output phage was grown for all nine peptides using the same protocol as described in 2.7.4 above. The phage was then screened against all peptides and all screens involved screening the output phage for each peptide with the peptide it was panned against (positive control, six replicates) as well as screening against the other six peptides (negative controls), this was for the seven related peptides. For the two related peptide, the other peptide (6 replicates) served as the negative control for each one. 96 well Pierce™ Streptavidin coated plates were used for the screens as described for rounds 1 and 2 but with no subtraction steps and using pH shift to elute bound phage. Phage was transferred to sterile eppendorfs and 40% glycerol was added and then stored at -80°C until needed. Eluted phage was also titred.

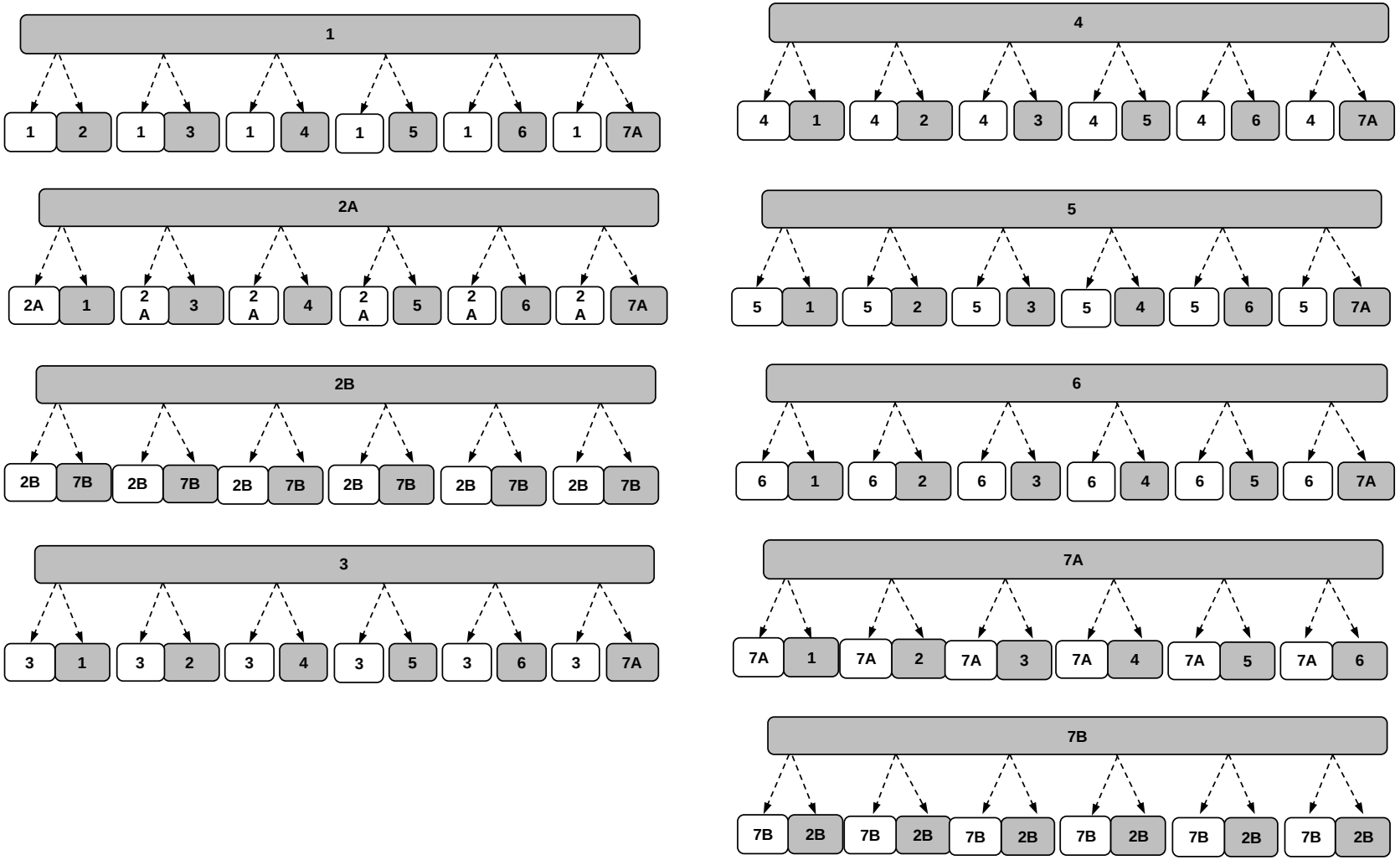


Figure 2:8: Schematic representation of V_HH – peptide screening after 3 rounds of panning. Each phage-V_HH sub library isolated against one of peptides 1, 2A, 3, 4, 5, 6, or 7A (large grey rectangles) were screened against themselves (6 replicates, white squares) and against each of the six control non-target

peptides (grey squares). For peptides 2B and 7B (large grey rectangles) were screened against themselves (positive sample, six replicates, white squares) and the other peptide (negative sample, six replicates, grey squares).

2.8.8 NGS analysis of V_HH-peptide panning

The data was analysed in a similar way to that described in section 2.78 where 7 datasets for related peptide targets from the 56 kDa gametocyte antigen were screened and analysed against each other (6 comparative analyses for each peptide target). In addition, two other target peptides that were related to each other from the 56 kDa gametocyte antigen were screened and analysed against each other (6 sets of analyses for each target peptide). The HCDR3s that had a Z score of above 3 and were seen in at least 3 barcodes out of 6 were selected as described in subsection 2.7.8. The HCDR3s that had the top 5 Z scores were selected. The final list of CDR3s were screened to see that were not also enriched in datasets for panning targeting other peptides, if found to be enriched in panning targeting other peptides, they were not selected.

2.8.9 Rescue and screening of V_HH-peptide clones

Primers for V_HH synthesis of V_HH-peptide panning were designed using the same protocol described in section 2.7.9 and phagemid clones were synthesized the same way as in section 2.7.10. Phage were grown for ELISA using the protocol described in section 2.7.7.2 and phage ELISA was carried out exactly as described in 2.7.7.1.

Table 2:10: Inverse PCR primers for phagemid synthesis of VHH selected against oocyst wall peptides

lLQqKIPRSNKLHIKP	ILQQKIPRSNKLHIKP	TCTAACAAACTGCACATCAAACCGTGGGGACAAGGGACGCAG	ACGCGGGATTTTCTGCTGCAGGATCGCAGCGCAATAGTAGAT
GTNFDMSVMSqLHNLLRFEqS	GTNFDMSVMSQLHNLLRFEQS	AGCTGCACAACCTGCTGCGTTTCGAACAGTCTTGGGGACAAGGGACGCAG	GAGACATAACAGACATGTCGAAGTTGGTACCCGCAGCGCAATAGTAGAT
MPISNPGLQLMTSNDG	MPISNPGLQLMTSNDG	CAGCTGATGACCTCTAACGACGGTTGGGGACAAGGGACGCAG	CAGACCCGGGTTAGAGATCGGCATCGCAGCGCAATAGTAGAT
SLNYPRTqHRRKQKNE	SLNYPRTQHRRKQKNE	CACCGTCGTAAACAGAAAAACGAATGGGGACAAGGGACGCAG	CTGGGTACGCGGGTAGTTCAGAGACGCAGCGCAATAGTAGAT
MPNYITPQNVKFLECR	MPNYITPQNVKFLECR	AACGTTAAATTCCTGGAATGCCGTTGGGGACAAGGGACGCAG	CTGCGGGGTGATGTAGTTCGGCATCGCAGCGCAATAGTAGAT
VKGQTSGRKNYVNGMREARLq	VKGQTSGRKNYVNGMREARLQ	ACGTTAACGGTATGCGTGAAGCTCGTCTGCAGTGGGGACAAGGGACGCAG	AGTTTTTACGACCAGAGGTCTGACCTTTAACCGCAGCGCAATAGTAGAT
FPTSSSVAHLRPRSRC	FPTSSSVAHLRPRSRC	CACCTGCGTCCGCGTTCTCGTTGCTGGGGACAAGGGACGCAG	AGCAACAGAAGAAGAGGTCGGGAACGCAGCGCAATAGTAGAT
QWGVCLRLFIRVLLMGLIRLqW	QWGVCLRLFIRVLLMGLIRLQW	GTTCTGCTGATGGGTCTGATCCGTCTGCAGTGGTGGGGACAAGGGACGCAG	ACGGATGAACAGACGCAGGCAAACACCCCACTGCGCAGCGCAATAGTAGAT
qWFFqTSLRTHS	QWFFQTSLRTHS	TCTCTGCGTACCCACTCTTGGGGACAAGGGACGCAG	GGTCTGGAAGAACCACTGCGCAGCGCAATAGTAGAT
MPISNPGLQLMTSNDG	MPISNPGLQLMTSNDG	CAGCTGATGACCTCTAACGACGGTTGGGGACAAGGGACGCAG	CAGACCCGGGTTAGAGATCGGCATCGCAGCGCAATAGTAGAT
PHLKFPVLMFCqHHGS	PHLKFPVLMFCQHHGS	ATGTTCTGCCAGCACCACGGTTCTTGGGGACAAGGGACGCAG	CAGAACCGGGAATTTCAGGTGCGGCGCAGCGCAATAGTAGAT
HLVKRSqqNQTSCDVR	HLVKRSQQNQTSCDVR	AACCAGACCTCTTGCGACGTTCGTTGGGGACAAGGGACGCAG	CTGCTGAGAACGTTTAACCAGGTGCGCAGCGCAATAGTAGAT
YSCQNASVWTPM	YSCQNASVWTPM	TCTGTTTGGACCCCGATGTGGGGACAAGGGACGCAG	AGCGTTCTGGCAAGAGTACGCAGCGCAATAGTAGAT
RPAqIDASDSSLVqRq	RPAQIDASDSSLVQRQ	GACTCTTCTCTGGTTCAGCGTCAGTGGGGACAAGGGACGCAG	AGAAGCGTCGATCTGAGCCGGACGCGCAGCGCAATAGTAGAT
SEFHSKNQHqCLADTS	SEFHSKNQHQCLADTS	CACCAGTGCCTGGCTGACACCTCTTGGGGACAAGGGACGCAG	CTGGTTTTTAGAGTGGAATTCAGACGCAGCGCAATAGTAGAT

CGATqTRGRFITF PASPYASq	CGATQTRGRFITF PASPYASQ	TCACCTTCCCGGCTTCTCCGTACGCTTCTC AGTGGGGACAAGGGACGCAG	TGAAACGACCACGGGTCTGGGTAGCACCG CACGCAGCGCAATAGTAGAT
SKCLHTLSRSqLH PHFQFHDI	SKCLHTLSRSQLH PHFQFHDI	AGCTGCACCCGCACTTCCAGTTCCACGACA TCTGGGGACAAGGGACGCAG	GAGAACGAGACAGGGTGTGCAGGCATTTA GACGCAGCGCAATAGTAGAT
DKFLAqHVDqTFq qHTHYQDA	DKFLAQHVDQTF QQHTHYQDA	CCTTCCAGCAGCACACCCACTACCAGGAC GCTTGGGGACAAGGGACGCAG	TCTGGTCAACGTGCTGAGCCAGGAATTTGT CCGCAGCGCAATAGTAGAT
YYSIPLqDNTWV	YYSIPLQDNTWV	CAGGACAACACCTGGGTTTGGGGACAAGG GACGCAG	CAGCGGGATAGAGTAGTACGCAGCGCAAT AGTAGAT
AqPIRGKVTIVL	AQPIRGKVTIVL	AAAGTTACCATCGTTCTGTGGGGACAAGGG ACGCAG	ACCACGGATCGGCTGAGCCGCAGCGCAAT AGTAGAT
QYTqKKAGNqTKK PHPKRKAA	QYTQKKAGNQTK KPHPKRKAA	CCAAAAAACCGCACCCGAAACGTAAAGCTG CTTGGGGACAAGGGACGCAG	TCTGGTTACCAGCTTTTTTCTGGGTGTACT GCGCAGCGCAATAGTAGAT
DYGPCGCYLEQL VqGGCLGGIA	DYGPCGCYLEQL VQGGCLGGIA	CTGGTTCAGGGTGGTTGCCTGGGTGGTAT CGCTTGGGGACAAGGGACGCAG	CTGTTCCAGGTAGCAACCGCACGGACCGT AGTCCGCAGCGCAATAGTAGAT
ITAWGAPSQRDL	ITAWGAPSQRDL	CCGTCTCAGCGTGACCTGTGGGGACAAGG GACGCAG	AGCACCCCAAGCGGTGATCGCAGCGCAAT AGTAGAT
PAIqPTRSYARHG PPR	PAIQPTRSYARHG PPR	TACGCTCGTCACGGTCCGCCGCGTTGGGG ACAAGGGACGCAG	AGAACGGGTCGGCTGGATAGCCGGCGCA GCGCAATAGTAGAT
IERNERPDLPSER MNSATqSL	IERNERPDLPSER MNSATQSL	CTGAACGTATGAACTCTGCTACCCAGTCTC TGTGGGGACAAGGGACGCAG	ACGGCAGGTCCGGACGTTCGTTACGTTCG ATCGCAGCGCAATAGTAGAT
QHRPRPHRPTKR RKSY	QHRPRPHRPTKR RKSY	CCGACCAAACGTCGTAAATCTTACTGGGGA CAAGGGACGCAG	ACGGTGCGGACGCGGACGGTGCTGCGCA GCGCAATAGTAGAT
VNSKRKHWHRSR	VNSKRKHWHRSR	CACTGGCACCGTTCTCGT TGGGGACAAGGGACGCAG	TTTACGTTTAGAGTTAACCGCAGCGCAATA GTAGAT
KMPqTPNKKPHR KWKR	KMPQTPNKKPHR KWKR	AAACCGCACCGTAAATGGAAACGTTGGGGA CAAGGGACGCAG	TTTGTTCGGGGTCTGCGGCATTTTCGCAGC GCAATAGTAGAT
HKSRDGKTSKNH qLLKQKHLK	HKSRDGKTSKNH QLLKQKHLK	ACCACCAGCTGCTGAAACAGAAACACCTGA AATGGGGACAAGGGACGCAG	TTTTAGAGGTTTTACCGTCACGAGATTTGT GCGCAGCGCAATAGTAGAT
SqPFGPPMYYKL	SQPFGPPMYYKL	CCGATGTACTACAAACTGTGGGGACAAGG GACGCAG	CGGACCGAACGGCTGAGACGCAGCGCAAT AGTAGAT

WaqYSKPPSTYVYYLGKRYLI	WAQYSKPPSTYVYYLGKRYLI	ACGTTTACTACCTGGGTAAACGTTACCTGATCTGGGGACAAGGGACGCAG	AGGTAGACGGCGGTTTAGAGTACTGAGCCCACGCAGCGCAATAGTAGAT
MLHSSEFlqHSYGDND	MLHSSEFIQHSYGDND	CAGCACTCTTACGGTGACAACGACTGGGGACAAGGGACGCAG	GATGAATTCAGAAGAGTGCAGCATCGCAGCGCAATAGTAGAT
ASSNSVHLSEqPLHHP	ASSNSVHLSEQPLHHP	TCTGAACAGCCGCTGCACCACCCGTGGGGACAAGGGACGCAG	CAGGTGAACAGAGTTAGAAGAAGCCGCAGCGCAATAGTAGAT
qTKANMKGLqTPGYGqLGLKF	QTKANMKGLQTPGYGQLGLKF	CCCCGGGTTACGGTCAGCTGGGTCTGAAATTCTGGGGACAAGGGACGCAG	TCTGCAGACCTTTCATGTTAGCTTTGGTCTGCGCAGCGCAATAGTAGAT
NRGHqSPIQYTRGMTGRGRHS	NRGHQSPIQYTRGMTGRGRHS	CCCGTGGTATGACCGGTCGTGGTCGTCACTCTTGGGGACAAGGGACGCAG	TGTACTGGATCGGAGACTGGTGACCACGGTTCGCAGCGCAATAGTAGAT
PEPTWRNAQVqFWSRLPTMRN	PEPTWRNAQVQFWSRLPTMRN	AGTTCTGGTCTCGTCTGCCGACCATGCGTAACTGGGGACAAGGGACGCAG	GAACCTGAGCGTTACGCCAGGTCGGTTCCGGCGCAGCGCAATAGTAGAT
MYERVYSLYYqLFSNLLMSDY	MYERVYSLYYQLFSNLLMSDY	AGCTGTTCTCTAACCTGCTGATGTCTGACTACTGGGGACAAGGGACGCAG	GGTAGTACAGAGAGTAAACACGTTCGTACA T CGCAGCGCAATAGTAGAT
YYLSVqHGAVCTSDSNNNDAK	YYLSVQHGAVCTSDSNNNDAK	GCACCTCTGACTCTAACAACAACGACGCTAAATGGGGACAAGGGACGCAG	AAACAGCACCGTGCTGAACAGACAGGTAGTACGCAGCGCAATAGTAGAT
YTGLRWLWqPITCqMPDSNLC	YTGLRWLWQPITCQMPDSNLC	TCACCTGCCAGATGCCGGACTCTAACCTGTGCTGGGGACAAGGGACGCAG	TCGGCTGCCACAGCCAACGCAGACCGGTGTACGCAGCGCAATAGTAGAT
RNHLPKqRKRIPKqNT	RNHLPKQRKRIPKQNT	AAACGTATCCCGAAACAGAACACCTGGGGACAAGGGACGCAG	ACGCTGTTTCGGCAGGTGGTTACGCGCAGCGCAATAGTAGAT
KLQqTYVVRSqASRSGCMMCT	KLQQTYVVRSQASRSGCMMCT	AGGCTTCTCGTTCTGGTTGCATGATGTGCACCTGGGGACAAGGGACGCAG	GAGAACGAACAACGTAGGTCTGCTGCAGTTTCGCAGCGCAATAGTAGAT
NRGHqSPIQYTPYFNRqLFNH	NRGHQSPIQYTPYFNRQLFNH	CCCCGTACTTCAACCGTCAGCTGTTCAACCACTGGGGACAAGGGACGCAG	TGTACTGGATCGGAGACTGGTGACCACGGTTCGCAGCGCAATAGTAGAT
WRVTHRHHqPHRRRPR	WRVTHRHHQPHRRRPR	CAGCCGCACCGTCGTCGTCCGCGTTGGGGACAAGGGACGCAG	GTGGTGACGGTGGGTAACACGCCACGCAGCGCAATAGTAGAT
SqPFGPPMYYKL	SQPFGPPMYYKL	CCGATGTACTACAAACTGTGGGGACAAGGGACGCAG	CGGACCGAACGGCTGAGACGCAGCGCAATAGTAGAT
SIPTHPPMTLTFHNQR	SIPTHPPMTLTFHNQR	ACCCTGACCTTCCACAACCAGCGTTGGGGACAAGGGACGCAG	CATCGGCGGGTGGGTCGGGATAGACGCAGCGCAATAGTAGAT

2.9 Assessment and optimisation of bioinformatics analysis methods

2.9.1 scFv Flanking region changes

The typical flanking regions around the Griffin scFvs HCDR3 are 'VYYCAR' at the 5' end and 'WGQGT' at the 3' end. In chapter 3, the bioinformatics analysis permitted single amino acid changes [^amino acid] in the expected HCDR3 flanking sequences. Only the heavy chain was amplified in preparation for the NGS analysis and further degeneracy could be permitted within the flanking regions without contaminating the data with light chain sequences. In **chapter 7**, the Griffin bioinformatics data analysis permitted zero and two changes in the flanking regions and the left and right flanking sequences text files were changed accordingly. The text files are shown in Appendix VI.

2.9.2 Using V_HH flanking region changes to predict error rates caused by amplification and sequencing

The error rates caused by amplification and sequencing of the conserved V_HH library were assessed by permitting up to 1 or 2 changes within the flanking sequences in both V_HH/oocyst lysate and V_HH/peptide panning. The original datasets allowed no amino acid sequence change in each of the flanking regions (IYYCAA and WGQGT) because the V_HH library had a conserved V_HH scaffold and varied only in the HCDR3s. Changes in the number of the HCDR3s identified might point to sequencing errors. The left and right flanking sequence text files were changed as required and are shown in Appendix VII.

2.9.3 Reanalysing V_HH data using percentage frequency and comparison of percentage frequency of known scFvs binders versus V_HH selected rescues

To determine the possibility of a candidate frequency-based bioinformatics analysis changing the candidate binders that were identified, the V_HH NGS datasets of **chapter 5** (panning against oocyst lysates) and **chapter 6** (panning against distinct peptide regions of Gam56 protein), were reanalysed by ranking HCDR3s according to percentage frequency in positive replicates. This would select for binders without imposing comparative enrichment criteria in the bioinformatic steps. The frequency values of scFvs from **chapter 3** that were true binders were also determined. These frequency values were scrutinised to find out whether percentage frequency of scFvs have any relationship with positive ELISA results while comparing to published results to find what percentage of frequency reflect true binders.

2.9.4 Reanalysing V_HH data using an alternative *in silico* analysis

Another *in silico* analysis was performed to further identify HCDR3s that were binding to oocysts of the seven *Eimeria* species during NGS analysis but cannot be verified by ELISA possibly due to the latter having a lower assay sensitivity. In this analysis, HCDR3s were selected in a training cohort of samples using the criteria 1) Z scores >3 in positive replicates 2) those identified in at least 2 out of 3 positive replicates which served as the 'training' cohort. HCDR3s identified that met these criteria were then screened in a test cohort (n=3) of samples under the same criteria.

2.10 Statistical analysis

All statistical analysis was carried out using GraphPad Prism software version 7 (version 7 San Diego, CA). For the comparison of rescued antibody clones with controls, samples were analysed in duplicates or triplicates on ELISA plates and the average value taken. Data were analysed by two-way ANOVA, and were considered to be of statistical significance when the p value is ≤ 0.05. Data are presented as mean ± SD and differences among groups were determined using Tukey's multiple comparative test

3. The isolation of antibodies to *Eimeria* using conventional phage display and Next generation phage display technology

3.1 Introduction

Poultry coccidiosis caused by the protozoan parasite *Eimeria* is a major economic problem for the poultry industry worldwide causing substantial losses (Blake and Tomley, 2014). Given that the parasite is predominantly host specific and immunity is species specific with each species varying in impact and pathogenicity, accurate diagnostics at species level are essential for the control of coccidiosis. Effective and accurate species level diagnostic assays are important because they have major implications for control especially in the development and application of vaccines given that species of *Eimeria* are propagated and included in current live vaccines, making them expensive (Shirley *et al.,* 2005).

There is a heavy reliance on traditional methods of diagnosis such as observation for clinical signs, faecal microscopy and lesion scoring even though these methods are time consuming, highly subjective, require expertise for species-level diagnosis and they are not very efficient at diagnosing sub-clinical infections (Haug *et al.,* 2007).

In terms of molecular tests, Barkway *et al.* (2015) developed loop mediated isothermal amplification (LAMP) assays to successfully diagnose *Eimeria* infections at the species level in poultry which is less subjective and does not require expensive laboratory equipment. However, this technique involved carrying out a post-mortem examination and cutting out sections of the intestine that are predilection sites for the individual *Eimeria* species and scraping out mucosal cells for use as the source of DNA template for the analysis. While this is a major improvement in the species level diagnosis of poultry coccidiosis in terms of cost, ease of use and with no major equipment requirements, it still involves a time-consuming sampling process. Being able to know and dissect out predilection sites of infecting *Eimeria* species in chickens also still requires expertise.

There is a need to develop an easier, quicker, cheaper, high through put assay for speciating *Eimeria* infections in poultry that will help differentiate infecting *Eimeria* species and ideally that can be applied in the field. This is particularly required because of the tight economic margin prevalent in present day poultry production.

Surface display technology dates back to 1985 when George P. Smith discovered that bacteriophage (phage) coat proteins could be modified to express recombinant peptides (Smith, 1985). Since then, phage display has been a widely used and successful platform for antibody discovery and screening. Phage display has enabled the isolation of many monoclonal antibodies (MAbs) against a wide variety of antigens from synthetic and naïve combinatorial libraries (Clementi *et al.,* 2012). Phage has

the advantage of bypassing the need to immunize animals hence removing bias towards immunodominant epitopes (Griep, 1999).

Phage display coupled with next generation sequencing allows for a thorough characterization of the phage-displayed library sequences isolated by biopanning which has the potential to enhance ligand identification (Rentero Rebollo *et al*., 2014, Ngubane *et al*., 2013).

In this study, we sought to isolate antibodies to individual species of *Eimeria* of poultry, which would be used to develop high throughput lab-based diagnostics, with the longer-term aim to develop in-field diagnostics. We approached the study using the Griffin library; a single-pot semi-synthetic library to select for antibodies against oocysts from the seven species of *Eimeria*. Single-pot libraries have the advantage of being able to select antibodies to a large number of targets because of their unbiased nature. Biopanning, which is the process of affinity selection, was carried out and phage particles were selected based on their binding ability to whole oocysts. Typically 3-5 cycles of selection are necessary to get the desired level of enrichment (Bradbury and Marks, 2004, Parola *et al.,* 2018). The strategy of panning employed in this study was aimed at maximising enrichment of antibody to whole oocysts and hence five rounds of panning were carried out. The analysis involved traditional random colony picks of phage and also sequencing of genes from bound phage from all five rounds of panning against all species of *Eimeria.* For NGS data, Z score analysis was used to rank the HCDR3s according to the extent of their enrichment against each *Eimeria* species oocysts and comparison was made between enrichment during growth only phases compare to growth+selection phases.

3.2 Results

3.2.1 scFv Phage display; panning for binders to chicken *Eimeria* oocysts

3.2.1.1 Quantification of Eimeria oocysts

The first batch of *Eimeria* oocysts obtained for this research were quantified and numbers of oocysts for each of the seven species were between $3.5x10^7$ and $3.3x10^8$ in 50 ml (Table 3.1).

Table 3:1: Concentrations of oocysts in *Eimeria* samples counted with the haemocytometer

Species	Concentration per ml	Total oocyst number (in 50 ml)
E. mitis	6.6×10^6/ml	**3.3×10^8**
E. necatrix	1.4×10^6/ml	**6.8×10^7**
E. acervulina	4.9×10^6/ml	**2.5×10^8**
E. brunetti	7.0×10^5/ml	**3.5×10^7**
E. maxima	2.2×10^6/ml	**1.1×10^8**
E. praecox	1.0×10^6/ml	**5.0×10^7**
E. tenella	2.9×10^6/ml	**1.4×10^8**

3.2.1.2 Preparation and titre of phage

The Griffin phage was prepared for panning experiments. Phage were acid precipitated and titred, the titre was 10^{13} PFU/ml. At other times, phage supernatant was used without precipitation for screening polyclonal and monoclonal phage as well as the subsequent panning rounds titres were found to be around 10^{10} - 10^{11} PFU/ml. These titres are as expected when using exphage as the helper phage (Baek *et al.,* 2002).

3.2.1.3 Panning Rounds

Phage selection was carried out against all seven species of whole oocysts for 5 rounds of panning. Round one input phage was phage from the Griffin library while input phage for subsequent panning rounds was always a pool of the output phage from panning against the seven *Eimeria* species in the preceding round. Input phage had titres of between 8.26×10^{10} and 7.4×10^{13} PFU/ml. The eluted phage across all panning rounds and *Eimeria* species were calculated and the average taken from at least three species and titres were between 8.7×10^5 and $4.3x\ 10^7$ PFU/ml (Table 3.2). The phage titres indicated enrichment over each of rounds 1 to 4.

Table 3:2: Panning results of Griffin library against *Eimeria* oocysts

Panning round	**No of oocysts**	**Input phage (PFU/ml)**	**Average output phage (PFU/ml)**	**Bound fraction (10^{-4}) (% recovery)**	**Enrichment from previous round**
1	4×10^5	7.4×10^{13}	3.4×10^7	0.459	-
2	4×10^5	8.2×10^{11}	8.6×10^6	10.49	22.9
3	4×10^5	2.1×10^{11}	3.5×10^7	166.7	15.89
4	4×10^5	1.8×10^{11}	4.3×10^7	238.9	1.433
5	4×10^5	8.26×10^{10}	8.7×10^5	33.5	0.140

Where % phage bound = (output/input) X 100

Enrichment = fold increase of % phage bound compared to the previous round (Abi-Ghanem *et al.,* 2008).

3.2.1.4 Preparation of DNA for Ion Proton Sequencing

DNA was extracted from bacterial glycerol stocks from the original Griffin library (1 sample) and each of the 5 rounds of panning against the seven species of *Eimeria* (35 samples) and also from each of the grown phage used as input phage for each round of panning (5 samples). DNA was extracted from all samples (totalling 41) for use as template for Heavy chain CDR3 (HCDR3) amplifications. The grown input phage for the five rounds of panning and DNA from the 35 samples from the rounds of panning as well as the original Griffin library stock were quantified using the Nanodrop. Extracts were labelled E# for species and R# for rounds of panning. DNA concentrations for all samples were between 16 and 150 µg/ml.

The phagemid DNA was used as template for the amplification of a ~350 bp amplicon spanning the HCDR3 region. Representative samples are shown in figures 3.1 and 3.2 for the 2-step PCR process. A unique barcode sequence was added to each sample along with Ion torrent sequencing tags.

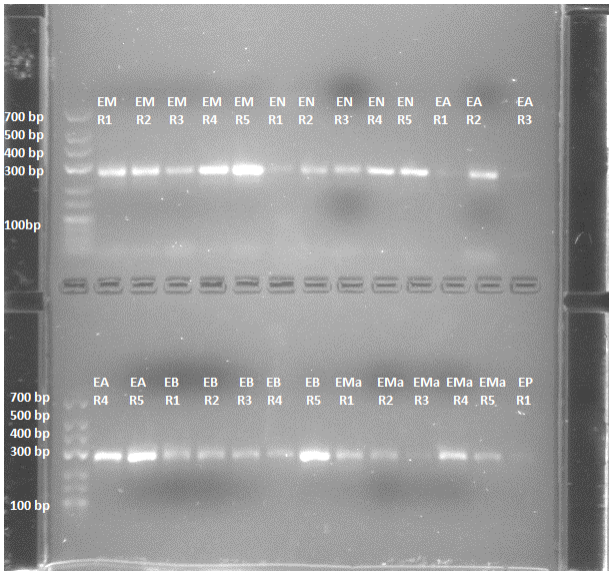


Figure 3:1: An example of first step PCR products for NGPD: *Eimeria*. 10 µl of amplified PCR products were mixed with 2 µl of DNA loading dye and run on a on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Samples were labelled as E# R# representing *species* and panning rounds, respectively, and molecular markers are indicated in bp. EM; *E. mitis* EN; *E. necatrix* EA; *E. acervulina* EB; *E. brunetti* EMa; *Eimeria maxima* EP; *Eimeria praecox*. The round 1 PCR products were then purified and used as a template for round 2 PCR.

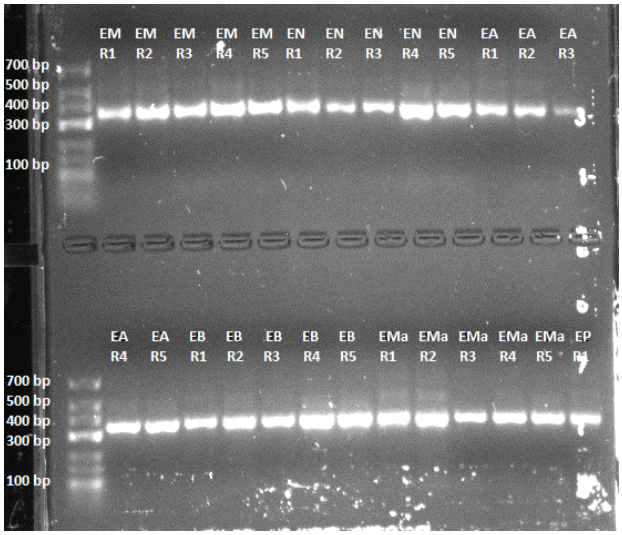


Figure 3:2: An example of second step PCR products for NGPD: 10 µl of amplified PCR products were mixed with 2 µl of DNA loading dye and run on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Samples are labelled as E# R# representing *Eimeria* species and panning rounds, respectively, and molecular markers are indicated in bp. EM; *E. mitis* EN; *E. necatrix* EA; *E. acervulina* EB; *E. brunetti* EMa; *E. maxima* EP; *E. praecox*.

The 41 samples and 1 water control were relabelled 1-43 (barcode 42 was excluded as it has previously been shown to be non-specific) reflecting their barcode primers. *Eimeria mitis* (round 1-5) were tagged with barcodes 1-5, *E. necatrix* (round 1-5) were tagged with barcodes 6-10, *E. acervulina* (round 1-5) were tagged with barcodes 11-15, *E. brunetti* (round 1-5) were tagged with barcodes 16-20, *E. maxima* (round 1-5) were tagged with barcodes 21-25, *Eimeria praecox* (round 1-5) were tagged with barcodes 26-30, *E. tenella* (round 1-5) were tagged with barcodes 31-35. Phage used for round 1-5 were tagged with barcodes 36-40. Griffin library in bacteria was tagged with barcode 41. The water control was tagged with barcode 43. The PCR products from round 2 were then quantified by fluorometry using Qubit and an equal contribution by concentration from each sample was used to produce a total pool of 132 µl, containing 3075 ng DNA.

The pooled DNA sample was purified using Agencourt®AMPure®XP PCR purification kit and then re-quantified by Fluorometry using Qubit, the final concentration was of 55.0 µg/ml representing a 73.3% recovery of sample after purification. The purified sample was visualised on a 2% Agarose Gel (figure 3.3).

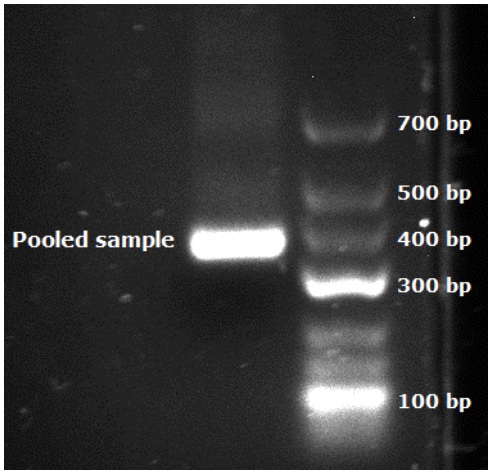


Figure 3:3: Pooled PCR amplicon from phagemid DNA. Pooled PCR amplicon from phagemid DNA. After 2nd stage PCR amplification, the PCR products were quantified by Qubit 2.0 Fluorometer and the DNA from all 41 barcoded samples were pooled in an equal ratio so that the reads will be equalised during NGS. The sample was purified using Agencourt®AMPure®XP and then analysed on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611).

3.2.2. Conventional screening for scFv phage binders

3.2.2.1. Antigen preparation

Whilst panning was carried out against whole intact oocysts, this antigen format does not allow high throughput screening of phage clones. To facilitate the screening, oocyst lysates were used as the antigen. Lysates were produced for each of the

Eimeria species and the protein content determined by Bradford assay (Figure 3.4). Lysates contained between 0.0376 and 0.1560 mg/ml of protein.

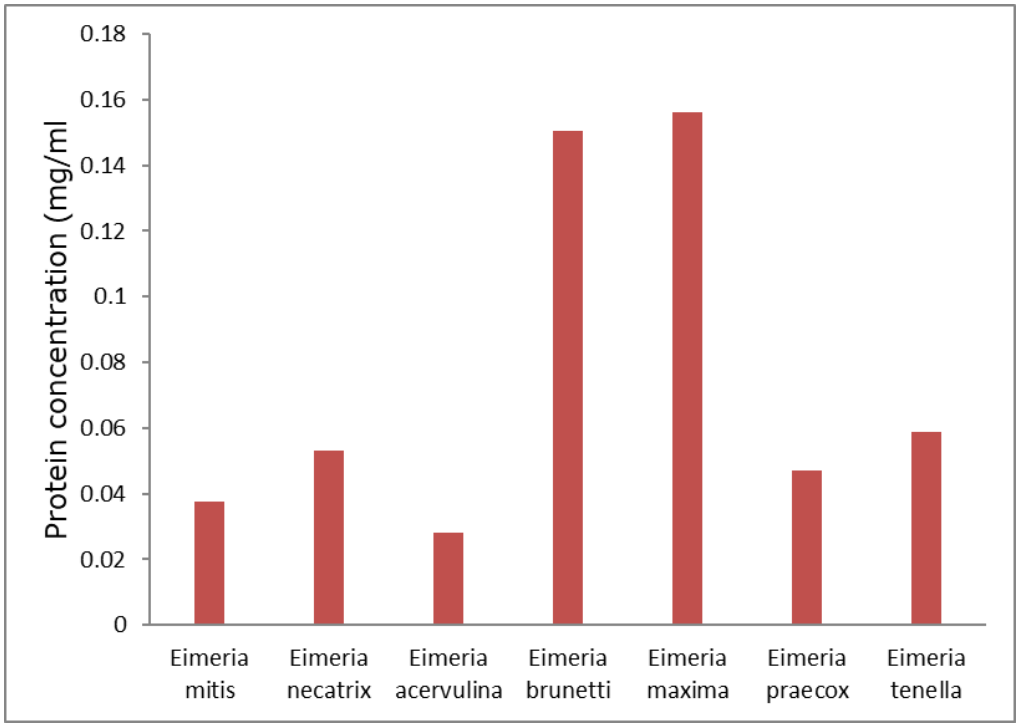


Figure 3:4: Protein concentration of the lysed oocysts of individual *Eimeria* species These were derived from a Bradford protein assay.

3.2.2.2 Polyclonal phage ELISA

Polyclonal phage ELISA was carried out using oocyst lysates as antigen. Phage grown from the Griffin library as well as all five rounds of panning were screened via ELISA. Assay was carried out in Nunc Maxisorp® plates. The signals produced were background signals (data not shown).

3.2.2.3 Colony pick ELISA

Single colonies were picked from round 5 of panning, ten from each of the seven species were grown, phage produced and screened in an ELISA using oocyst lysate antigen. Signals produced in the ELISA were background signals (data not shown).

3.2.2.4 Diversity of the single colony picks assessed by BstNI digestion patterns

In order to estimate the diversity of clones that were picked and screened, the antibody genes were analysed by BstNI digestion and determination of the digestion patterns. An initial PCR was carried out to amplify the genes. The PCR products were

ran on 2% agarose gel to confirm amplicon size which was ~ 1kb (Figure 3.5). BstNI digestion patterns observed were not readily distinguishable with the exception of EP 7 and EP 8 indicating that the panning may have resulted in the isolation of a single dominant clone especially since the clones were picked from the 5th round of panning (Figure 3.6).

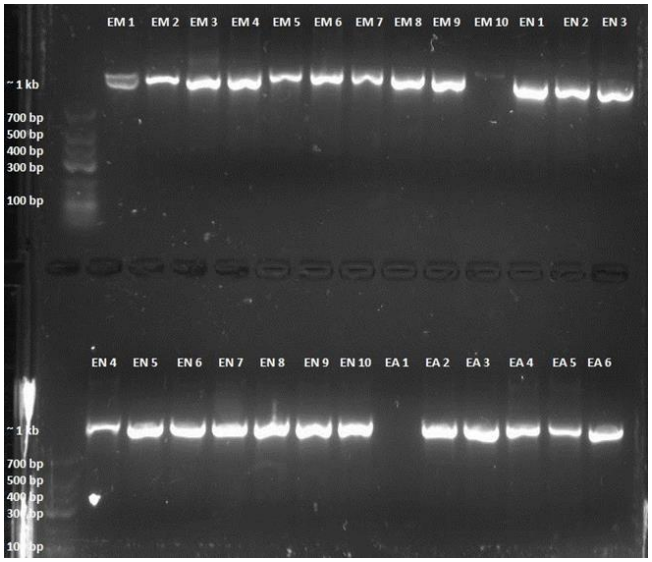


Figure 3:5: An example of amplification of scFv genes for analysis by BstNI digestion. Samples on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Molecular markers are indicated in bp and kb and samples are labelled as E1-10# representing *Eimeria* species and clone, respectively.

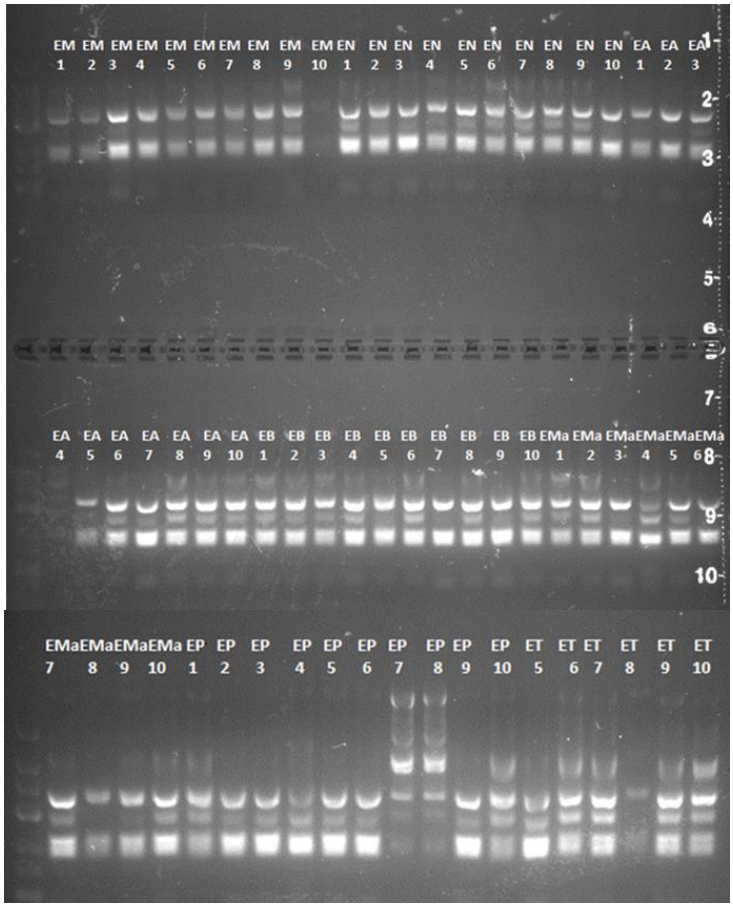
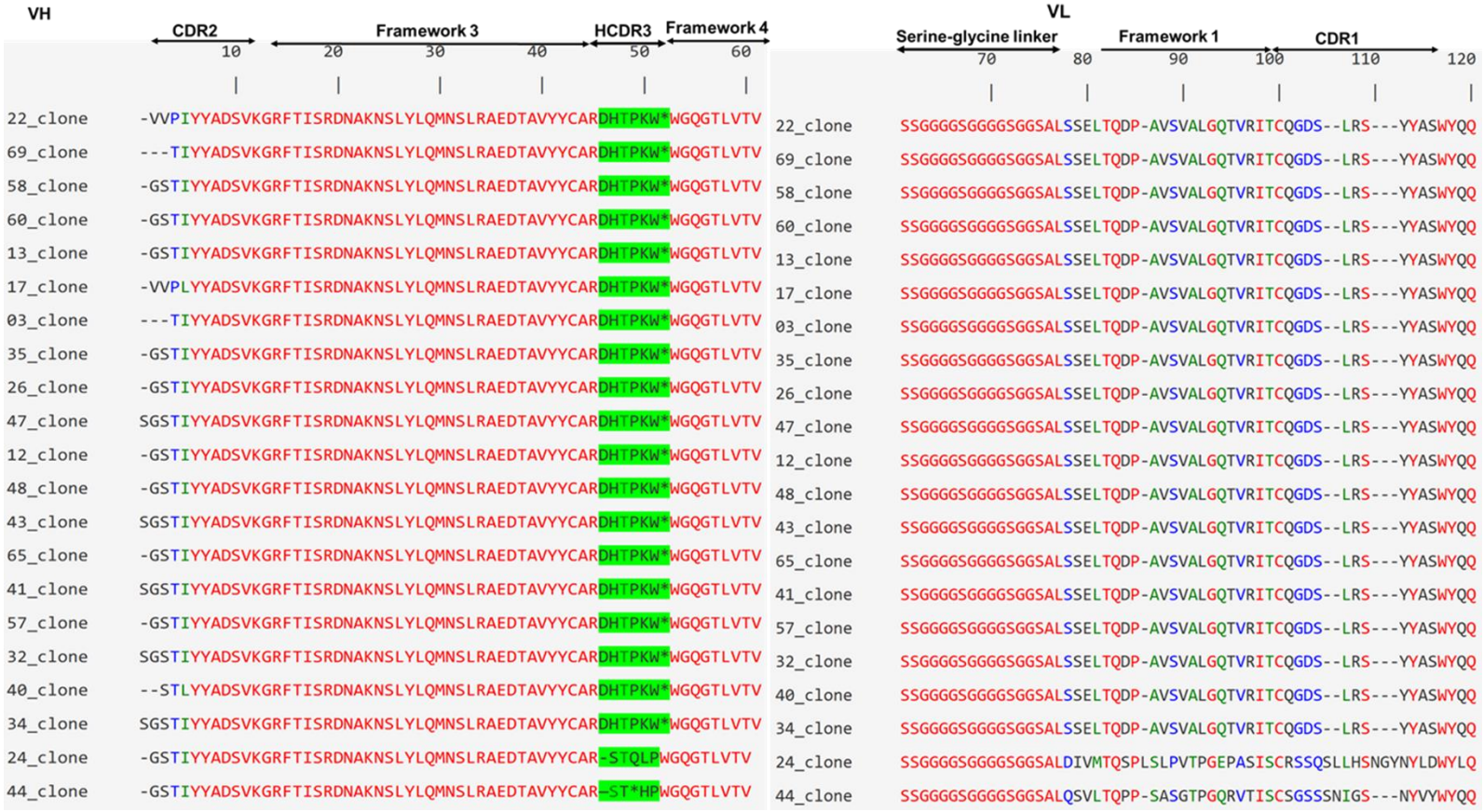


Figure 3:6: Analysis of BstNI digestion pattern of PCR amplicons of scFv genes from the 70 single clones picked after adding 100 µg/ml of BSA, 5 units of BstNI buffer and enzyme followed by 2 hours incubation at 37°C. Samples were analysed in 3% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Samples are labelled as E# 1-10# representing *Eimeria* species and clone, respectively. EM; *E. mitis* EN; *E. necatrix* EA; *E. acervulina* EB; *E. brunetti* EMa; *E. maxima* EP; *E. praecox* ET; *E. tenella*

3.2.2.5 Confirmation of clones by Sanger Sequencing

Given that digestion pattern of seventy single clones failed to clearly distinguish clones, 23 (1/3rd) of the 70 single clones were randomly selected and Sanger sequenced and when translated, 21 out of the 23 clones had the HCDR3 sequence DHTPKWq. Two other clones identified were STQLP (EA 2) and STqHP (EMa 4) (Figure 3.7). This data suggested that about 90% of the selected clones were the same clone after 5 rounds of panning (Table 3.5). This may well reflect that the diversity of clones is greatly reduced with higher numbers of panning rounds (Derda *et al*., 2011).


VH
CDR2
Framework 3
HCDR3
Framework 4
10
20
30
40
50
60
22_clone -VVPIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
69_clone ---TIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
58_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
60_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
13_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
17_clone -VVPLYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
03_clone ---TIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
35_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
26_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
47_clone SGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
12_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
48_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
43_clone SGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
65_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
41_clone SGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
57_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
32_clone SGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
40_clone --STLYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
34_clone SGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDHTPKW*WGQGTLVTV
24_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR-STQLPWGQGTLVTV
44_clone -GSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR-ST*HPWGQGTLVTV
VL
Serine-glycine linker
Framework 1
CDR1
70
80
90
100
110
120
22_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
69_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
58_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
60_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
13_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
17_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
03_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
35_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
26_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
47_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
12_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
48_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
43_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
65_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
41_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
57_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
32_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
40_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
34_clone SSGGGGSGGGGSGGGSALSSELTQDP-AVSVALGQTVRITCQGDS--LRS---YYASWYQQ
24_clone SSGGGGSGGGGSGGGSALDIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQ
44_clone SSGGGGSGGGGSGGGSALQSVLTQPP-SASGTPGQRVTISCSGSSSNIGS---NYVYWYQQ

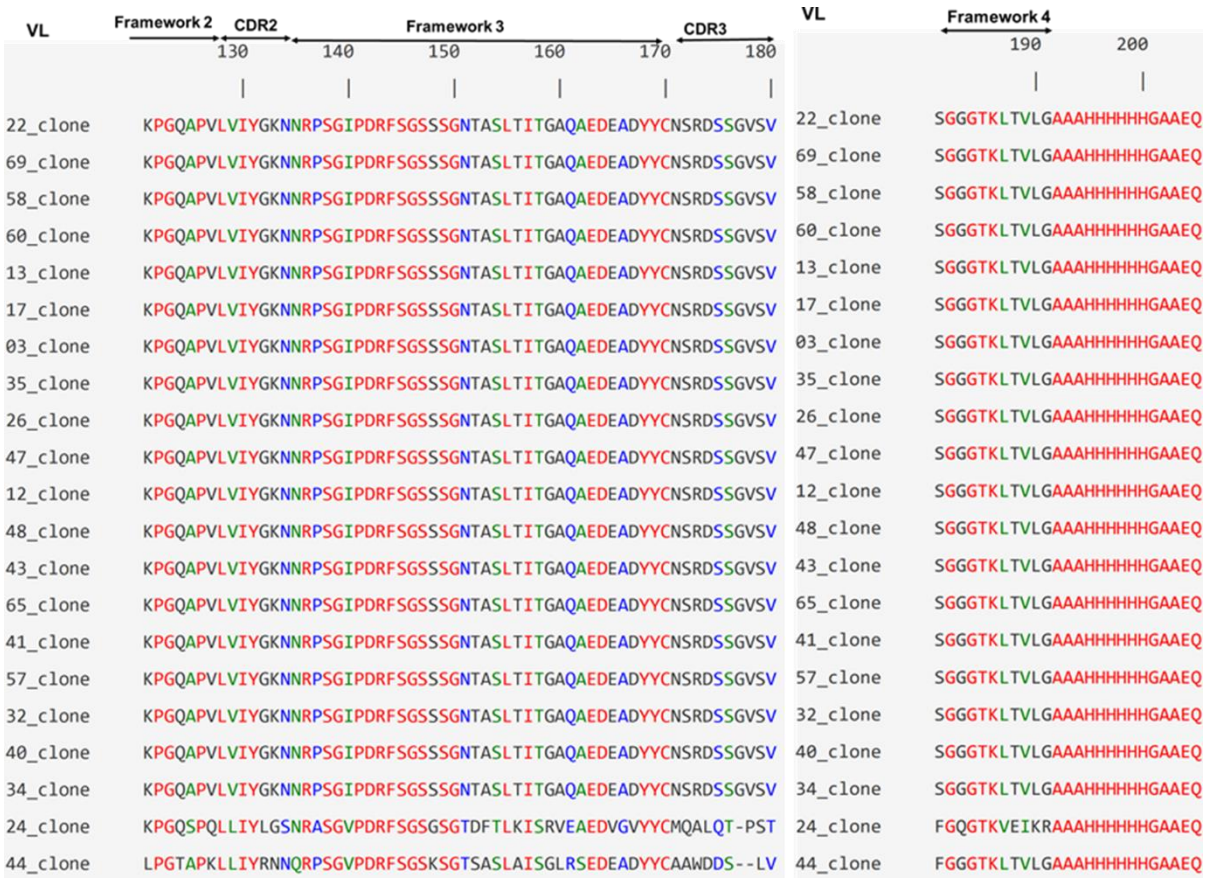


Figure 3:7: Deduced amino acids sequences of scFv antibody fragments from single colony picks raised against *Eimeria* oocysts. The V_H (above) and V_L (below) segments of the scFvs were sequenced as described in subsection 2.3.5.1.5 in Materials and methods **chapter 2.** The HCDR3 sequences are highlighted in green. The GC-rich sequence that links V_H and V_L segments is indicated as well as the framework regions and complementarity determining regions (CDRs), numbering was performed according to Kabat.

3.2.3 Next generation phage display

3.2.3.1 Data Analysis and scFv rescue

The aim of analysis of the next generation sequencing data was to identify enriched scFvs and to quantify them for each species (Figure 3.8). In the first set of analysis (summarised in Figure 3.9), the Z scores were generated based on the frequency and ratio of binding when comparing one sample to another. An initial script was written to select only the scFvs that were present at 5 reads or more, and also produced a Z score of >5 (indicating that they were enriched) in at least two of the panning rounds (when compared to the preceding panning round). Comparisons were made of round 2 panning against round 1 panning, round 3 panning against round 2 panning, round 4 panning against round 3 panning and round 5 panning against round 4 panning for all species and Z scores generated (Table 3.4).

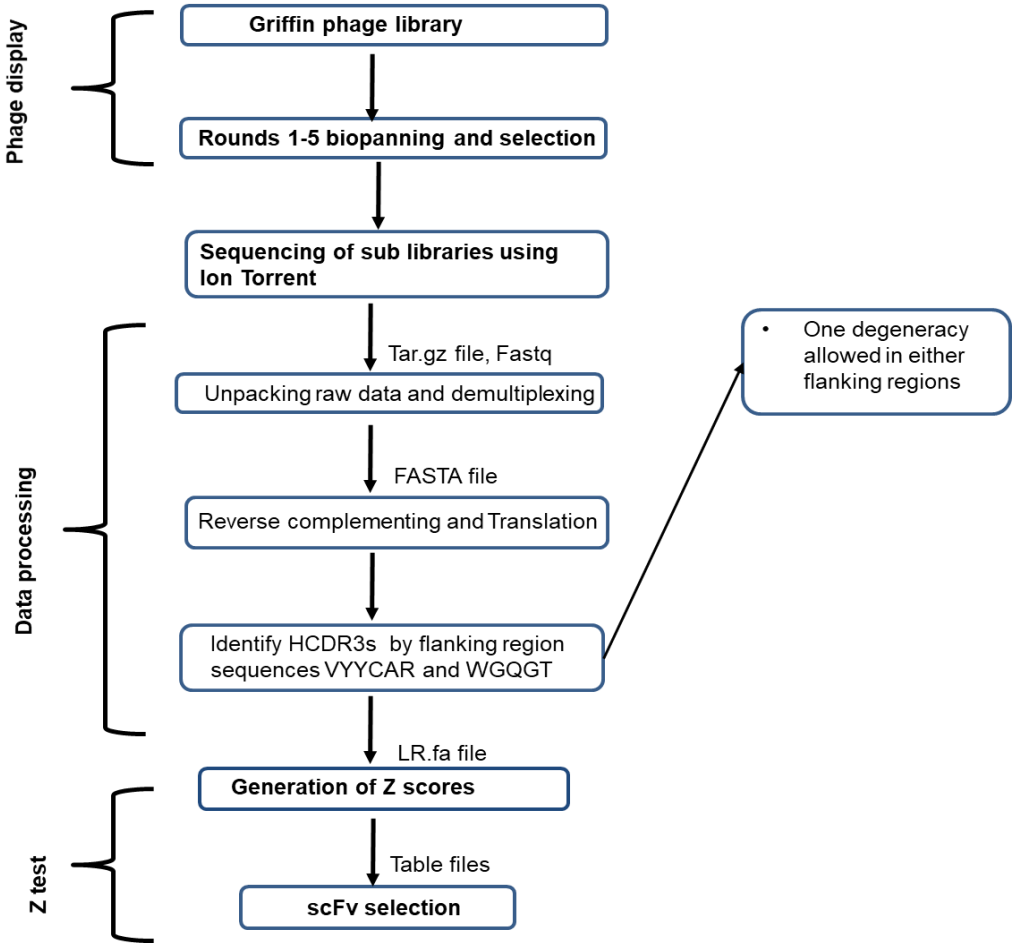


Figure 3:8: Schematic representation of phage display panning followed by analysis of NGS data. Each step is indicated on the left, with data file types indicated between each stage of analysis. Panning experiments were performed over five rounds of selection of phage antibodies. Sub libraries were sequenced by Ion Torrent sequencing and raw data was received as a tar.gz library file and extracted as FASTQ files. Sequence reads were separated according to barcodes by demultiplexing to form FASTA files. They were translated and reverse complemented. HCDR3

sequences were identified by the flanking motifs VYYCAR and WGQGT allowing one degeneracy. A two proportion Z-test was performed to produce Z-scores for each HCDR3. HCDR3 sequences were then selected for their enrichment during selection rounds. HCDR3s that showed enrichment in panning rounds and were enriched only during the selection phases of panning were further selected. Finally, scFv that displayed enrichment against limited number of species were selected.

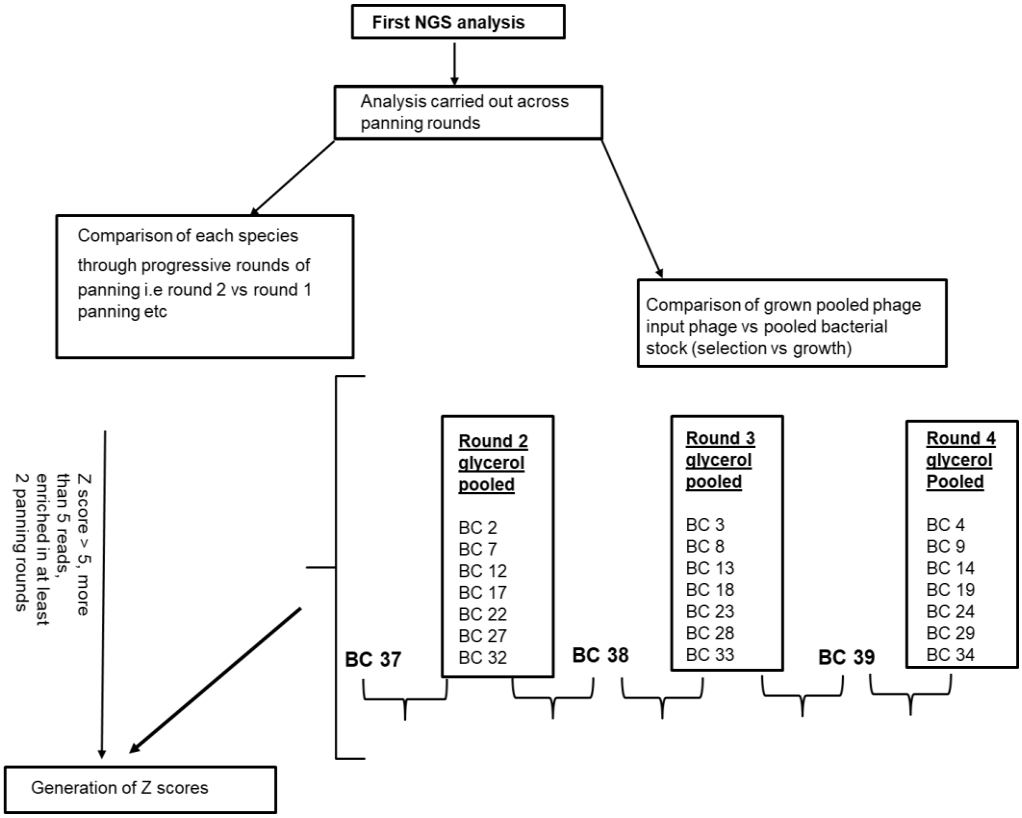


Figure 3:9: Workflow of first strategy employed for the analysis of NGS data. Comparison of each species through progressive panning rounds were carried out and the HCDR3s with Z scores higher than 5, enriched in at least 2 panning rounds and having at least 5 reads were selected. All pooled barcodes of output phage from selection rounds of panning were compared with the growth phase barcodes BC 37, BC 38 and BC 39 (having pooled them earlier in the panning process). Z scores were generated based on the comparisons.

Further analysis was then performed on these scFvs to select for scFvs that were enriched during selection phases of panning and not growth-only phases. A comparison was made of grown pooled input phage for each round of panning and the pooled bacterial stock output from the previous round which served to compare enrichment through growth phases (enrichment between a bacterial stock and the resulting input phage, designated GG) and selection + growth phases (enrichment between the input phage and the next bacterial stock after panning, designated SG)

of each of the scFvs selected so far. Since the comparison was carried out over several rounds, Z scores that were positive (enrichment during SG), negative (no enrichment during GG), positive (enrichment during SG) and negative (no enrichment during GG) in that order signified that they were enriched during interaction with oocysts and not just in growth phases (Table 3.4). All scFvs that did not meet the criteria were not selected. This was aimed at identifying the scFvs that were enriched in each round of panning and not just those that had a growth advantage. The 43 HCDR3 which met these criteria and their specificities are listed in Table 3.3. From this list, those that were specific to a single species, or a limited number of species were selected. This was also based on those having the highest Z scores with a particular specificity. Two species; *E. brunetti* and *E. praecox* didn't have any scFvs specific to them. Using these criteria, the list of 20 scFvs were generated (Table 3.4). The selected scFvs were rescued from the polyclonal phage sub-libraries produced during panning using inverse PCR (Spiliotopoulos *et al.,* 2015). Each scFv chosen to be rescued was rescued from the DNA extracts of the polyclonal phage sub library that had the highest frequency for that particular scFv (Figures 3.7 and 3.8). The frequencies of each scFv within their most enriched experiment are detailed in Table 3.6 and ranged from 0.02 to 3.09% of phage populations.

Phosphorylated PCR primers were designed based on each scFv's specific HCDR3 region which were paired with forward framework 4 (FR4) primers (**chapter 2**, materials and methods subsection 2.3.7). For the scFvs that had five or less amino acids, an alternative FR4 primer was paired with the unique CDR3 primer in order to extend the CDR3 primer into FR4.

Table 3:3: The 43 enriched scFvs and their specificities based on the NGS analysis selection criteria. A grey box indicates the specificity of the scFv. ScFvs were selected based on those that were enriched in at least 2 panning rounds (Z score of >5) and in selection phases not in the growth-only phases.

scFv	*E. mitis*	*E. necatrix*	*E. acervulina*	*E. brunetti*	*E. maxima*	*E. praecox*	*E. tenella*
DHTPKPq	■	■	■	■	■	■	■
DHTPKRq	■	■	■	■	■	■	■
DHTPKWq	■	■	■	■	■	■	■
DHTPKWQ	■	■	■		■	■	■
DRTPKWq	■	■	■	■	■	■	■
DGKPPSE	■		■		■	■	■
DGKPPSG							■
DHAPKWq	■	■	■	■	■	■	
DHMPKWq	■		■		■	■	
DHTLKWq			■		■	■	
DHTPKpq		■	■		■		
DHTPKqq			■	■	■	■	
DHTPKRq	■	■	■	■	■	■	■
DHTPKWo		■	■		■		
STqHP	■	■	■	■	■	■	■
STQLP	■	■	■	■	■	■	■
VDISAWSFME					■		
AWTVNQTTNY					■		
AYPA					■		
DRSYTACDTSK					■		
EDRqCKHG					■		
GGKPPSE					■		
LMVYKH					■		
LWFNY					■		
MIMDG					■		
MWAR					■		
QDFPA					■		
QTGMN					■		
RDLTqDMWR					■		
RRLA					■		
SPRLPFDY					■		
STAVTV					■		
VAADPFDY		■					
YIRYTSPV		■					
DKSSqGT	■						
KDVGVVPK	■						
ESSqMNS							■
SSDAP							■
STQHP							■
IHRMVYL			■				
HPMLGGLQGK					■		■
SAqHP			■				
TTALP						■	■
SRSKNYLWRSM		■		■	■		

Table 3:4: The scFvs selected for phagemid rescues after selection against oocysts. Blue boxes indicate enrichment of an scFv from one round of panning to the next. All scFvs were enriched during selection phases (green boxes) but not in growth only phases (grey boxes). Their apparent specificity against the different *Eimeria* species is also shown.

ScFv	Specificity	02vs01[1]	03vs02[2]	04vs03[3]	05vs04[4]	R2PHvsR2B[5]	R2BvsR3PH[6]	R3PvsR3B[7]	R3BvsR4P[8]
AWTVNQTTNY	*E. maxima*	6.4	13.73	-17.32	xx	54.94	-42.38	2.08	-9.03
DGKPPSG	*E. tenella*	5.2	11.75	-7.97	-8.08	20	-16.14	20.78	-5.97
DKSSqGT	*E. mitis*	-0.66	12.48	6.99	-18.75	75.43	-54.13	55.74	-25.8
DRYSTACDTSK	*E. maxima*	11.92	10.86	-20.3	-1	6.59	-2.81	2.11	-12.68
EDRqCKHG	*E. maxima*	6.52	7.99	-14.07	xx	3.09	-7.96	1.59	-4.24
ESSqMNS	*E. tenella*	10.99	29.92	-25.8	-14.84	38.23	-30.29	50.71	-17.38
VAADPFDY	*E. necatrix*	35.76	11	-44.7	-1	35.75	-9.48	25.86	-21.42
YIRYTSPV	*E. necatrix*	9.43	8.09	-15.62	xx	9.43	-6.15	14.44	-7.53
STQHP	*E. tenella*	25.62	80.37	-70.15	-39.89	123.2	-111.33	131.87	-67.49
SSDAP	*E. tenella*	7.14	13.36	-17.32	-7.6	46.38	-40.01	29.1	-16.22
SPRLPFDY	*E. maxima*	7.68	31.21	-33.73	-2.09	106.2	-26.91	1.49	-37.89
QTGMN	*E. maxima*	13.08	11.75	-22.17	-1	45.02	-22.77	2.18	-20.78
LMVYKH	*E. maxima*	5.39	10.58	-13.68	-1	22.88	-1.94	3.37	-10.98
KDVGVVPK	*E. mitis*	5.2	6.02	-8.12	xx	10.47	-11.57	1.79	-4.52
IHRMVYL	*E. acervulina*	6.31	11.25	-23.4	-1	55.02	-28.91	5.9	-16.84
HPMLGGLQGK	*E. maxima*	9.06	8.75	-15.65	-1.73	29.03	-20.25	2.76	-9.54
	E. tenella	5.2	6.06	-10.1	xx	29.03	-20.25	2.76	-9.54
SRSKNYLWRSM	*E. necatrix*	7	19.8	-21.6	-7.39	67.79	-65.68	62.08	-26.67
	E. brunetti	15.4	17.61	-18.48	-3.53	67.79	-65.68	62.08	-26.67
	E. maxima	7	43.21	-43.02	-8.44	67.79	-65.68	62.08	-26.67
RDLTqDMWR	*E. maxima*	12.13	11.9	-20.93	-3	51.4	-57.5	5.62	-20.36
TTALP	*E. praecox*	-2	7.21	21.8	-25.23	97.75	-85.98	68.98	-40.46
	E. tenella	14.47	36.39	-46.25	-21.37	97.75	-85.98	68.98	-40.46
SAqHP	*E. acervulina*	-1.73	5.29	8.05	-2.48	1.81	-8.94	18.39	-2.33

[1]Round 2 panning versus round 1 panning [2]Round 3 panning versus round 2 panning [3]Round 4 panning versus round 3 panning [4]Round 5 panning versus round 4 panning [5]Round 2 input phage versus round 2 bacterial stock (growth+selection phases) [6]Round 2 bacterial stock versus round 3 input phage (growth only phase) [7]Round 3 input phage versus round 3 bacterial stock (growth+selection phases) [8]Round 3 bacterial stock versus round 4 input phage (growth only phase), [xx] scFv was not found in that panning round

Table 3:5: Percentage frequency of the conventionally picked scFvs in the round 3 and 5 panning rounds in the NGS analysis. Green highlight represents the most frequent clone which constituted over 90% of the 5th panning round.

Species/panning rounds[1]	DHTPKWq[2]	STQLP[3]	STqHP[4]
E. mitis Rd 3	0.0152	0.011161	0.004582
E. mitis Rd 5	94.97145	0.090527	2.101463
E. necatrix Rd 3	48.22387	0.787788	6.146705
E. necatrix Rd 5	91.7641	0.234299	5.303911
E. acervulina Rd 3	25.36691	0.21225	1.447962
E. acervulina Rd 5	92.40877	0.22588	4.427293
E. brunetti Rd 3	65.48298	0.3497	2.559171
E. brunetti Rd 5	96.71434	0.065268	1.292457
E. maxima Rd 3	17.38644	0.22715	1.319281
E. maxima Rd 5	94.62543	0.170525	2.723774
E. praecox Rd 3	7.610627	0.046874	0.561986
E. praecox Rd 5	95.94472	0.147139	1.794253
E. tenella Rd 3	64.23593	0.581749	7.218855
E. tenella Rd 5	93.24491	0.178326	3.913582

[1] Species of *Eimeria* oocysts and panning rounds; either round 3 or round 5
[2] Frequency of conventionally picked motif DHTPKWq in different *Eimeria* species in rounds 3 and 5 of panning rounds
[3] Frequency of conventionally picked motif STQLP in different *Eimeria* species in rounds 3 and 5 of panning rounds
[4] Frequency of conventionally picked motif STqHP in different *Eimeria* species in rounds 3 and 5 of panning rounds

Table 3:6: Frequency of each scFv rescued within their most enriched experiment

HCDR3 sequence[1]	**Frequency**[2]	**Total read number**[3]	**Percentage frequency**[4]
AWTVNQTTNY	4450	744960	0.60
DGKPPSG	239	800481	0.03
DKSSqGT	4109	800481	0.51
DRSYTACDTSK	1472	657754	0.22
EDRqCKHG	650	895018	0.07
ESSqMNS	1363	863087	0.15
VAADPFDY	1993	846421	0.24
YIRYTSPV	244	846421	0.03
STQHP	10773	798000	1.35
SSDAP	2079	799615	0.26
SPRLPFDY	20299	657777	3.09
QTGMN	5422	800481	0.68
LMVYKH	1012	800481	0.13
KDVGVVPK	761	744960	0.10
IHRMVYL	5742	800481	0.72
HPMLGGLQGK	2321	895018	0.26
SRSKNYLWRSM	2164	657754	0.33
RDLTqDMWR	9847	752802	1.31
TTALP	4842	800481	0.60
SAqHP	502	3147964	0.02

[1] The scfv to be rescued

[2] The number of reads of the scFv in the experiment it's most abundant

[3] The total number of reads of all clones in that experiment

[4] The percentage frequency of the scFv compared to other clones

3.2.3.2: Optimisation of Inverse PCR for clone rescue

Non-specific PCR products were obtained after the first attempt at inverse PCR using an initial denaturing reaction conducted at 95°C for 2 minutes followed by 25 cycles of 95°C for 30 seconds, 50°C for 30 seconds and an extension time of 5 minutes at 72°C.

Asymmetric PCR was then carried out in which the HCDR3 primer was added initially and after 10 cycles, the universal framework 4 primer was added, this approach gave specific PCR products for some clones.

An alternative PCR method was also then carried out, with each scFv to be rescued having an annealing temperature 10°C lower than the Tm of the HCDR3 primer in the first 10 cycles and then the temperature was increased to 5°C lower than each scFv HCDR3 primer Tm in the last 18 cycles carried out, this approach also gave specific PCR products for some clones.

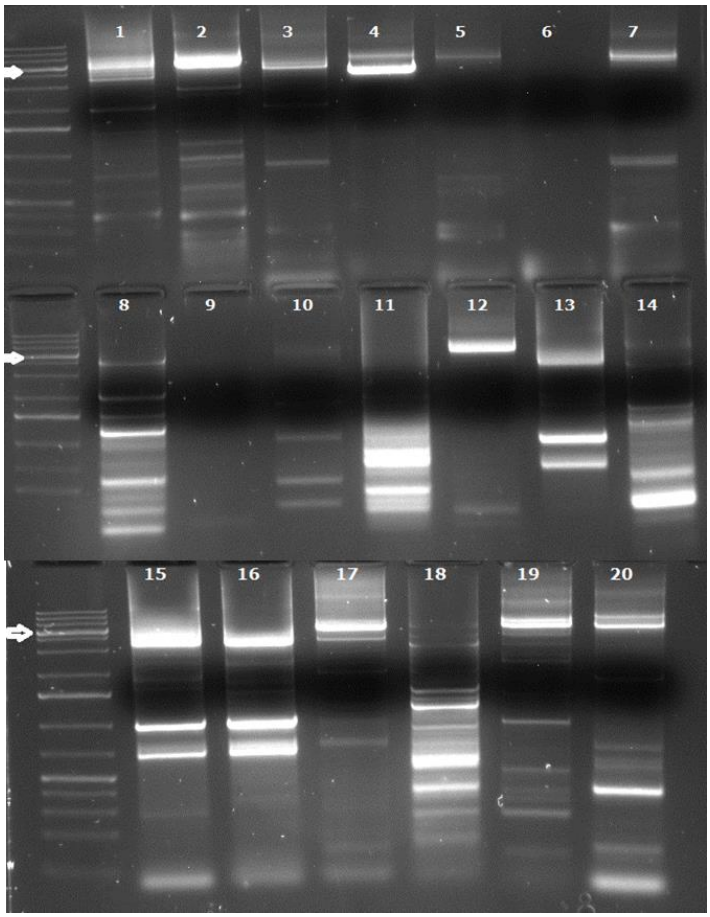


Figure 3:10: Inverse PCR products for scFv rescues. The entire reaction (50 µl) PCR products were loaded onto 2% (w/v) agarose gel with a KB+ ladder (SM1333) at 100 volts. Products were expected around 5kb (Indicated by white arrow next to the ladder). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). The scFv sequences are listed and each sequence number corresponds to the number labelled on the gel. Those listed as 'failed' produced no amplicons after inverse PCR under any of the conditions tested. (1) AWTVNQTTNY,

(2) DGKPPSG, (3) DKSSqGT, (4) DRSYTACDTSK, (5) EDRqCKHG, (6) ESSqMNS **failed**, (7) VAADPFDY, (8) YIRYTSPV, (9) STQHP **failed,** (10) SSDAP **failed,** (11) SPRLPFDY **failed,** (12) QTGMN, (13) LMVYKH, (14) KDVGVVPK **failed,** (15) IHRMVYL, (16) HPMLGGLQGK, (17) SRSKNYLWRSM, (18) RDLTqDMWR **failed**, (19) TTALP (20) SAqHP. Inverse PCR products with a correct band at ~5kb were extracted from the agarose gel and purified. The products were then ligated microdialysed to remove excess salts before transformation into electro competent TG1 cells.

Inverse PCR for rescue of target scFvs was employed and was carried out as described in subsection 3.2.2.2. From the first NGS analysis that compared enrichment of scFvs in growth and selection phases, twenty scFvs were selected for rescue out of which six of the clones failed to amplify during inverse PCR using any of the PCR methods (Figure 3.10). The gel fragments from the 14 clones were gel purified and DNA quantified and they were within the range of 9.7-20.1 ng/µl. These were ligated overnight, microdialysed and transformed in bacteria and 2 clones failed to produce colonies following transformation. Colonies from the 12 remaining targets were grown and miniprepped and quantified using fluorometry and the DNA obtained ranged between 27.4-210 ng/µl.

They were Sanger sequenced and 11 were confirmed to have the right inserts and flanking regions. The 11 clones rescued were; AWTVNQTTNY, DGKPPSG, DKSSqGT, DRSYTACDTSK, VAADPFDY, QTGMN, KDVGVVPK, HPMLGGLQGK, SRSKNYLWRSM, TTALP, SAqHP. YIRYTSPV had the correct HCDR3 sequence but not the flanking sequences. The sequences across the primer binding and ligation site are shown in Figure 3.11.

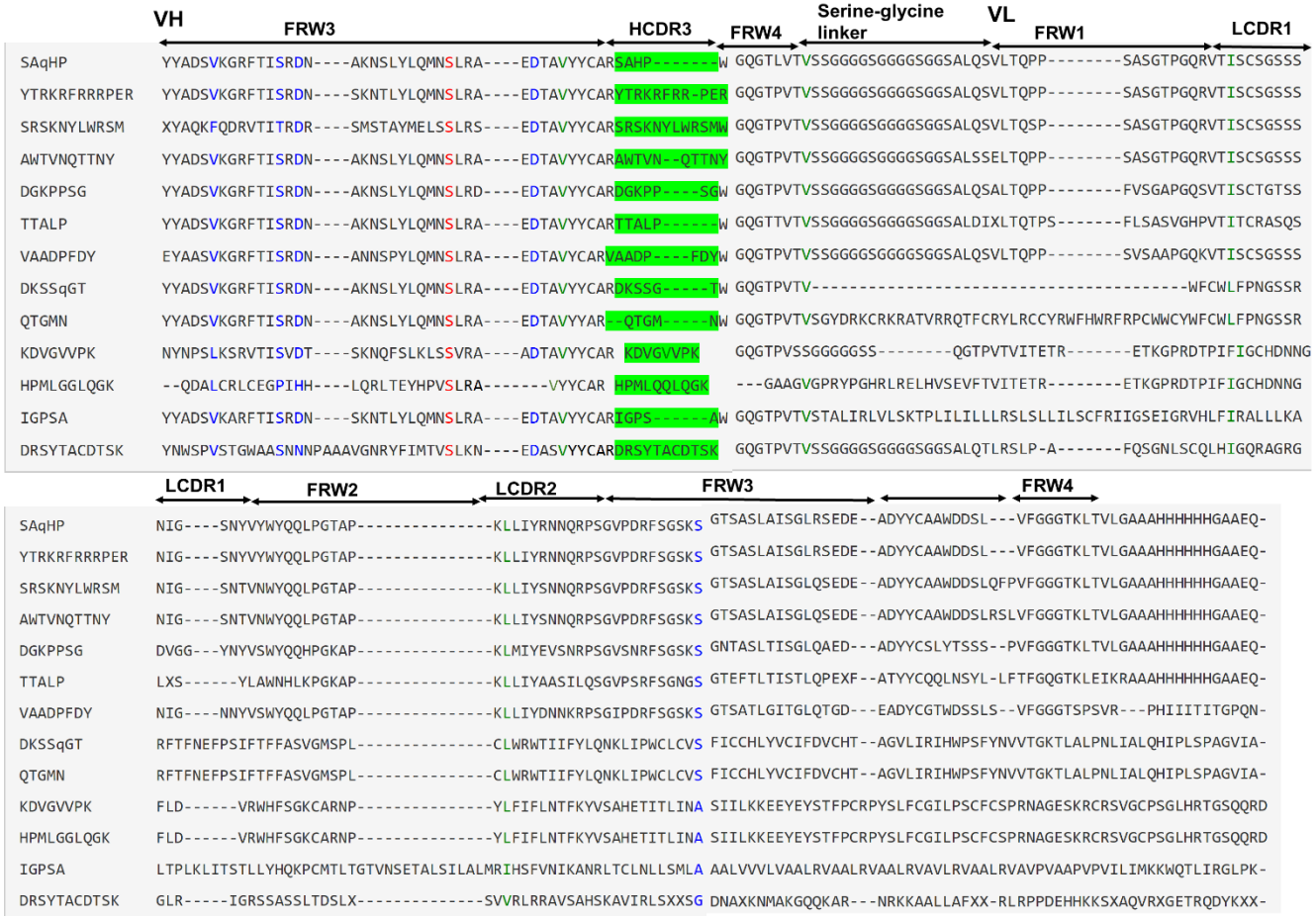


Figure 3:11: Deduced amino acids sequences of the VH and VL of the 13 NGS rescued scFv antibody fragments raised against *Eimeria* oocysts. The V_H (above) and V_L (below) segments of the scFvs were sequenced as described in subsection 2.3.5.1.5 in Materials and methods **chapter 2.** The HCDR3 sequences are highlighted in green. The GC-rich sequence that links V_H and V_L segments is indicated as well as the framework regions and complementarity determining regions (CDRs), numbering was performed according to Kabat.

3.2.4. An alternative NGS analysis to enhance recovery of species-specific clones

An alternative NGS analysis was carried out to try and maximise the chances of finding species-specific scFvs. Analysis was carried out from the data of the 3rd panning round because the frequency of one clone was over 90% of the 5th panning round making it mostly monoclonal. Also clones that had been rescued showed sufficient enrichment by the third panning round (Table 3.4). Using different bioinformatics analysis, the NGS data compared each scFv enriched against each species versus the other 6 species to generate 6 Z scores for each scFv (Figure 3.12). The Z scores were ranked in a descending order from those that had the highest to the lowest Z scores. The scFvs that had been isolated previously from the colony picks and the initial NGS analysis that compared selection and growth were removed as well as scFvs that showed enrichment to other species. Selected motifs from each of the species that had only one sequence change and appeared in multiple data sets were identified and discarded because of the possibility of them binding to the same epitope. The top ten scFvs ranked according to Z scores that met the criteria were then selected for each species (Table 3.7).

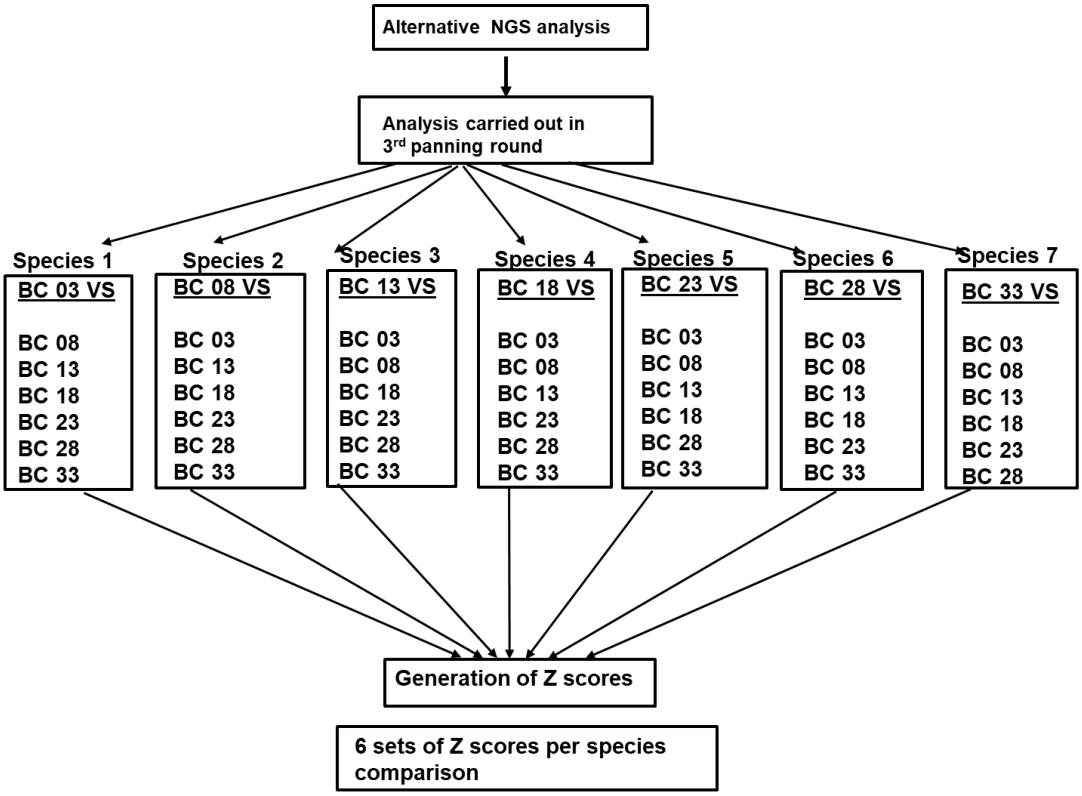


Figure 3:12: Workflow of second strategy employed for the analysis of NGS data. Analysis was carried out using data from 3rd panning round. Each species (positive) was compared with the other six species (negative control) and Z scores were generated. The top 10 HCDR3s that had the highest Z scores were selected.

Table 3:7 scFvs selected for rescue from the NGS analysis in all seven *Eimeria* species for the second set of NGS analysis**.** The figures in the columns show the Z scores when the particular scFv isolated against a particular species was compared to its abundance when selected against the other 6 species.

***E. mitis* scFv**	**Z scores for species 1 vs species 2**	**Z scores for species 1 vs species 3**	**Z scores for species 1 vs species 4**	**Z scores for species 1 vs species 5**	**Z scores for species 1 vs species 6**	**Z scores for species 1 vs species 7**
ANACID	98.48	98.47	98.48	98.48	98.46	98.48
RWRAMR	94.21	94.21	94.13	94.21	94.21	94.19
SEYCEKTPGG	88.16	88.18	88.18	88.18	88.18	88.16
LWRNYYSS	78.96	78.94	78.08	78.96	78.96	78.96
CGNVN	55.07	59.88	58.38	57.71	59.84	57.8
TCTTLPIGLQA	49.72	57.12	56.35	55.68	57.12	56.02
DDTINVEV	38.85	39.92	39.36	33.14	44.32	49.5
KHICRFDY	30.62	30.62	30.62	30.25	30.62	30.62
VWSTESI	26.81	27.99	27.69	24.9	27.99	27.76
PHYVLM	10.77	14.73	13.18	13.23	5.66	14.62

***E. necatrix* scFv**	**Z scores for species 2 vs species 1**	**Z scores for species 2 vs species 3**	**Z scores for species 2 vs species 4**	**Z scores for species 2 vs species 5**	**Z scores for species 2 vs species 6**	**Z scores for species 2 vs species 7**
RQTKYWYDCSD	78.33	78.33	78.33	78.33	78.33	78.33
GYRFCHNSRD	60.01	60.01	60.01	59.71	60.01	60.01
ELPPTPITS	59.39	59.39	59.39	59.39	59.39	59.39
AFEWKTRVLF	35.56	35.97	35.97	35.92	35.97	35.97
RRPDLLM	22.99	21.39	14.73	25.01	25.59	28.56
LESTSV	19.54	20.05	21.15	20.49	19.14	21.15
CWKGQG	18.97	19.89	19.98	19.98	19.9	19.98
VNPTKFDY	17.18	18.47	19.57	19.4	19.16	19.57
WVPAGWY	15.27	15.27	15.27	15.27	15.27	15.27
FEWRPPTSCQG	11.71	10.86	11.71	11.71	11.71	11.71

***E. acervulina* scFv**	**Z scores for species 3 vs species 1**	**Z scores for species 3 vs species 2**	**Z scores for species 3 vs species 4**	**Z scores for species 3 vs species 5**	**Z scores for species 3 vs species 6**	**Z scores for species 3 vs species 7**
ARWRAEK	57.83	54.01	65.06	68.1	66.79	69.86

FCVSHFDY	46.4	48.02	46.86	48.53	48.59	48.59
PSYWRFDY	44.33	44.33	44.33	44.33	44.33	44.33
CAPEE	25.73	27.71	27.71	27.71	27.4	27.71
ICSPRATRRQ	22.72	22.83	22.83	22.7	22.83	22.83
DQHWQPLAMS	21.05	21.29	21.29	15.52	19.7	16.31
EKTWDFDY	17.64	17.64	17.64	17.64	17.64	17.64
QQNENSD	16.03	16.19	16.19	16.19	16.19	16.19
RDQSHKQEMS	14.83	18.09	18.09	18.09	17.38	18.09
ASNCERE	14.28	16.95	17.18	16.03	16.93	17.18

***E. brunetti* scFv**	**Z scores for species 4 vs species 1**	**Z scores for species 4 vs species 2**	**Z scores for species 4 vs species 3**	**Z scores for species 4 vs species 5**	**Z scores for species 4 vs species 6**	**Z scores for species 4 vs species 7**
NHMRTFDY	94.32	94.32	94.32	94.32	94.32	94.32
KWNSGRDAD	71.11	71.08	71.11	71.05	71.11	71.11
APRWDI	63.07	63.09	63.09	63.06	63.09	63.09
FAAMCDDV	58.25	58.25	58.18	58.25	58.25	58.25
TGVENWTL	53.99	53.99	53.99	53.99	53.99	53.99
MAEPPLTTHT	50.44	50.44	50.44	50.44	50.44	50.44
HSQVPAVAPqF	50.12	50.12	50.12	49.49	50.12	38.12
IWGPQFDY	49.26	49.26	49.22	49.26	49.26	49.22
RADRASF	48.79	48.79	48.67	46.75	48.79	47.29
RAILSRNGLHC	45.49	45.49	45.49	45.49	45.49	45.49

***E. maxima* scFv**	**Z scores for species 5 vs species 1**	**Z scores for species 5 vs species 2**	**Z scores for species 5 vs species 3**	**Z scores for species 5 vs species 4**	**Z scores for species 5 vs species 6**	**Z scores for species 5 vs species 7**
SPRMF	88.59	93.8	81.44	91.61	91.83	93.06
SFTLVMDHPS	73.01	73.02	73.02	73.02	73.02	73.02
NRYSKT	72.55	73.98	71.51	47.29	73.17	73.56
RLFQVE	44.09	46.64	40.67	46.83	43.37	46.83
ASRDPKMNQVL	43.36	43.36	43.36	43.36	43.36	43.36
MTTVFR	43.13	43.13	43.09	43.13	43.13	43.13
WRMPTP	34.94	34.94	34.94	34.94	34.46	34.94

ENTMRGFGL	31.97	31.97	31.97	21.91	29.8	31.39
FMVGDG	31.26	32.23	30.6	29.04	31.89	32.81
VEGPRFDY	27.68	31.85	24.33	11.01	31.45	31.97

***E. praecox* scFv**	**Z scores for species 6 vs species 1**	**Z scores for species 6 vs species 2**	**Z scores for species 6 vs species 3**	**Z scores for species 6 vs species 4**	**Z scores for species 6 vs species 5**	**Z scores for species 6 vs species 6**
KELIQPTV	44.97	44.72	44.93	44.97	44.25	44.61
YTRKRFRRPER	39.92	37.72	35.75	29.06	25.34	34.64
PWPTPNQ	38.68	35.72	40.39	38.61	40.58	40.74
SGSPPFDY	34.25	34.25	34.25	34.25	34.25	34.25
LRNGKSHMMRTV	34.16	36.72	30.36	37.45	28	36.14
PAGSRTL	32.14	33.81	33.25	33.85	30.5	33.85
HRPKQGKA	30.82	35.56	29.53	36.24	15.44	35.14
RFIMSHS	28.77	29.05	28.68	29.05	29.05	29.05
LNTNDV	27.27	30.15	30.15	23.39	28.68	30.15
TRLRPTNLKV	25.18	25.9	25.3	25.9	25.83	25.9

***E. tenella* scFv**	**Z scores for species 7 vs species 1**	**Z scores for species 7 vs species 2**	**Z scores for species 7 vs species 3**	**Z scores for species 7 vs species 4**	**Z scores for species 7 vs species 5**	**Z scores for species 7 vs species 6**
IGPSA	132.77	132.77	132.76	132.77	132.75	132.77
LVNRSSNEQG	48.53	48.5	48.53	48.53	48.53	48.53
LAPSLSERPV	47.74	47.74	47.74	47.74	47.74	47.74
KRAHGRNEPA	44.84	44.84	44.84	44.84	44.84	44.84
GGFSMTGLF	39.36	39.36	39.32	39.36	39.36	39.36
ASYRGH	19.27	19.27	19.27	19.27	19.17	19.01
TIAIGFDY	17.58	17.58	17.58	17.58	17.58	17.58
SHQTqWQSSIAE	17.39	19.73	19.98	20.4	12.62	20.4
RILDLDT	15.46	15.46	15.38	15.46	15.46	15.46

3.2.4.1 ScFv clone rescue by inverse PCR – sanger sequencing of resulting clones

From the alternative NGS analysis for the rescue of species-specific scFvs, inverse PCR was carried out as for the first set of rescues and described in subsection 3.2.2.2. Sixty-nine clones were targeted, having selected 9 scFvs for species 7 and 10 each for the other species. Of the 69 clones, 13 failed to be amplified with any of the PCR conditions used, hence 56 clones were extracted, gel purified and DNA quantified and DNA concentrations obtained were between 6.3-16.8 ng/µl. Ligations were carried out in batches and two batches of twenty each were ligated, microdialysed and transformed and the DNA obtained after the colonies were grown and miniprepped and were between 19.3-201 ng/µl. Only 29 target clones of the 40 produced colonies and when sent for Sanger sequencing to confirm the clones, many of the sequences were aberrant having framework shifts and many sequencing errors. Only two scFvs had the correct sequence, flanking regions and no sequencing errors across the primer binding and ligation site; YTRKRFRRRPER (with possible specificity to *E. praecox*) and IGPSA (with possible specificity to *E. tenella*). The cloning attempts were made from samples where the frequencies of the scFvs in their most abundant experiments were between 0.02 and 3.95 (Table 3.8). Cloning of the scFvs were very inefficient and time consuming and several attempts at rescuing the clones failed. It was also noted that this difficulty in rescuing scFv clones from the Griffin library was observed by others in the research group (Dr Rose Reader, personal communication). Therefore, the rescues were not pursued further, the main aim was then to analyse the clones already obtained.

Table 3:8: Frequency of each scFv rescued within their most enriched panning round for the second set of rescues

HCDR3 sequence[1]	Frequency[2]	Total clone number[3]	Percentage frequency[4]
SEYCEKTPGG	7715	979476	0.79
ANACID	9604	979476	0.98
RWRAMR	8796	979476	0.90
CGNVN	3561	4408371	0.08
TCTTLPIGLQA	3242	4408371	0.07
DDTINVEV	38724	979476	3.95
LWRNYYSS	6195	979476	0.63
KHICRFDY	936	4408371	0.02
PHYVLM	6885	979476	0.70
VWSTESI	782	4408371	0.02
AFEWKTRVLF	1292	846421	0.15
GYRFCHNSRD	3585	800481	0.45
RQTKYWYDCSD	6089	800481	0.76
RRPDLLM	855	846421	0.10
ELPPTPITS	3512	800481	0.44
FEWRPPTSCQG	137	846421	0.02
LESTSV	477	846421	0.06
CWKGQG	399	846421	0.05
VNPTKFDY	383	846421	0.05
WVPAGWY	233	846421	0.03
ARWRAEK	4970	772672	0.64
FCVSHFDY	2354	772672	0.30
PSYWRFDY	1960	772672	0.25
ICSPRATRRQ	521	772672	0.07
DQHWQPLAMS	453	772672	0.06
EKTWDFDY	311	772672	0.04
ASNCERE	2058	772672	0.27
CAPEE	767	772672	0.10
QQNENSD	262	772672	0.03
RDQSHKQEMS	327	772672	0.04
NHMRTFDY	8809	895018	0.98
KWNSGRDAD	5028	895018	0.56
APRWDI	3963	895018	0.44
FAAMCDDV	3380	895018	0.38
TGVENWTL	2905	895018	0.32
MAEPPLTTHT	2537	895018	0.28
HSQVPAVAPqF	2505	895018	0.28
IWGPQFDY	2420	895018	0.27
RADRASF	2374	895018	0.27
RAILSRNGLHC	2065	895018	0.23

SPRMF	8776	751925	1.17
NRYSKT	5469	751925	0.73
RLFQVE	2187	751925	0.29
WRMPTP	1219	751925	0.16
ENTMRGFGL	1021	751925	0.14
FMVGDG	1107	751925	0.15
VEGPRFDY	1021	751925	0.14
ASRDPKMNQVL	1875	698420	0.27
MTTVFR	1855	698420	0.27
SFTLVMDHPS	5292	698420	0.76
KELIQPTV	2017	793614	0.25
YTRKRFRRPER	9717	657754	1.48
PWPTPNQ	1656	793614	0.21
SGSPPFDY	1171	793614	0.15
LRNGKSHMMRTV	6510	657754	0.99
PAGSRTL	1144	793614	0.14
HRPKQGKA	6977	657754	1.06
RFIMSHS	843	793614	0.11
TRLRPTNLKV	670	793614	0.08
LNTNDV	908	793614	0.11
KRAHGRNEPA	2005	752802	0.27
LVNRSSNEQG	2348	752802	0.31
LAPSLSERPV	2272	752802	0.30
IGPSA	17224	752802	2.29
SHQTqWQSSIAE	416	863087	0.05
RILDLDT	276	863087	0.03
ASYRGH	371	863087	0.04
TIAIGFDY	309	863087	0.04
GGFSMTGLF	1546	752802	0.21

[1] The scfv to be rescued
[2] The number of reads of the scFv in the experiment it's most abundant
[3] The total number of reads of all clones in that experiment
[4] The percentage frequency of the scFv compared to other clones

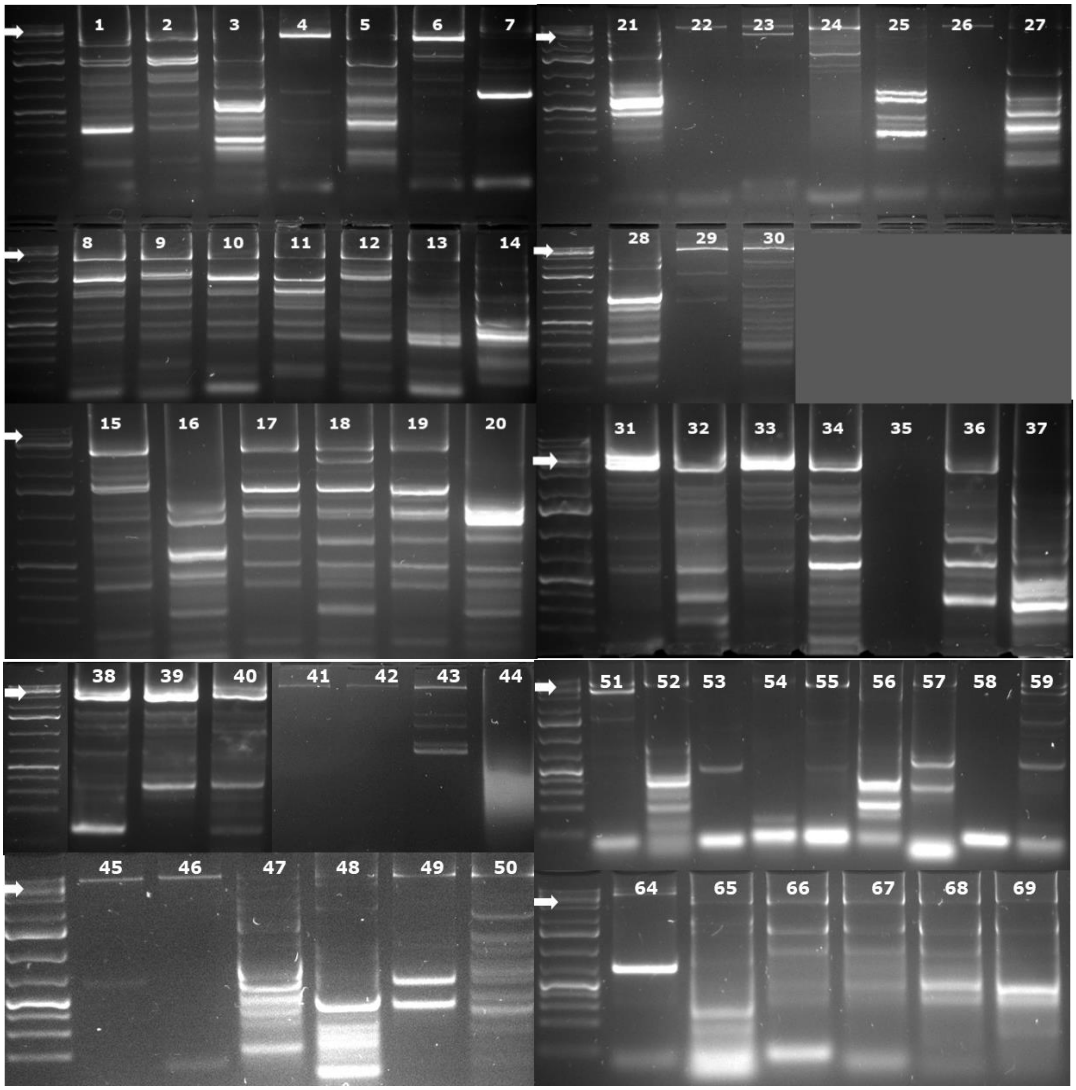


Figure 3:13: Inverse PCR products for rescue of possible species-specific scFv rescues. The entire reaction (50 µl) PCR products were loaded onto 2% (w/v) agarose gel with a KB+ ladder (SM1333) at 100 volts. Products were expected around 5kb (Indicated by white arrow next to ladder). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). The scFv sequence are listed and each sequence number corresponds to the number on the gel. Those listed as 'failed' produced no/very weak amplicons after inverse PCR. (1) SEYCEKTPGG, (2) ANACID, (3) RWRAMR, (4) CGNVN, (5) TCTTLPIGLQA, (6)

DDTINVEV, (7) LWRNYYSS **failed**, (8) KHICRFDY, (9) PHYVLM, (10) VWSTESI, (11) AFEWKTRVLF, (12) GYRFCHNSRD, (13) RQTKYWYDCSD, (14) RRPDLLM, (15) ELPPTPITS, (16) FEWRPPTSCQG **failed**, (17) LESTSV, (18) CWKGQG, (19) VNPTKFDY,
(20) WVPAGWY **failed,** (21) ARWRAEK **failed**, (22) FCVSHFDY, (23) PSYWRFDY, (24) ICSPRATRRQ, (25) DQHWQPLAMS **failed,** (26) EKTWDFDY, (27) ASNCERE **failed,** (28) CAPEE **failed,** (29) QQNENSD, (30) RDQSHKQEMS, (31) NHMRTFDY, (32) KWNSGRDAD, (33) APRWDI, (34) FAAMCDDV, (35) TGVENWTL **failed,** (36) MAEPPLTTHT, (37) HSQVPAVAPqF, (38) IWGPQFDY, (39) RADRASF, (40) RAILSRNGLHC (41) SPRMF, (42) NRYSKT, (43) RLFQVE, (44) WRMPTP **failed,** (45) ENTMRGFGL, (46) FMVGDG, (47) VEGPRFDY **failed,** (48) ASRDPKMNQVL, (49) MTTVFR, (50) SFTLVMDHPS (51) KELIQPTV, (52) YTRKRFRRPER, (53) PWPTPNQ **failed**, (54) SGSPPFDY **failed,** (55) LRNGKSHMMRTV, (56) PAGSRTL, (57) HRPKQGKA, (58) RFIMSHS **failed**, (59) TRLRPTNLKV, (64) IGPSA, (65) SHQTqWQSSIAE, (66) RILDLDT, (67) ASYRGH, (68) TIAIGFDY, (69) GGFSMTGLF

Three clones were recovered from conventional handpicking of single clones and 13 clones were rescued from the two NGS analyses carried out from the sequenced sub-libraries; 11 in the initial analysis comparing enrichment in selection and growth phases and two from the species-specific enrichment analysis. The clones were screened via immunoassay to determine target specificity to determine if clones that show enrichment *in silico* are actually binding to the target. As polyclonal and picked monoclonal phage did not bind to oocyst lysates, these clones were analysed using a spin down ELISA that centrifuges whole oocysts between binding and washing steps (refer to subsection 2.6.8.1 in **chapter 2**). This assay is the most similar to the panning protocol. The development of alternative and higher throughout assays were attempted and details of the assays tried and their optimisation steps are described in detail in **chapter 4.**

3.2.4.2 Testing isolated scFvs in a spin down immunoassay to detect whole oocysts

Conventionally picked motif, DHTPKWq was screened including an anti-BSA control via spin down ELISA and the data analysed using a two-way ANOVA. Differences among binding of the test scFv compared to the anti-BSA scFv against a particular species of oocyst were then determined using a post hoc Tukey's multiple comparative test. DHTPKWq was significantly higher than anti-BSA at $p<0.0001$ when binding to four species (*E. necatrix, E. brunetti, E. maxima and E. tenella*) and *E. mitis* at $p =0.0003$. It did not bind to *E. acervulina* ($p =0.9994$) or *E. praecox* ($p =0.9256$). This assay was repeated a second time and DHTPKWq displayed a significantly higher binding than the anti-BSA scFv ($p<0.0001$) in four species (*E. necatrix, E. acervulina, E. brunetti and E. tenella*) and bound significantly higher compared to the anti-BSA scFv at $p =0.0285$ in *E. mitis* and $p=0.0010$ in *E. maxima*. It did not bind significantly to *E. praecox* at $p=0.8661$ (Figure 3.14). The results of both assays showed consistency of binding to *E. necatrix, E. brunetti, E. maxima and E. tenella* and *E. mitis*. Binding of the antibody to *E. acervulina* showed different results in both assays and no binding activity was observed to *E. praecox* in both assays. This isolated scFv, being the most abundant, accounting for 90% of the round 5 phage pool, was used to start the optimisation of the assay developed for the isolated scFvs (details in **chapter 4**)

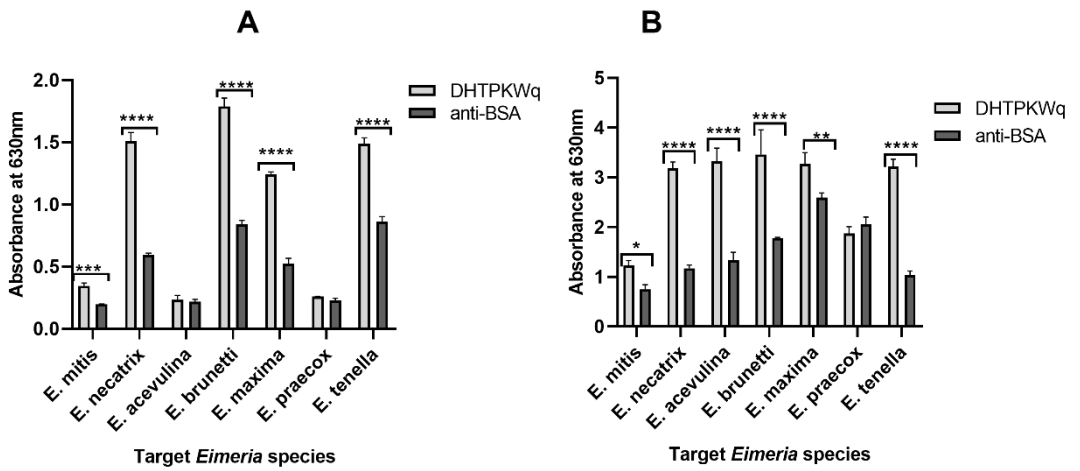


Figure 3:14: Binding of conventionally picked motif DHTPKWq to species of *Eimeria* oocysts. scFv phage were produced for DHTPKWq and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. In both assays monoclonal scFv phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

Conventionally picked motif STQLP including an anti-BSA negative control were screened, analysed and differences in binding amongst species were determined exactly as described above. STQLP displayed significantly higher binding than anti-BSA at p<0.0001 when binding to six species (*E. necatrix, E. acervulina, E. brunetti, E. maxima, E. praecox and E. tenella*). It did not bind to *E. mitis* significantly higher than anti-BSA control at p =0.9937. When the assay was repeated, STQLP bound to *E. mitis* significantly higher than anti-BSA at p =0.007 and binding to the other six species significantly higher than anti-BSA at p <0.0001 (Figure 3.15). The results of both assays show consistency of binding to all species of *Eimeria* except *E. mitis*.

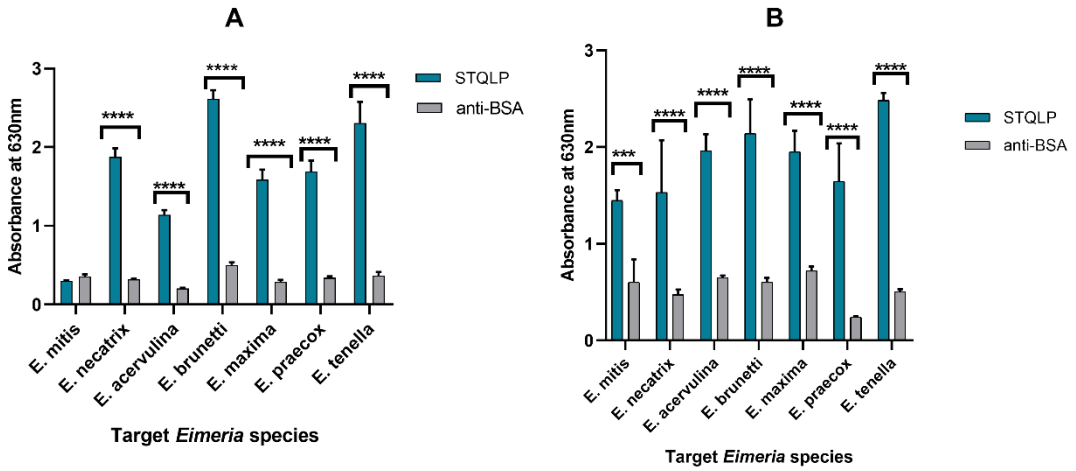


Figure 3:15: Binding of conventionally picked motif STQLP to species of *Eimeria* oocysts. scFv phage were produced for STQLP and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. In both assays monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10 minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

Conventionally picked motif STqHP including an anti-BSA negative control were screened, analysed and differences in binding amongst species were determined exactly as described above. STqHP displayed significantly higher binding than anti-BSA at p<0.0001 when binding to six species (*E. necatrix, E. acervulina, E. brunetti, E. maxima, E. praecox and E. tenella*) and to *E. mitis* at p =0.006. When the assay was repeated, STqHP displayed significantly higher binding compared to anti-BSA at p<0.0001 when binding to *E. mitis*, *E. necatrix*, *E. brunetti* and *E. tenella*. It was binding to *E. maxima* significantly at p =0.0029. It did not bind significantly higher to *E. acervulina* (p =0.0510) and *E. praecox* (p =0.5230) (Figure 3.16). The results of the assay showed consistency of binding to *E. necatrix*, *E. brunetti*, *E. maxima* and *E. tenella*. Binding to *E. mitis* and *E. acervulina* and *E. praecox* gave different results in both assays.

Similarly, rescued motif SRSKNYLWRSM was screened alongside a BSA negative control and significant differences in binding against all species at p<0.0001 comparing SRSKNYLWRSM and BSA control were observed (Figure 3.17). This assay was repeated at least four times and the results remained consistent.

Incubation time with substrate was reduced to 3 minutes because of the speed of colour development.

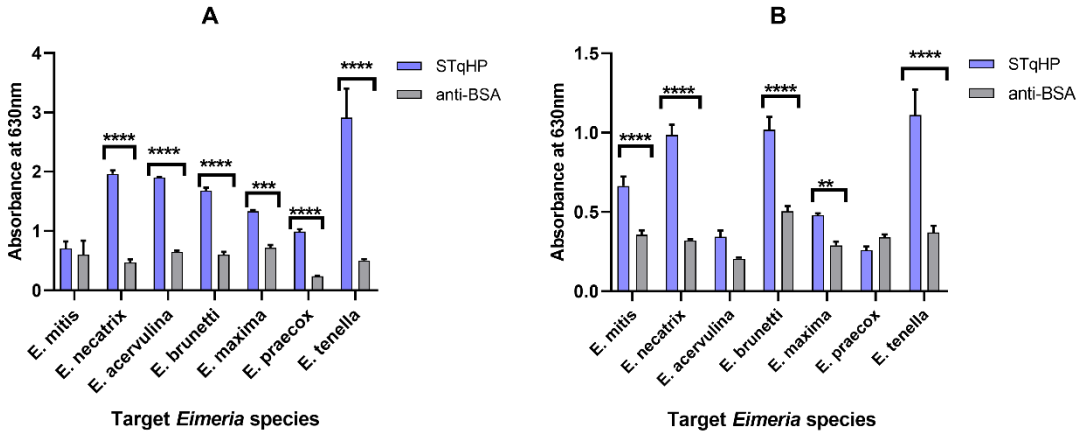


Figure 3:16: Binding of conventionally picked motif STqHP to species of *Eimeria* oocysts. scFv phage were produced for STqHP and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10 minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

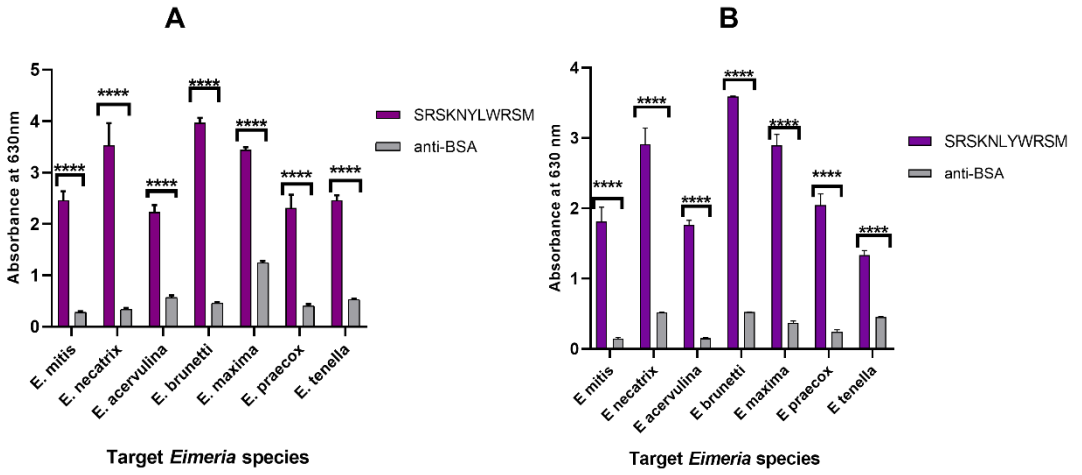


Figure 3:17: Binding of rescued motif SRSKNYLWRSM to species of *Eimeria* oocysts. scFv phage were produced for SRSKNYLWRSM and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 3-minute incubation before absorbance was

read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

Rescued motif SAqHP displayed significantly higher binding (p <0.0001) to *E. mitis*, *E. acervulina*, *E. maxima*, *E. praecox* and *E. tenella*. It did not bind significantly to *E. necatrix* (p =0.8735) and *E. brunetti* (p =0.9828). When the assay was repeated, SAqHP displayed significantly higher binding to *E. mitis* (p<0.0001), *E. brunetti* (p =0.0052), *E. maxima* (p =0.0051), *E. praecox* (p =0.0007) and *E. tenella* (0.0061) (Figure 3.18). It did not display significantly higher binding to *E. necatrix* (p =0.7278). This assay was repeated again and SAqHP displayed significantly higher binding to *E. mitis* (p<0.0001) and *E. praecox* (p =0.0008). It did not display significantly higher binding to the other species *E. necatrix* (p =0.7122), *E. acervulina* (p =0.8621), *E. brunetti* (0.9921) and *E. tenella* (p =0.9966). Because the assays were variable, this assay was repeated one more time and SAqHP displayed significantly higher binding to only *E. mitis* at p<0.0001. It did not display significantly higher binding compared to anti-BSA to other species *E. necatrix* (p =0.9955), *E. acervulina* (p =0.9566), *E. brunetti* (p=0.9776), *E. maxima* (p =0.6705), *E. praecox* (p =0.9998) and *E. tenella* (p =0.2259). There was consistency of strong binding to only *E. mitis* in all four repeats of the assay. There was binding to *E. praecox* three out of the four repeats. There was no significantly higher binding to *E. necatrix* in all four repeats. Binding to the other species were variable.

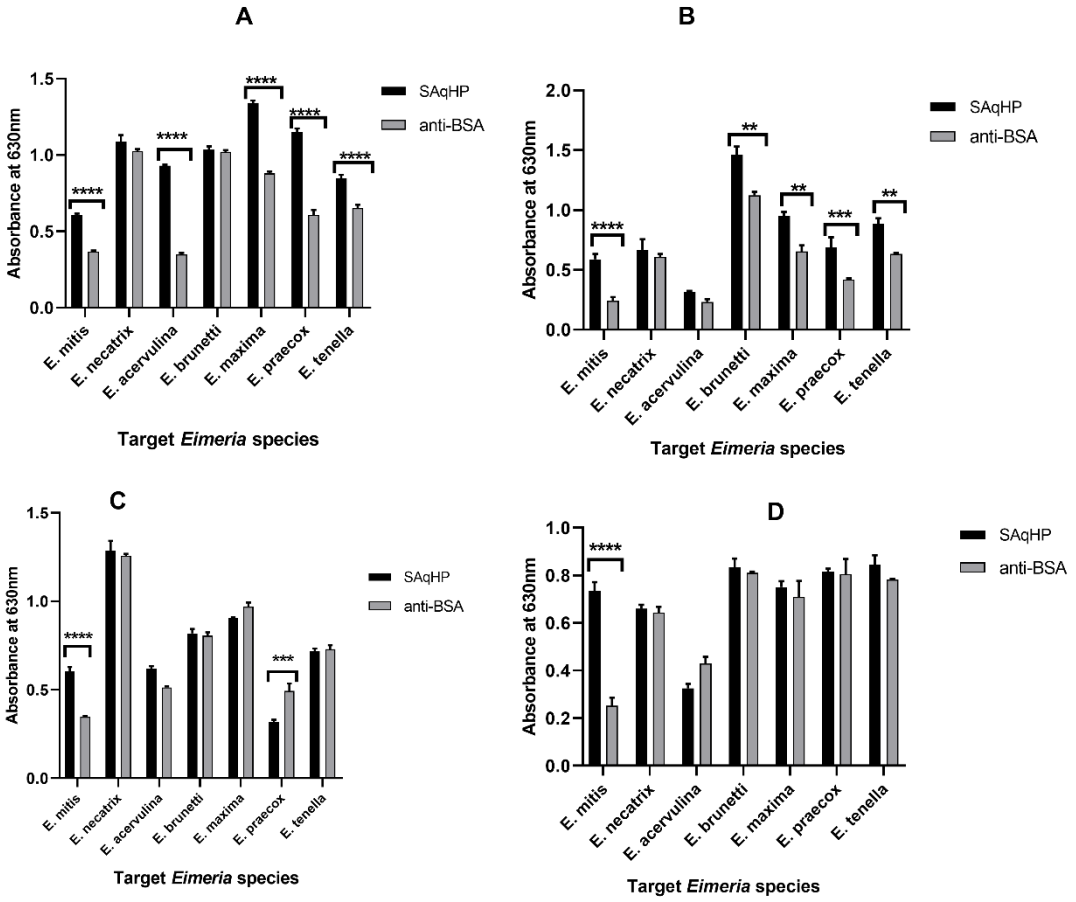


Figure 3:18: Binding of rescued motif SAqHP to species of *Eimeria* oocysts. scFv phage were produced for SRSKNYLWRSM and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. In all four repeats, monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10 minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A), (B), (C) and (D) represent repeat assays.

Rescued motif DGKPPSG displayed a significantly higher binding compared to anti-BSA when binding to two species (*E. brunetti* (p =0.0008) and *E. maxima* (p =0.0007). It did not display significantly higher binding compared to anti-BSA in the other species; *E. mitis* (p =0.4178), *E. necatrix* (p =0.9998), *E. acervulina* (p =0.3124), *E. praecox* (p =0.0226) and *E. tenella* (p =0.9024) (Figure 3.19). This assay was repeated and it was found to be binding to *E. acervulina* (p =0.0006) and *E. brunetti* (p<0.0001). A second repeat of the assay showed that DGKPPSG was not binding to

any of the *Eimeria* species significantly higher than the anti-BSA control, *E. mitis* (p =0.9643), *E. necatrix* (p>0.9999), *E. acervulina* (p =0.9861), *E. brunetti* (p>0.9999), *E. maxima* (p =0.9868), *E. praecox* (p>0.9999) and *E. tenella* (p>0.9999).

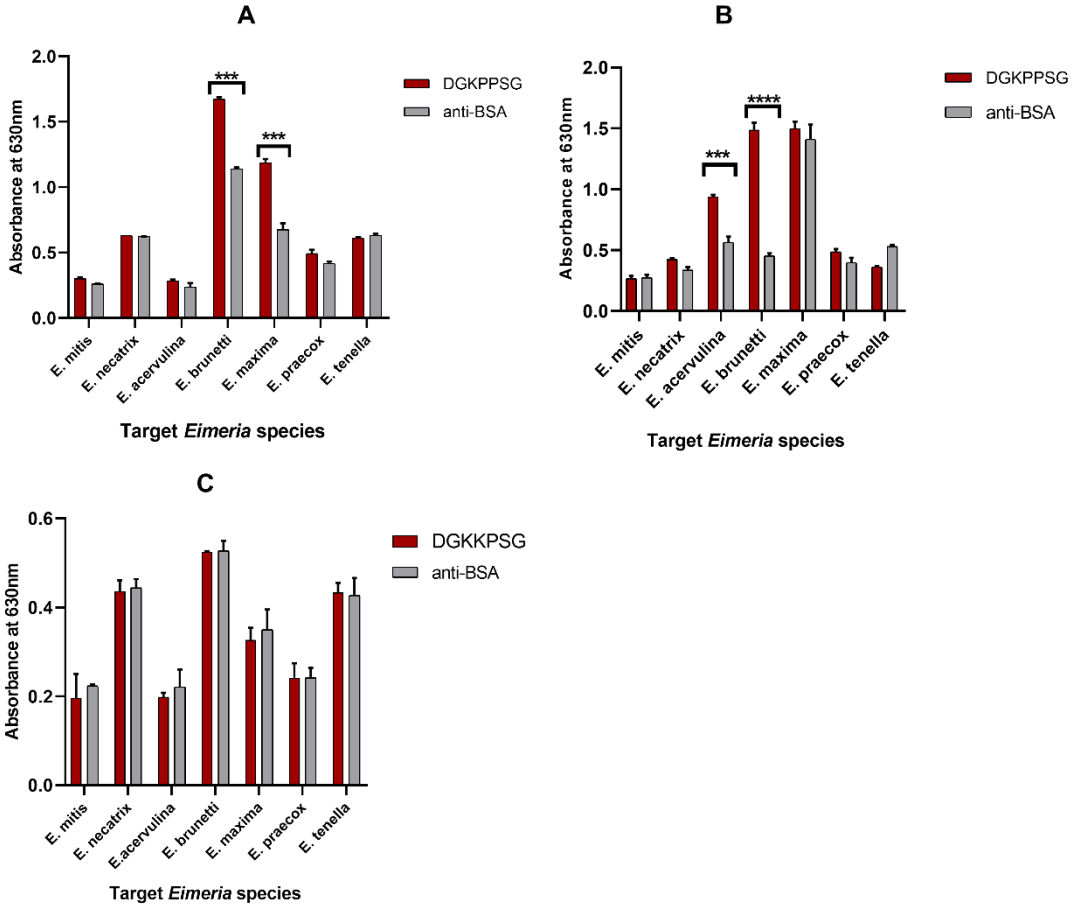


Figure 3:19: Binding of rescued motif DGKKPSG to species of *Eimeria* oocysts. scFv phage were produced for DGKKPSG and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. Data represents the mean and standard deviation of two replicates. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). (A), (B) and (C) represent repeat assays.

Rescued motif HPMLGGLQGK was significantly higher than anti-BSA when binding to *E. necatrix* (p =0.00520) and *E. tenella* (p =0.0265). There was no significant difference when binding to other species; *E. mitis* (p>0.9999), *E. acervulina* (p>0.9999), *E. brunetti* (p =0.1276), *E. maxima* (p =0.9957) and *E. praecox*

(p>0.9999) (Figure 3.20). When this assay was repeated, HPMLGGLQGK was not significantly higher than anti-BSA when binding to any species; *E. mitis* (p =0.9997), *E. necatrix* (p =0.9876), *E. acervulina* (p =0.5576), *E. brunetti* (p =0.2763), *E. maxima* (p>0.9999), *E. praecox* (p>0.9999) and *E. tenella* (p =0.5643).

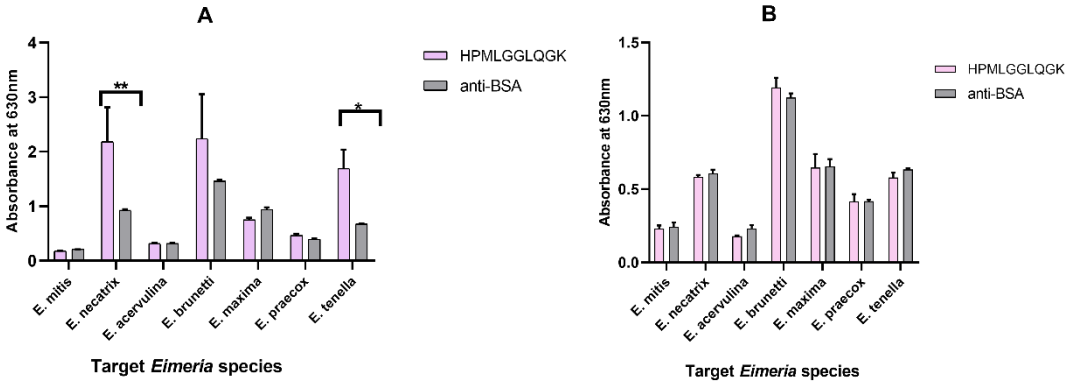


Figure 3:20: Binding of rescued motif HPMLGGLQGK to species of *Eimeria* oocysts. scFv phage were produced for HPMLGGLQGK and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

The binding of rescued motif AWVTVNQTTNY was compared against an anti-BSA control and AWVTVNQTTNY was significantly higher than anti-BSA when binding to *E. necatrix* (p =0.0008) and *E. maxima* (p =0.0007). There was no significant difference when binding to other species; *E. mitis* (p =0.9993), *E. acervulina* (p =0.9903), *E. brunetti* (p>0.9999), *E. praecox* (p>0.9999) and *E. tenella* (p>0.9999) (Figure 3.21). When this assay was repeated, AWVTVNQTTNY did not display significantly higher binding compared to anti-BSA when binding to all species; *E. mitis* (p =0.4357), *E. necatrix* (p =0.9876), *E. acervulina* (p =0.5576), *E. brunetti* (p =0.2763), *E. maxima* (p>0.9999), *E. praecox* (p>0.9999) and *E. tenella* (p =0.5643).

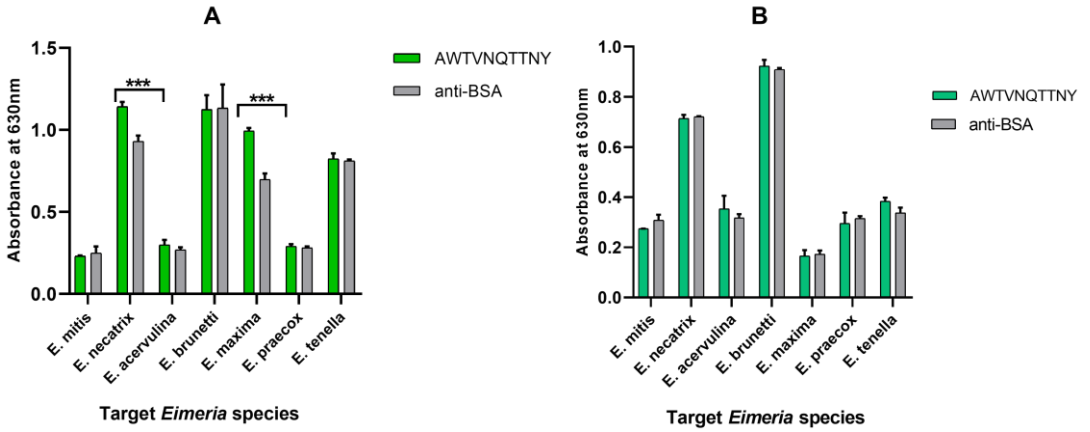


Figure 3:21: Binding of rescued motif AWTVNQTTNY to species of *Eimeria* oocysts. scFv phage were produced for AWTVNQTTNY and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10 minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. p ≤ 0.001 (***). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

The binding of rescued motif DRSYTACDTSK was compared against an anti-BSA control and DRSYTACDTSK was significantly higher than anti-BSA when binding to *E. necatrix* (p>0.0001), *E. brunetti* (p>0.0001) and *E. maxima* (p =0.0137). There was no significant difference when binding to other species; *E. mitis* (p>0.7473), *E. acervulina* (p>0.9999) *E. praecox* (p =0.1836) and *E. tenella* (p =0.9799) (Figure 3.22). When this assay was repeated DRSYTACDTSK displayed significantly higher binding compared to anti-BSA when binding to *E. tenella* (p<0.0001). It did not bind significantly higher compared to anti-BSA in other species *E. mitis* (p =0.8956), *E. necatrix* (p =0.9992), *E. acervulina* (p =0.0627), *E. brunetti* (p =0.2300), *E. maxima* (p =0.9857) and *E. praecox* (p =0.6395).

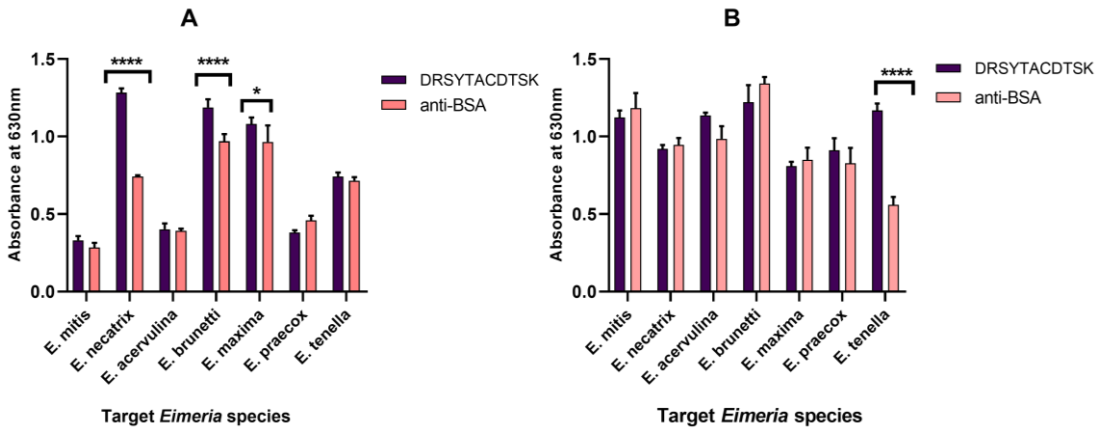


Figure 3:22: Binding of rescued motif DRSYTACDTSK to species of *Eimeria* oocysts. scFv phage were produced for DRSYTACDTSK and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. In both occasions, monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. Data represents the mean and standard deviation of two replicates. P ≤ 0.05 (*), p≤ 0.0001 (****). (A) and (B) represent repeat assays.

The binding of rescued motif QTGMN was compared against an anti-BSA control and QTGMN was significantly higher (p<0.0001) when binding to *E. brunetti*. It did not bind significantly higher than anti-BSA when binding to *E. mitis* (p>0.9999), *E. necatrix* (p>0.9999), *E. acervulina* (p =0.7935), *E. maxima* (p>0.9999), *E. praecox* (p =0.1797) and *E. tenella* (p =0.8456) (Figure 3.23). When this assay was repeated, QTGMN was significantly higher than anti-BSA when binding to *E. maxima* (p<0.0001). There was no significant difference when binding to other species; *E. mitis* (p =0.9982), *E. necatrix* (p>0.9999), *E. acervulina* (p =0.9941), *E. brunetti* (p>0.9999), *E. praecox* (p =0.6090) and *E. tenella* (p>0.9999).

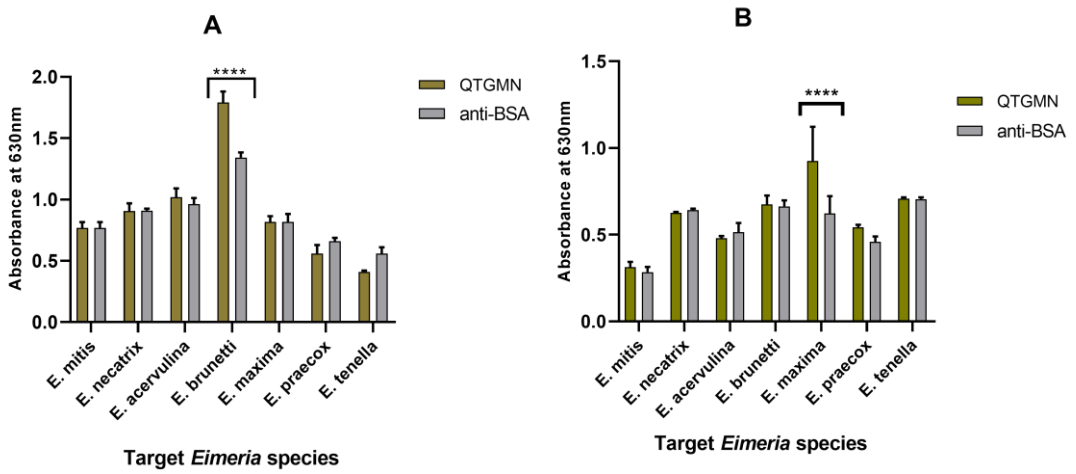


Figure 3:23: Binding of rescued motif QTGMN to species of *Eimeria* oocysts. scFv phage were produced for QTGMN and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

From the second set of analysis to find species-specific motifs. Two motifs were rescued with the correct sequence IGPSA and YTRKRFRRRPER. YTRKRFRRRPER was screened via ELISA alongside an anti-BSA control. YTRKRFRRRPER binding was significantly higher than anti BSA scFv (p<0.0001) in six species (*E. necatrix, E. acervulina, E. brunetti, E. maxima, E. praecox and E. tenella*) and significantly higher in *E. mitis* (p = 0.0037) (Figure 3.24). This assay was repeated and the motif displayed significantly higher binding compared to anti-BSA (p<0.0001) in three species *E. mitis*, *E. acervulina* and *E. tenella*, it was significantly higher in *E. necatrix* (p =.0025), *E. brunetti* (p =0.0003), *E. maxima* (p =0.0008) and *E. praecox* (p =0.0029).

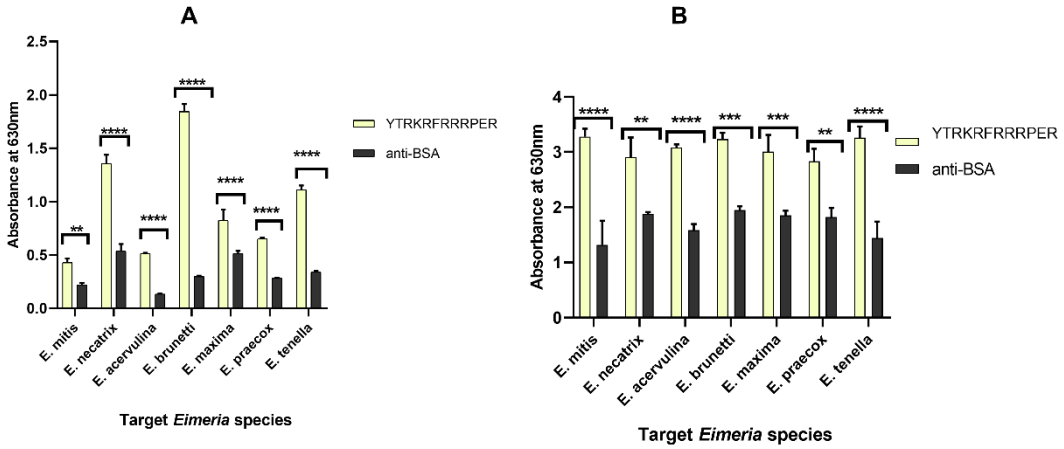


Figure 3:24: Binding of rescued motif YTRKRFRRRPER to species of *Eimeria* oocysts. scFv phage were produced for YTRKRFRRRPER and the anti-BSA negative control. Whole oocysts 10^5 were put in duplicate sterile Eppendorf tubes for both test phage and anti-BSA control phage. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. (Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates. (A) and (B) represent repeat assays.

When the binding of rescued motifs DKSSqGT, TTALP, VAADPFDY, KDVGVVPK and IGPSA (from the second set of rescues) were compared against an anti-BSA control, they were not significantly higher than the anti-BSA control for all species (data not shown). The assay was repeated for motifs DKSSqGT and IGPSA and similar results were obtained (data not shown). Screening of motifs were then not pursued further.

ScFvs were tabulated according to their specificities and binding strengths in the ELISA assays. (Table 3.9). From the ELISAs, six scFv candidates that showed consistent binding/ strong binding signals were selected and are represented in Table 3.14.

Table 3:9: Specificity and binding strengths of conventionally picked and NGS rescued motifs

scFv[1]	***E. mitis***	***E. necatrix***	***E. acervulina***	***E. brunetti***	***E. maxima***	***E. praecox***	***E. tenella***
DHTPKWq	Yes (***)	Yes (****)	No	Yes (****)	Yes (****)	No	Yes (****)
DHTPKWq	Yes (*)	Yes (****)	Yes (****)	Yes (****)	Yes (**)	No	Yes (****)
STQLP	No	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)
STQLP	Yes (***)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)
STqHP	No	Yes (****)	Yes (****)	Yes (****)	Yes (***)	Yes (****)	Yes (****)
STqHP	Yes (****)	Yes (****)	No	Yes (****)	Yes (**)	No	Yes (****)
SAqHP	Yes (****)	No	Yes (****)	No	Yes (****)	Yes (****)	Yes (****)
SAqHP	Yes (****)	No	No	Yes (**)	Yes (**)	Yes (***)	Yes (**)
SAqHP	Yes (****)	No	No	No	No	Yes (***)	No
SAqHP	Yes (****)	No	No	No	No	No	No
SRSKNYLWRSM	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)
SRSKNYLWRSM	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)
YTRKRFRRPER	Yes (**)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)	Yes (****)
YTRKRFRRPER	Yes (****)	Yes (**)	Yes (****)	Yes (***)	Yes (***)	Yes (**)	Yes (****)
DKSSqGT	No	No	No	No	No	No	No
DKSSqGT	No	No	No	No	No	No	No
DGKPPSG	No	No	No	Yes (***)	Yes (***)	No	No
DGKPPSG	No	No	Yes (***)	Yes (****)	No	No	No
DGKPPSG	No	No	No	No	No	No	No
VAADPFDY	No	No	No	No	No	No	No
TTALP	No	No	No	No	No	No	No
KDVGVVPK	No	No	No	No	No	No	No

HPMLGGLQGK	No	Yes (**)	No	No	No	No	Yes (*)
HPMLGGLQGK	No	No	No	No	No	No	No
AWTVNQTTNY	No	Yes (***)	No	No	Yes (***)	No	No
AWTVNQTTNY	No	No	No	No	No	No	No
DRSYTACDTSK	No	Yes (****)	No	Yes (****)	Yes (*)	No	No
DRSYTACDTSK	No	No	No	No	No	No	Yes (****)
QTGMN	No	No	No	Yes (****)	No	No	No
QTGMN	No	No	No	No	Yes (****)	No	No
IGPSA	No	No	No	No	No	No	No
IGPSA	No	No	No	No	No	No	No

[1] Candidate scFvs selected from conventional colony picks or successful rescued after identification in NGS datasets

Dark green highlight: Binding strength **** (p value ≤ 0.0001) or *** (p value ≤ 0.001)

Light green highlight: Binding strength ** (p value ≤ 0.01) or * (p value ≤ 0.05)

Yellow highlight – no binding

Table 3:10: Method of isolation and specificity in ELISA assays and NGS analysis of selected candidate scFvs

scFv	**Method of isolation**	**Apparent specificity based on spin down ELISA**							**Apparent specificity based on NGS analysis**						
		1	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
DHTPKWq	Conventional pick	+	+	+/-	+	+	-	+	+	+	+	+	+	+	+
STQLP	Conventional pick	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+
STqHP	Conventional pick	+/-	+	+/-	+	+	+/-	+	+	+	+	+	+	+	+
SRSKNYLWRSM	NGS rescue	+	+	+	+	+	+	+	-	+	-	+	+	-	-
SAqHP	NGS rescue	+	-	+/-	+/-	+/-	+/-	+/-	-	-	+	-	-	-	-
YTRKRFRRRPER	NGS rescue	+	+	+	+	+	+	+	-	-	-	-	-	+	-

1 (*E. mitis*), 2 (*E. necatrix*), 3 (*E. acervulina*), 4 (*E. brunetti*), 5 (*E. maxima*), 6 (*E. praecox*), 7 (*E. tenella*)

Green highlight (binding to species), Red highlight (Not binding to species), Purple highlight (showing variable binding)

3.3 Discussion

Phage display technology is a potentially powerful technique that can enable the identification of target specific ligands without the need for animal immunisations (Smith, 2019). It has been a very successful method of isolating antibodies to different targets from diverse libraries and can allow for the enrichment of binders with very defined binding specificity (Rouet *et al*., 2012).

In this study, we have demonstrated that using conventional phage display and NGPD, antibodies can be isolated against oocysts of the seven economically important species of *Eimeria* in poultry. The process of phage isolation based on affinity is carried out through biopanning rounds which is a critical step for tailoring the isolation of ligands with specific or desired binding properties. Typically between 2-5 rounds are carried out to isolate binders from large single pot libraries (Zhang *et al.,* 2011, Yang *et al.,* 2017). Biopanning usually relies on the immobilisation of target antigen on a solid support and target molecules have been immobilised via passive adsorption and capture system with each method of antigen presentation having its advantages and disadvantages. Direct adsorption on polystyrene is a simple method yet there could be conformational changes upon binding to the well. Capture systems have been used especially by biotinylation of the antigen and capture on streptavidin coated plates, which have resulted in improved display due to the antigen not being in direct contact with the plastic (Ledsgaard *et al.,* 2018). Here, Griffin library scFv phage was panned against whole intact individual *Eimeria* oocysts of the seven species of poultry in solution. Whole oocysts were used as the target for this study because they are the most abundant form of the parasite and are present in poultry litter, making them a readily available sample type for immunoassays (Waldenstedt *et al.,* 2001, Long *et al.,* 1975, Gadelhaq *et al.,* 2018). Also, panning on intact cells have been reported to have the advantage of isolating antibodies against native epitopes of antigens in as close as possible state to that found in the field (Abi-Ghanem *et al.,* 2008). When antibodies are isolated that are close to the state of field antigens, field diagnostic developed can easily be applied without further treatment of oocysts.

This study applied conventional phage display and NGPD to find antibodies to *Eimeria* oocysts in poultry. In the first round of panning selection, the seven species of *Eimeria* oocysts were panned in parallel using the naïve Griffin library and bound phage was eluted using direct infection with TG1 bacteria. The seven polyclonal phage-antibody sub-libraries selected from the first round of panning were pooled and

grown together and were used to pan against the seven *Eimeria* oocysts individually in the next round. Five panning rounds were carried out and in each panning round, seven sub-libraries were produced. Random colony picks were performed from the round 5 panning glycerol stock. A total of seventy single clones were picked. Analysis of the diversity of the single colonies picked revealed that a single clone accounted for up to 90% of the clones picked. Initial ELISA was performed using oocyst lysates on Nunc Maxisorp® plates and when this failed to identify any positive binders, whole oocysts were used on Nunc Maxisorp® plates (data not shown) and no binders were identified. It was discovered that oocysts were not being immobilised on the plates and so ELISAs were performed in eppendorf tubes to mimic how the panning was carried out. Three different scFvs identified from sequencing the single clones were screened and were found to bind to *Eimeria* species. In order to find further binders to whole oocysts, the eluted phage were also used as template to amplify the HCDR3 genes which were then sequenced by NGS. Analysis of NGS data was performed using two approaches, the first one was performed to compare selection rounds and growth phases. The frequency and ratio of HCDR3 sequences in each sub library were analysed by a two-proportion Z-test through progressive rounds of panning i.e. establishing scFv enrichment against each individual *Eimeria* species after each round of panning and further selecting scFvs that were only enriched during selection phases of the panning. According to Z score ranking, 43 HCDR3s were highly enriched against the different *Eimeria* species oocysts in the *in-silico* assessment and some were found in panning against multiple species. It is interesting to note that all the candidate scFvs selected with the exception of DKSSqGT had been enriched to only the third panning round. The NGS strategy identified two identical HCDR3s as having different binding characteristics; STQHP was binding to only *E. tenella* while STqHP appeared to be pan-specific. STQHP was not successfully rescued hence the full scFv sequence could not be compared to STqHP to determine if they are different outside their HCDR3s. Three of the HCDR3s that had just 1 amino acid difference were compared against one another (data not shown). SAqHP (binding consistently to only *E. acervulina*), STqHP (pan-specific) and STQLP (pan-specific). SAqHP and STqHP had identical sequences outside their HCDR3s differing in 1 amino acid in their HCDR3s but had very different binding properties. This may not be surprising as substantial changes in binding specificity of scFvs by single amino acid exchange have been observed (Winkler *et al*., 2000). STQLP when compared to SAqHP and STqHP had different sequences in the framework regions and CDRs. An scFv having multiple amber stop codons is likely to be displayed at a much lower frequency than one with the same sequence with a Q codon replacing the stop codon. Being the

same antibody having different concentrations which may show different binding specificities.

The scFvs were not enriched in the 4th and 5th panning rounds. This bioinformatics analysis strategy was similar to that of the published method of (Sasso *et al*., 2015) on recovery of scFv clones from screening phage libraries against cells expressing a protein involved in hepatitis C infection. In the study, the authors compared enrichment profiles through panning rounds. Interestingly, that study also found that clones reached their *maxima*l enrichment at round 3 of panning. (Abi-Ghanem *et al*., 2008) in the characterization of scFvs against *E. tenella* sporozoites also found enrichment reached its maximum in the 3rd round of panning.

An alternative NGS analysis was carried out to improve the chances of finding species-specific HCDR3s by generating Z scores from the comparison of each HCDR3s selected against a particular species with its isolation against the other six species. These selected HCDR3s were then subjected to another analysis whereby motifs that had only either one or two sequence change and were found in multiple data sets (for the different *Eimeria* species) as different motifs were removed as they may bind the same epitope. This was carried out because in the previous analysis, many HCDR3s that had just one or two sequence changes emerged as HCDR3s having top Z scores.

The top ten HCDR3s with the highest Z scores were selected for each species. However, this analysis did not get rid of all of the pan-specific clones as some of the pan-specific HCDR3s that had been selected in the first analysis appeared in the second analysis and they were removed before selecting the top ten HCDR3s that were showing species-specificity and had not been selected previously.

Conventional screening methods which involve picking up to a few hundred clones, screening via ELISA and Sanger sequencing to confirm the clones was performed in the present study enabled the comparison of conventional phage display and NGPD. Typically, picking of clones are carried out from the final panning round which ideally should be the most enriched with clones of potentially higher affinity to the target. Previous studies on NGPD have indicated the validity of also using conventional phage display to identify ligands to various targets (Ladner *et al.,* 2004, Bruin *et al.,* 1999, Yang *et al.,* 2017). (Abi-Ghanem *et al*., 2008) employed phage display using an immune library against *E. tenella* sporozoites in five panning rounds and isolated antibodies to *E. tenella*.

Conventional random colony picks however, have a limitation of providing a small perspective ~0.01% of the complete sequence space which can easily be dominated by nonspecific sequences and may lead to the identification of false positives or

exclude the identification of target-binding sequences (Liu *et al.,* 2015). Also, the degree of enrichment during the biopanning process cannot be controlled, it frequently results in a limited number of clones especially in the later selection rounds (Yang *et al.,* 2017). It is not surprising that by the 5th panning round in this study, an antibody constituted about 90% of the handpicked clones because diversity decreases in later rounds of selections and in some cases it is largely monoclonal (Rouet *et al.,* 2018).

In the study by (Ngubane *et al*., 2013), just as in the present study, 5 panning rounds were performed and an 80% clonal dominance was observed in the 5th panning round but unlike the present study, conventional colony picking methods failed to isolate it. Clonal dominance may reflect true selection for high affinity binders but may also reflect certain properties such as superior growth advantage or expression (Rouet *et al.,* 2018). It is not uncommon for higher affinity binders to be present at low frequency, which may not be detectable using conventional screening methods such as random picks followed by ELISA (Rouet *et al.,* 2012).

Conventional screening methods are usually adequate to identify dominant clones after selection, but NGS sequencing of sub-libraries allows far greater depth and insights into the sub-library diversity (Barreto *et al.,* 2019, Rouet *et al.,* 2012). Often, the most promising clones are missed when such limited numbers of clones are analysed and false positive clones are a problem when isolating binders (AC't Hoen *et al.,* 2012). In this study, conventional screening methods enabled us to find three binders to oocysts. These binders were of higher frequencies compared to other HCDR3s, they were among the top ten binders based on frequency across panning rounds in all species.

In this study, Sanger sequencing of 23 random clones revealed that one clone DHTPKWq was dominant in round 5 panning (Figure 3.7).

In the present study, following NGS analysis phage were rescued using inverse PCR from panning sub-libraries where they are most enriched (Spiliotopoulos *et al.,* 2015). Several techniques have been previously described for phage rescue from enriched libraries. The studies by (Ravn et al., 2010b, Ravn et al., 2013, Yang et al., 2017) described the amplification and rescue of candidate scFvs of interest from their enriched sub-libraries using a two-step PCR reaction. DNA for the VH and VL domains of the scFv were amplified separately in a PCR reaction and then assembled using an overlap PCR using primers that target the specific HCDR3 sequence. The PCR product was then digested and cloned back into the vector. The sensitivity of the technique was not fully determined but using this approach, specific scFv clones of interest at a level of 0.5% frequency were recovered successfully. Alternatively,

(Zhang *et al*., 2011) described the use of a biotinylated probe complimentary to the HCDR3 of the phage DNA which bound the HCDR3 from the phage pool through hybridisation. The DNA that has been captured was then converted to dsDNA and transformed into electrocompetent cells. Again, this study did not determine the sensitivity of the technique but clones with a 1% frequency in the background library were successfully rescued. An alternative method was also described by (D'angelo *et al*., 2014) and developed by (Hoskins *et al*., 2005) which involved an inverse PCR strategy similar to the one used in the present study. The study by (D'angelo *et al*., 2014) divided the HCDR3 sequence between the two primers which extended into the adjacent framework regions. With this technique, clones that had a frequency of 1.9% in the library were successfully rescued. This approach also suggested that clones that were present at levels down to 0.5% could be isolated. Rescue of specific clones in this study was performed using a very sensitive method described by (Spiliotopoulos *et al*., 2015). The difference between this strategy and that described by (D'angelo *et al*., 2014) was that while (D'angelo *et al*., 2014) divided the HCDR3 sequence between both primers which extended to the adjacent frameworks, (Spiliotopoulos *et al*., 2015) described using a primer that covered the entirety of the HCDR3 region together with a degenerate framework 4 primer which was applied to a range of scFv candidates. The PCR product was then ligated together to form the phagemid DNA (Spiliotopoulos *et al.,* 2015). The phage rescue methodology was applied with 100% success to scFvs with a wide range of HCDR3 lengths, the smallest being just 4 amino acids, and frequencies that were as low as 0.0025%.

Here, out of a total of 90 clones set to be rescued from both sets of NGS analysis, only 13 of the isolated scFvs were successfully rescued and confirmed via Sanger sequencing, as the sequences obtained often contained frameshifts, truncated scFvs or mutations. The first set of rescues which were 20 scFv candidates were performed in a slightly different strategy as that described by (Spiliotopoulos *et al*., 2015). Primers used for the rescues were specific to each HCDR3 and each framework 4 region for all the candidate rescues unlike the degenerate framework 4 primer that could be applied to a range of candidates as described by (Spiliotopoulos *et al*., 2015). Using this strategy, eleven (55%) candidate scFvs were successfully rescued. A candidate scFv with frequency as low as 0.02% was successfully rescued at the first attempt (Table 3.6). For the second set of rescues, the exact strategy described by (Spiliotopoulos *et al*., 2015) was carried out (Figure 2.4, **chapter 2**). This strategy was employed as a large number of scFvs candidates were targeted. Only two (< 3%) out of 69 candidate scFvs were rescued successfully. One of these (IGPSA) had a HCDR3 of just 5 amino acids and so specific HCDR3 and framework 4 primers were

used, as the degenerate framework 4 primer could not be used for scFvs having less than 6 amino acid sequences (refer to Table 2.6, **chapter 2**). The frequencies of the successful rescues were also observed to be relatively higher (1.48% and 2.29%) than all but one of the other rescue targets (mostly < 1%). It is clear that the combination of the degeneracy of the framework 4 primer used for the second set of rescues as well as the low frequency of the candidate scFvs and low stringency of inverse PCR settings resulted in a low recovery rate of target scFvs (3% of the 69 targets). The data also shows that even with clone specific primer sets (24 clones in total) only 50% were successfully rescued. This presents a considerable limitation to the use of NGS analysis to identify clones of interest from naïve/semi-synthetic scFv libraries.

NGS data sets have been analysed using different approaches which could either be based on comparison of frequency of a clone within the enriched sub library relative to the whole library (Ravn et al., 2010b), comparison of growth and selection stages (Sasso *et al.,* 2015), or comparison of positive and negative panning reactions in which the same library that is panned against a target is panned against an unrelated ligand and Z scores generated (Naqid *et al.,* 2016b). (Miyazaki *et al*., 2015) compared the frequency of clones in panning rounds versus the naïve library in analysis of NGS data sets. (Rentero Rebollo *et al*., 2014) in the identification of target-binding peptide motifs by high-throughput sequencing described the use of frequency of motif isolation to identify enriched peptide binders to different protein targets.

In the present study, even though five panning rounds were carried out, round 5 were mostly dominated by a single clone. Round 5 frequency data compared against round 3 (Table 3.5) showed a percentage frequency of between 92-97% of a single clone in the round 5 panning compared to between 0.0152-65% of the same clone in the round 3 panning.

In this study, the traditional phage display technique which involved hand picking random colonies from enriched panning sub-libraries to find binders to the target was carried out alongside NGPD. In both methods of phage display, binders were identified. Conventional phage display is typically paired with NGPD to give an insight into the binding properties of the phage libraries but the depth NGS offers makes it superior to conventional phage display for finding binders to any specific target. (Yang *et al*., 2017) in comparing conventional phage display and NGPD using 3 chicken immune libraries to identify binders to prostate-specific antigen (PSA), found that the scFv clones retrieved from NGS analysis included all clones identified by conventional phage display as well as many additional clones not picked during the colony screening process. (Miyazaki *et al*., 2015) using an immunised V_HH library, found 13

antigen specific binders via conventionally colony picking but also noted a higher number of binders were identified after deep sequencing.

From the combination of colony picks and both sets of NGS rescues, 16 scFvs were isolated. Following the development of a spin down ELISA (discussed in detail in **chapter 4**), screening of isolated clones was performed (**chapter 2** subsection 2.6.8.1). The spin down assay proved to be technically challenging as there was always the possibility of losing oocysts during wash steps leading to varied results. SRSKNYLWRSM scFv which had high display levels and produced very consistent results likely because it was not affected by lose of oocysts to the same extent compared to the others given its high display level.

Also, wash steps might not have been very efficient because pipetting out all of the wash buffer at every step was not possible without loss of oocysts, and so background signals were relatively high. The assay was also not high throughput making it difficult to assay a large number of clones. Assays were repeated at least two times for most clones except for three clones TTALP, KDVGVVPK and VAADPFDY that did not show any binding to oocysts of any species when the first assay was performed.

Conventionally picked motif DHTPKWq that was dominant at round 5 was found to bind 5 species of *Eimeria* oocysts consistently, was found to be binding to oocysts of *E. acervulina* variably and not binding to *E. praecox*. STQLP bound to six species of oocysts when assayed twice but bound inconsistently to *E. mitis.* Similarly, STqHP bound inconsistently to *E. mitis*, *E. acervulina* and *E. praecox* but bound in repeat assays to other species. Across 4 repeat assays, SAqHP showed consistent binding to *E. mitis* in repeat assays, no binding to *E. necatrix* and variable binding to the other species. Two rescued motifs SRSKNYLWRSM from the first set of rescues and YTRKRFRRPER from the second set of rescues bound to all species of *Eimeria* albeit the former produced stronger signals. All other clones did not show reproducible binding to any *Eimeria* species across 2 to 3 assay repeats. Five clones (SRSKNYLWRSM, SAqHP. DHTPKWq, STQLP and STqHP) were selected for further assay development. YTRKRFRRPER was not selected for assay development as it had the same binding pattern as SRSKNYLWRSM which was a far stronger binder.

Taken together, the bioinformatics approach used in this current study as well as the conventional panning were successful in isolating binders to *Eimeria* oocysts. The use of Z scores to rank and analyse samples is an efficient way of analysing gene sequences produced from NGS. The analysis of growth and selection patterns across panning rounds aided in the isolation of binders to *Eimeria* oocysts proving that it is a

valid strategy of analysis of NGS data. It has also been used with success in some NGS published methods (Sasso *et al.,* 2015).

The alternative NGS analysis could not be validated as the scFvs were not rescued as intended, of the two that were rescued, one did not show any binding activity towards oocysts, the other bound to all species of oocysts. It was also noted that neither analysis method completely eliminated the pan-specific scFvs. Panning and NGS analysis were geared towards isolating species-specific binders with panning performed against individual species, and two out of the six binders identified showed some consistent differential binding to different species. However, no scFvs that bound consistently to a single *Eimeria* species were identified. It may be that pooling input phage between panning rounds rather than carrying out panning against the 7-target species completely independently may have resulted in the selection of scFvs against epitopes displayed on all or most species as they would be present across samples and therefore at overall higher levels than species-specific epitopes. Panning each species independently with subtraction of binders against the other species may be a more stringent and valid approach to obtain species-specific binders and would be considered as the next panning approach.

In conclusion, this chapter describes the successful discovery of antibodies to *Eimeria* oocysts aimed at developing species-specific diagnostics. This chapter demonstrating that phage display and NGPD are valid methods for the isolation of binders to *Eimeria* oocyst surfaces. The major limitations of the approach were the low efficiency of the scFv rescue strategy and the technical difficulties with screening scFvs against whole oocysts. At the time when this chapter was conducted, full gene synthesis was not a financially viable option given the large number of targets that were to be rescued. Some studies have performed full gene synthesis but both had a small number of intended rescues (Saggy *et al*., 2012b, Lövgren *et al*., 2016). A technique for obtaining full length sequences using NGS platform has been described (Raftery *et al.,* 2019). In theory, this could be combined with full length scFv gene synthesis to overcome difficulties in scFv candidate binder recovery, this may be something to consider in the future.

The spin down ELISA assay enabled the screening of the candidate scFvs but it was only reproducible to the binder; SRSKNLYWRSM that had very high display levels. The possibility of losing oocysts during wash steps makes the assay difficult to reproduce, this may be of particular concern where scFvs have relatively poor display levels and/or have relatively low affinities. The next chapter aimed to develop an assay for the isolated candidate scFvs that could be more easily reproducible and possibly higher throughput.

4. Assay development for the detection of antibodies to *Eimeria* oocysts of poultry.

4.1 Introduction

Serological and genetic based techniques such as enzyme variation, Southern blot analysis and PCR had been developed beginning from 1975 for the diagnosis of *Eimeria* infections in chickens but have not been widely adopted because they were limited by being either technically challenging, time consuming or were not able to differentiate all seven species of *Eimeria* (Shirley, 1994, Shirley, 1975, Thebo *et al.,* 1998, Ellis and Bumstead, 1990, Procunier *et al.,* 1993, Morgan *et al*., 2009). Chemotherapy using in-feed anticoccidiostats has been and is a major way infection is prevented and controlled in most commercial poultry establishments, especially broiler production (Amare *et al.,* 2012). However, there has been negative views about the use of drugs in food animals over the years and this has led to consumers calling for their ban. Live vaccines which are a good alternative for the control of this parasite are not widely used because of costs, partly due to the vaccine being made up of a mixture of all 7 *Eimeria* species.

The accurate identification of infecting *Eimeria* species has major implications for control as well as for studying their epidemiology. By being able to identify species that infect poultry in any outbreak will enable better control of the disease by targeted vaccination and since all species are not equally economically important, control strategies can be adapted for each species. Adequate control measures cannot be implemented without a good knowledge of species that infect both backyard and commercial flocks. There is therefore a need to develop an easier, cheaper and faster test that will help differentiate infecting *Eimeria* species and ideally that can be applied in the field.

The aim of this chapter was to develop and validate an assay for the detection of antibodies to *Eimeria* oocyst in poultry. Antibodies had been isolated against oocysts using NGPD as discussed in **chapter 3**. Recombinant antibodies have been successfully used in diverse diagnostic platforms ranging from standard immunoassays to microfluidics, nanoparticles, DNA integrated assays and lateral-flow assays (Ch'ng *et al.,* 2016). Antibodies are a crucial component of any diagnostic development platform because of their diverse applications (Kuhn *et al.,* 2016, Ch'ng *et al.,* 2016). It was hoped that the diagnostic would be cheap, sensitive, species-specific and easily applicable in the field.

Here, the study looks at optimising an assay for *Eimeria* oocysts using the antibodies isolated in **chapter 3**.

4.2 Results

4.2.1. Conventional screening of scFv phage binders

4.2.1.1 scFv polyclonal phage ELISA

The binding properties of the polyclonal phage was assessed using oocyst lysates as antigen. This was derived from bead beating and the protein content (between 0.0376 and 0.1560 µg/ml) from lysed oocysts was used at 20 µg/ml. Phage were grown from the Griffin library and from all five panning rounds pooled from each species (10^{11}-10^{12} PFU/ml). The assay used rabbit anti-M13-HRP antibody with a TMB substrate and it was conducted using Nunc MaxiSorp® 96 well plates. Absorbance signals produced after 30 minutes of adding the substrate was background level (that produced by the library) at ~ 0.2-0.3 (Figure 4.1).

A repeat ELISA included phage from the 5th panning round, a positive control phage-scFv (anti-BSA scFv-phage and BSA antigen), along with a *Salmonella* lysate) negative control antigen (assayed with the round 5 polyclonal phage). The anti-BSA scFv positive control produced high signals while the round 5 phage produced background signals (Figure 4.2). Data show that polyclonal phage do not bind non-denatured *Eimeria* oocyst lysate.

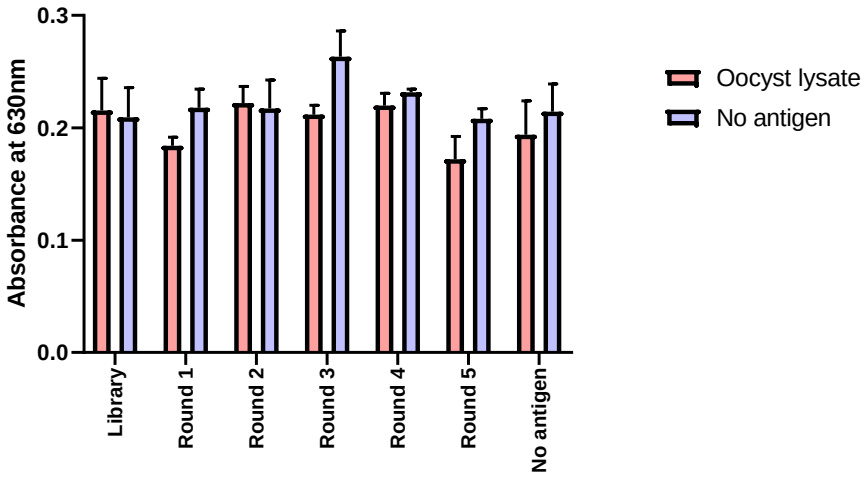


Figure 4:1: Polyclonal phage ELISA for *Eimeria* oocyst lysates. Oocyst lysates at 20 µg/ml or PBS were immobilised overnight in 100 µl aliquots on Nunc MaxiSorp® plate. scFv phage for each of 5 panning round (pooled from panning against each of the seven species of *Eimeria*) and Griffin scFv library (100 µl of ~ 10^{12} PFU/ml) were used to detect the immobilised oocyst lysate and binding detected using Anti-M13 HRP antibody. The TMB substrate was allowed 30 minutes incubation in the well before absorbance values were taken at 630 nm.

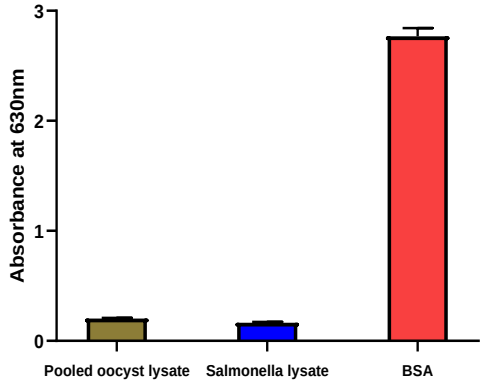


Figure 4:2: Polyclonal phage ELISA for oocyst lysates. scFv phage were produced from the 5th panning round (pooled from panning against each of the seven species of *Eimeria*) as well as anti-BSA positive control phage. Oocyst lysates, *Salmonella* lysates or BSA at 20 µg/ml were immobilised overnight in 100 µl aliquots on Nunc MaxiSorp® plate. Polyclonal scFv phage from panning with *Eimeria* oocysts were used to detect immobilised oocyst and *Salmonella* lysates while monoclonal scFv anti-BSA phage was used to detect BSA (100 µl of ~ 10^{12} PFU/ml) and those remaining after the wash steps were detected using Anti-M13 HRP antibody. The TMB substrate was allowed 30 minutes incubation in the well before absorbance values were taken at 630 nm.

4.2.2.2 Colony picking

Monoclonal phage ELISAs were carried out for all species. 10 single colonies were randomly picked from the round 5 panning glycerol stocks for each species, totalling 70 clones. Monoclonal phage preparations were made for each and the individual scFv binding properties were assessed by phage ELISA using oocyst lysates as antigen and an anti-M13-HRP antibody with a TMB substrate for detection of binding. Absorbance signals were compared against a monoclonal anti-BSA phage-scFv positive control and signals produced after 30 minutes of adding substrate were mostly very low at ~ 0.1-0.2, with a few (often the 10th clone for each species) showing 2X higher signals compared to other clones (Figure 4.3). From the clones that showed relatively higher signals (EM2, EM10, EN10, EMa10 and EP10), phage were grown to further investigate and to eliminate the possibility that the clones were relatively higher because of edge effects on the plate. Upon re-testing they all had background signals (data not shown).

Monoclonal phage ELISAs were then performed using acid precipitated phage that had on the average a ten-fold higher concentration of phage particles compared to

supernatant phage. Two clones were selected for testing; EM10 and EP10. Signals produced were background (data not shown).

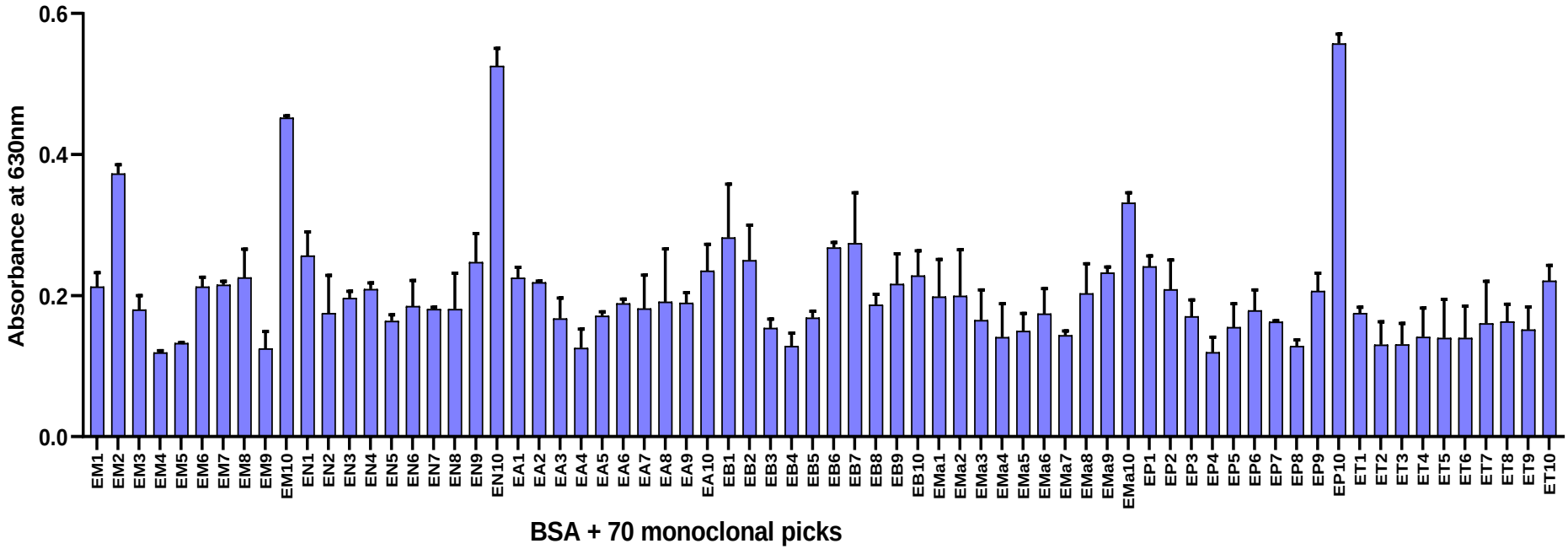


Figure 4:3: The binding of monoclonal phage antibodies to *Eimeria* oocyst lysates. Clones labelled EM1-10 represent *E. mitis* 1-10, clones labelled EN1-10 represent *E. necatrix* 1-10, clones labelled EA1-10 represent *E. acervulina* 1-10, clones labelled EB1-10 represent *E. brunetti*, clones EMa1-10 represent *E. maxima* 1-10, clones labelled EP1-10 represent *E. praecox* 1-10, clones ET1-10 represent *E. tenella* 1-10. scFv phage were produced from 70 randomly picked colonies from the 5th round of panning; 10 for each *Eimeria* species. Oocyst lysates or BSA was immobilised overnight in 100 µl aliquots at 20 µg/ml on Nunc MaxiSorp® plate. Monoclonal scFv phage (100 µl of ~10^{11}-10^{12} PFU/ml) for each individually picked colony were used to detect immobilised oocyst lysates while anti-BSA phage was used to detect BSA and those remaining after the wash steps were detected using Anti-M13 HRP antibody. The TMB substrate was allowed 30 minutes incubation in the well before absorbance values were taken at 630 nm. Absorbance value for BSA positive control was ~ 2.7 after 30 minutes of adding substrate (data not shown).

4.2.2 scFv Phage ELISA using sonicated and Nonidet®40 treated oocysts

As lysate produced by bead beating may result in membrane fragments that may not immobilise to Nunc MaxiSorp® plates, an alternative lysis method was attempted using Nonidet®40 and heating to release membrane components. Duplicate pooled oocysts (10^4) were used in two different treatments, the first treatment involved heating the oocysts for 15 minutes at 90°C with 10% (v/v) of Nonidet®40 while the second treatment involved heating oocysts for 10 minutes at 50°C with 5% (v/v) Nonidet®40. When proteins were quantified for each treatment using Bradford's assay, treatment 1 in different species produced 0.65-1.17 mg/ml while with the second treatment produced 0.96-1.40 mg/ml (Table 4.1).

Table 4:1: Concentration of oocyst proteins of two treatments of Nonidet®40 determined by Bradford's assay

Species [1]	**Treatment 1 (mg/ml)** [2]	**Treatment 2 (mg/ml)** [2]
E. mitis	0.967891	0.717791
E. necatrix	1.39573	1.167689
E. acervulina	0.962834	0.650149
E. brunetti	1.038485	0.692622
E. maxima	1.389665	1.078024
E. praecox	1.29505	0.835772
E. tenella	0.974855	0.667453

[1] Target *Eimeria* species

[2] Treatment of oocysts by heating for 15 minutes at 90°C with 10% (v/v) of Nonidet®40

[3] Treatment of oocysts by heating for 10 minutes at 50°C with 5% (v/v) Nonidet®40

The binding of scFv phage to oocyst lysates was assessed in an ELISA using Nonidet®40 treated oocyst lysate on 96 well plates. An average, this method produced a 10-fold higher protein content compared to lysing oocysts using a bead beater (refer to chapter 3, figure 3.4).

Monoclonal phage was produced from five clones SRSKNYLWRSM, STQLP, SAqHP, DHTPKWq and STqHP (at the time that this assay was attempted these clones were known to bind to whole oocysts using a spin down assay and this assay was attempted in a bid to develop a much higher throughput assay). An anti-BSA phage positive control was also produced. The phage were screened against 20 µg/ml of Nonidet®40 treated oocyst lysate from both treatments. Background signals (~0.2-0.3) were produced from the assay for each of the seven species of *Eimeria* (data not shown) from both treatments while anti-BSA positive control produced signals of ~0.8. The amount of protein used was doubled from 20 µg/ml to 40 µg/ml

while the other conditions remained the same but this did not improve the assay (data not shown). This may suggest that the antigen is only recognized when presented on the oocyst surface, is not soluble after processing by heating and treating with Nonidet®40 or does not immobilise on the Nunc MaxiSorp® plates.

4.2.3. Binding of whole oocysts on Nunc MaxiSorp® plates

Phage prepared from the DHTPKWq clone was used in an ELISA to determine its binding to whole oocysts. Using whole oocysts at 10^5/well and BSA at 20 µg/ ml the immunoassay was conducted on Nunc MaxiSorp® plates. The assay included oocysts detected with anti-BSA phage (negative control), oocysts detected with DHTPKWq phage, and no antigen with DHTPKWq phage (negative control), BSA detected with DHTPKWq phage (negative control) and no antigen with anti-BSA phage (negative control) and BSA detected with anti-BSA scFv phage (positive control). Signals produced for DHTPKWq phage binding whole oocysts were background ~ 0.1- 0.2, while the anti-BSA positive control phage produced an absorbance signal of 0.68 (Figure 4.4). To test whether oocysts were being immobilised the presence of oocysts were assessed by microscopic examination. It was found that no oocysts were being immobilised after washing equivalent to the ELISA protocol (discussed in detail in subsection 4.2.7).

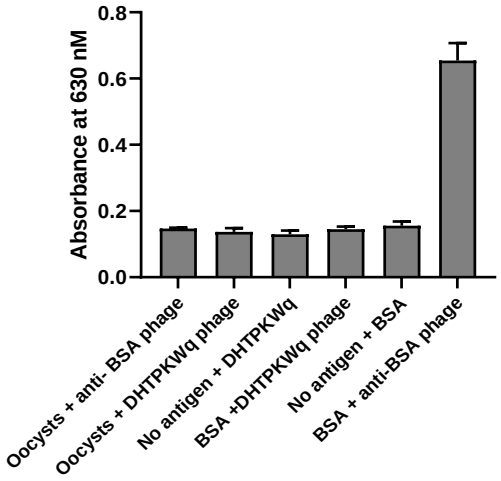


Figure 4:4: Monoclonal scFv phage ELISA against whole *Eimeria* oocysts. Whole oocysts 10^5, BSA at 20 µg/ml or PBS were immobilised overnight in 100 µl aliquots on Nunc MaxiSorp® plate. Monoclonal scFv phage DHTPKWq or anti-BSA phage were used to detect the immobilised oocyst and BSA (100 µl of ~ 10^{12} PFU/ml) and those remaining after the wash steps were detected with Anti-M13-HRP. The TMB substrate was allowed a 30-minute incubation before absorbance was read at 630 nm.

4.2.4 Optimisation of a spin down immunoassay to detect whole oocysts

Following the inability of whole oocysts to be immobilized on Nunc MaxiSorp® plates and the production of background signals in ELISAs carried out with oocyst lysates on Nunc MaxiSorp® plates, an ELISA was developed in solution using eppendorf tubes, spinning down between washes and pipetting out the wash buffer. Phage was prepared from the DHTPKWq clone and screened with pooled *Eimeria* species at 10^5 cells per species per tube and anti-BSA scFv phage was screened with an equal number of pooled *Eimeria* oocysts as a negative control. Even though the background absorbance signal produced was high, absorbance signal with DHTPKWq was significantly higher ($p = 0.0005$) than that of the anti-BSA after 10 minutes of adding TMB substrate and read at 630 nm wavelength (Figure 4.5).

Following this, it was thought to perform the assay with a varying number of oocysts to determine if excess oocysts were being used for the assay. Six variables were used; pooled oocyst (10^5) of each species + DHTPKWq phage, BSA+ DHTPKWq phage, pooled oocysts (10^5) of each species + anti-BSA phage, pooled oocyst (10^4) of each species + DHTPKWq phage, pooled oocyst (10^2) of each species + DHTPKWq phage and BSA with anti-BSA phage. Assays were performed in eppendorf tubes for assays involving oocysts while BSA was immobilised on Nunc MaxiSorp® plate. Signals produced for pooled oocyst of each species (all concentrations) + DHTPKWq was significantly higher than pooled oocysts (10^5) of each species + anti-BSA phage ($p<0.003$; Figure 4.6). There was a significant difference between pooled oocysts 10^5 + DHTPKWq phage and pooled oocyst 10^5 + anti-BSA phage hence the oocysts (10^5) were used for subsequent assays.

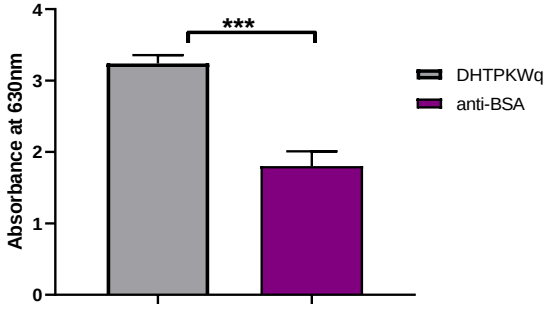


Figure 4:5: Binding of monoclonal scFv DHTPKWq to a pool of *Eimeria* oocysts. Whole oocysts (10^5) pooled from each of the seven species were put in sterile Eppendorf tubes. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal DHTPKWq phage or anti-BSA phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with Anti-M13-HRP. The TMB substrate was allowed a 10-minute incubation before absorbance was read at 630 nm. Statistical analysis was performed using unpaired t test analysis ($p = 0.005$). Data represents the mean and standard deviation of three

replicates.

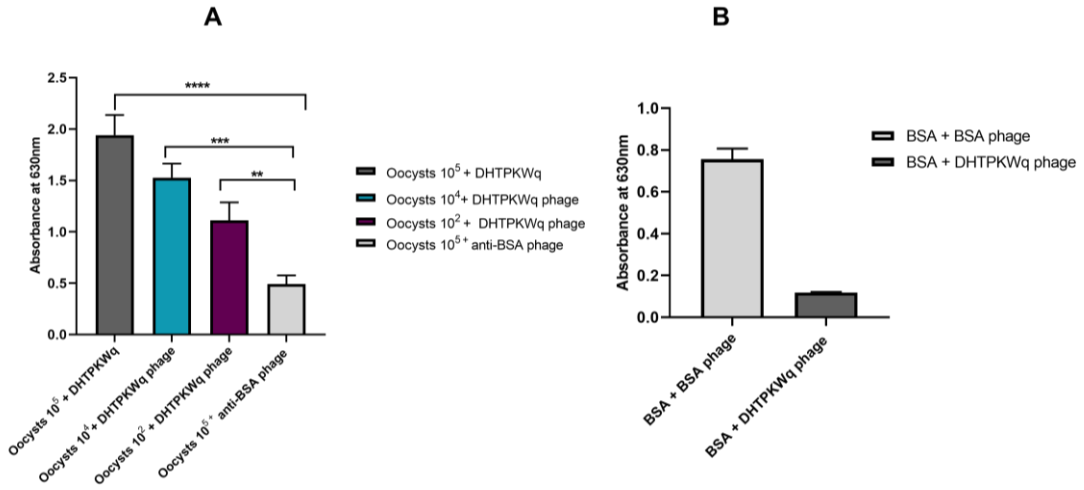


Figure 4:6: Binding of monoclonal scFv DHTPKWq to a pool of *Eimeria* oocysts. Oocysts; $10^{5,}$ 10^4, and 10^2 for each species were put in sterile eppendorf tubes while BSA was immobilised in100 µl aliquots on Nunc MaxiSorp® plates. Monoclonal scFv phage DHTPKWq or anti-BSA phage were used to detect the antigens (400 µl of ~ 10^{11} PFU/ml for oocysts in eppendorfs and 100 µl for BSA on Nunc MaxiSorp® plates). Those remaining after the wash steps were detected with an anti-M13 HRP antibody. The TMB substrate was allowed a 10-minute incubation before absorbance were read at 630 nm. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference among the test samples compared to the anti-BSA control. P ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). (A) represents oocysts assay in Eppendorf tubes (B) represents the control assay on Nunc MaxiSorp® plates. Data represents the mean and standard deviation of two replicates.

Following this, the assay was repeated without pooling of oocysts to determine if DHTPKWq bound to any particular species. Individual oocyst species (10^5) for each *Eimeria* species were put in sterile eppendorf tubes and the spin down ELISA was carried out (refer to chapter 2, subsection 2.6.8.1). Absorbance signals produced for DHTPKWq was higher than signals for anti-BSA for 6 out of 7 species after 5 minutes of adding TMB substrate and read at 630nm wavelength (Figure 4.7).

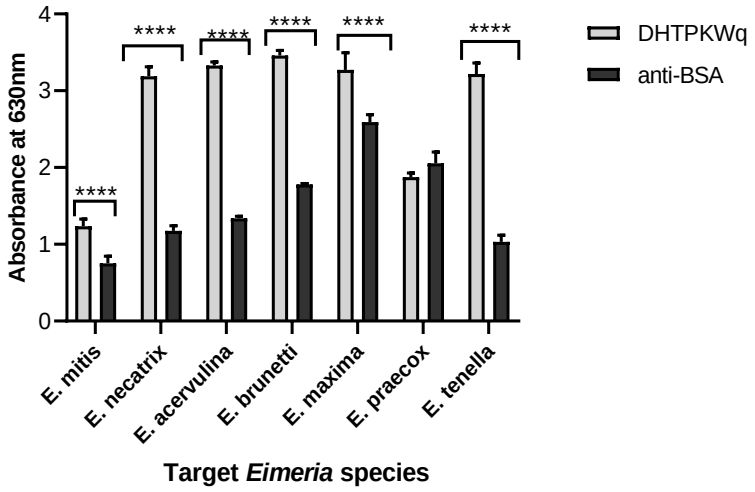


Figure 4:7: Binding of monoclonal scFv DHTPKWq to individual *Eimeria* oocysts. Whole oocysts 10^5 were put in duplicate sterile eppendorf tubes for both test phage and anti-BSA control phage. Oocysts were spun down and resuspended in 100 µl of PBS. Monoclonal DHTPKWq phage and anti-BSA phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised oocysts and those remaining after the wash steps were detected using anti-M13- HRP antibody. The TMB substrate was allowed a 10-minute incubation before absorbance values were taken at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of three replicates.

Similar assays were performed for other conventionally picked clones; STQLP and STqHP (refer to **chapter 3,** subsection 3.2.2.5) as well as 13 other scFv clones that were rescued after NGS analysis (refer to **chapter 3,** subsection 3.2.3.2). SRSKNYLWRSM and YTRKRFRRPER scFvs bound all 7 species of *Eimeria*, DHTPKWq bound 6 of the 7 species, STQLP and STqHP bound all 7 species but with some variability between assays. Combinations of these scFvs were selected for further assays.

The spin down ELISA allowed assessment of scFv-phage binding to whole oocysts but produced relatively high background. This was attributed to insufficient washing as a low level of washing was carried out to avoid the excessive loss of oocysts. Where very high signals were produced assays were consistent (for example with SRSKNYLWRSM) but with lower signals the assays could be variable. In addition, background phage may be less efficiently removed compared to soluble scFv

formats. As alternative approaches, filter tubes were used to aid the washing of the oocysts and/or the phage clones were produced as MBP-scFv fusion proteins.

4.2.5 Sub cloning Griffin scFv genes into the pMAL-p5X expression vector

Five scFv clones that were binding to *Eimeria* oocysts in the spin down ELISA assay were sub cloned into the pMAL-p5X expression vector and to produce soluble maltose binding protein (MBP)-scFv fusion exported to the periplasm of its host. Using restriction enzymes Nco 1 and Not 1 HF p5X was double digested. As a control, uncut P5X vector (500 ng) was analysed for comparison and cleavage was confirmed (Figure 4.8 [A]). The uncut p5X appeared slightly smaller than the cut p5X. The five scFv clones (DHTPKWq, STQLP, STqHP, SRSKNYLWRSM and SAqHP) were also double digested and visualised on an agarose gel to check for complete digestion (release for scFv genes from the phagemid vector) (Figure 4.8 [B]). The cut scFv and pMAL-p5X bands were gel purified and quantified using Qubit fluorometry producing between 7.2-8.8 ng/µl for the scFv genes and 29.3 ng/µl for the p5X. The vector was mixed with the Griffin scFv gene in a ratio of 2:1 and ligated, microdialysed to remove excess salts and transformed using electrocompetent cells. The next day, between 10-30 colonies were produced for each clone. Colonies were grown and sent for Sanger sequencing that confirmed that they had the correct inserts.

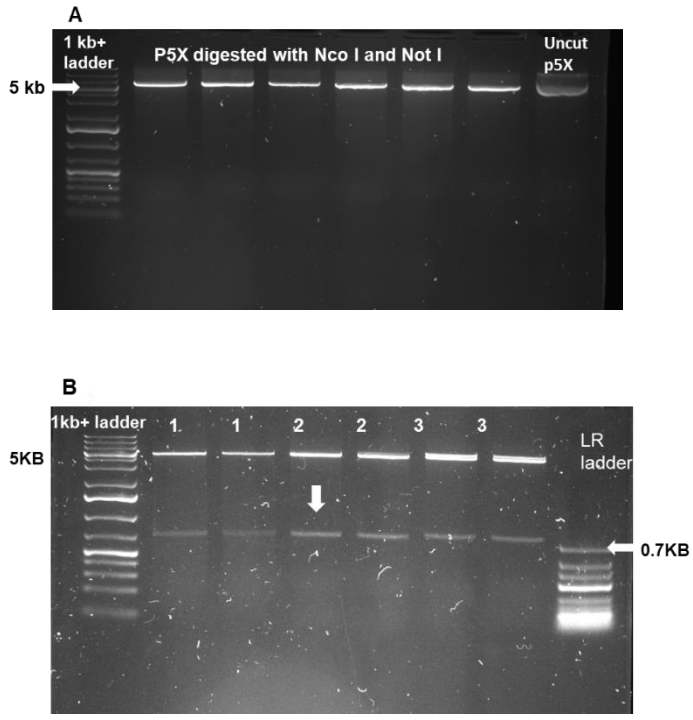


Figure 4:8: Analysis of the products of double digests of pMAL-p5 (A) and Griffin scFv (B) vectors. Restriction enzymes Nco 1 and Not 1 HF with Cutsmart® buffer were used to digest both vectors. Expected size of the pMAL-p5X vector is ~ 5

kb (A) while the scFv which is being released is ~ 800 bp (B), indicated by the white arrow (B). Ladders low range (right hand) and 1 kb plus (left side) were used. (A) Multiple cut p5X vector and uncut control (B) SRSKNYLWRSM (1), DHTPKWq (2) and STQLP (3). Analysis was performed on a 1% (w/v) agarose gel using 1 KB+ Ladder/ L-R ladder. Nancy-520 was used to stain the gel and samples were loaded using 6X loading buffer.

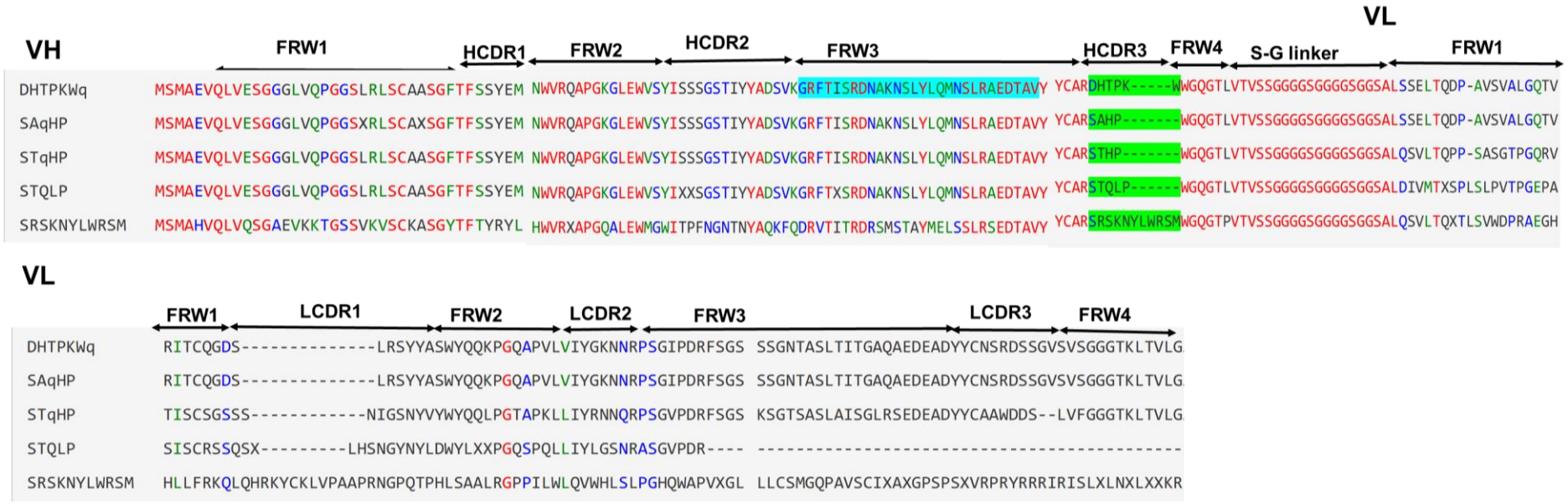


Figure 4:9: Deduced amino acids sequences of scFv antibody fragments cloned into the pMal-p5X vector. The V_H and V_L segments of the scFvs were sequenced as described in subsection 2.3.5.1.5 in Materials and methods **chapter 2.** The HCDR3 sequences are highlighted in green. The S-G sequence that links V_H and V_L segments is indicated as well as the framework regions and complementarity determining regions (CDRs), numbering was performed according to Kabat.

4.2.5.1 Analysis of scFv-MBP Expression

The expressed scFv proteins in the pMAL-p5X vector were extracted crudely from the periplasm of the cells. About 40 ml of crude periplasmic extract was obtained. The crude periplasmic extract was analysed to determine if the protein is expressed in the vector. This was carried out by western blot using the clones designated SRSKYNLWRSM and SAqHP. A commercial MBP was used as control as well as two replicates of the p5X vector. Western blot revealed the scFv fusions expressing in the vector (figure 4.10).

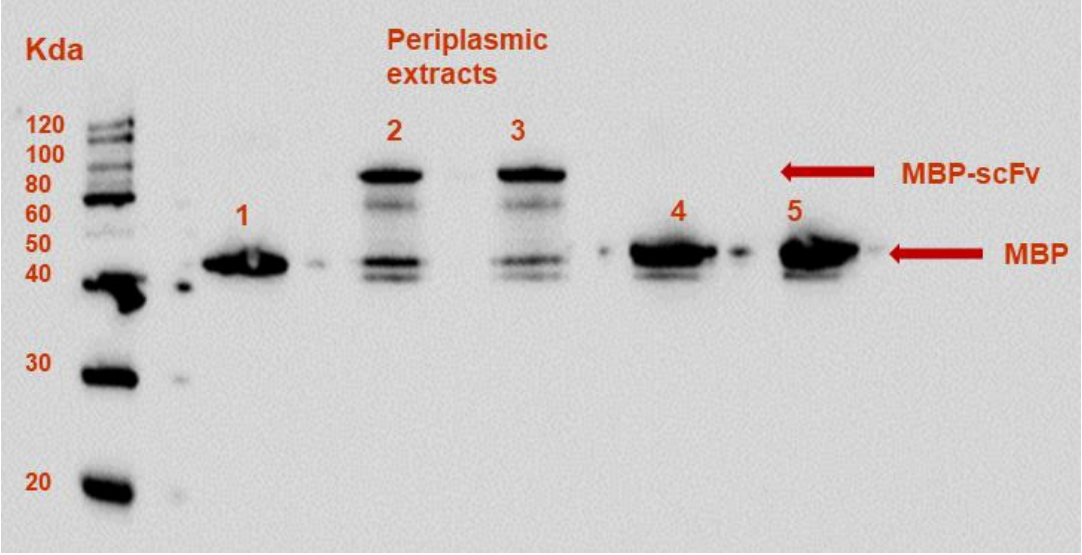


Figure 4:10: Western blot analysis of scFv-MBP fusion expression. MW ladder was Magic Marker, (1) Commercial MBP control, (2) SRSKNYLWRSM (3) SAqHP (4) P5X control first replicate (5) P5X control second replicate. Osmotic shock fluid containing the crude extract was mixed 1:1 with lysis buffer and 15 µl was loaded on NUPAGE gel. Anti-MBP (1:10000) was used to detect proteins while Goat Anti mouse-HRP (1:2000) was used to detect Anti-MBP. Detection was carried out using ECL substrate.

Based on the Sanger sequencing data as well as the western blot analysis, the clones had been fused to MBP vector (~ 60 kDa) (Figure 4.9). The crude extract was purified on amylose resin. The eluates were quantified using Bradford protein assay and the protein levels obtained were very low ranging from 4.10-5.15 µg/ml (Figure 4:10). Following the very low quantities of proteins obtained in the Bradford assay, analysis was not pursued further with the proteins. The binding properties of the clones were then tested as crude lysates in an ELISA.

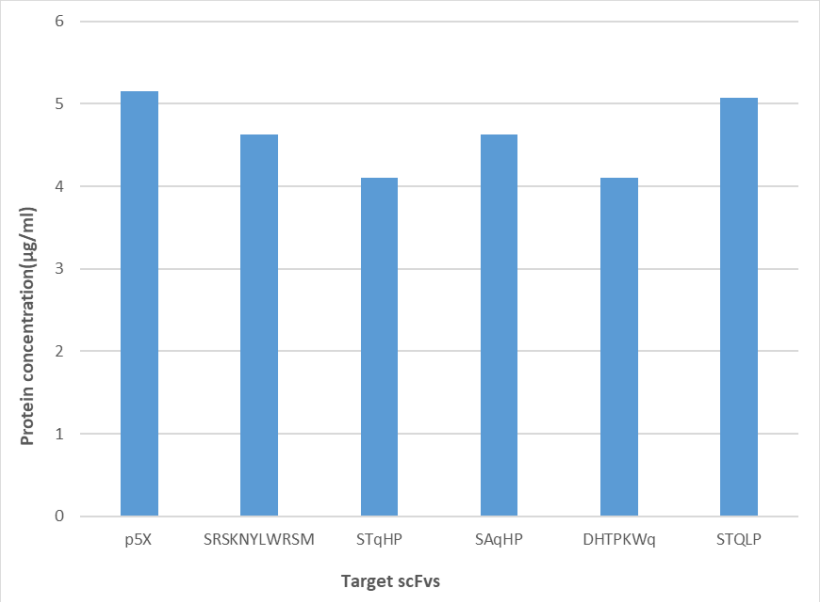


Figure 4:11: Protein concentration of the purified MBP-scFv. These were derived from the Bradford assay.

4.2.5.2 ScFv- MBP fusion crude lysates spin down ELISA

As only very low yields were obtained for the purified scFv-pMAL fusion, it was assessed whether the crude periplasmic extracts would detect the oocysts when using the spin down ELISA. For the clone designated SRSKNYWRSM and an MBP only control, spin down ELISA was carried out with 10^5 oocysts per eppendorf tube and binding detected with anti-MBP and goat anti-mouse-HRP antibodies. There was no significant difference between the absorbance signals produced after 10 minutes of adding substrate in the test clone and MBP only control for all species of *Eimeria* at p>0.9999 (*E. mitis*), p =0.9971 (*E. necatrix*), p =0.2085 (*E. acervulina*), p>0.9999 (*E. brunetti*), p =0.6150 (*E. maxima*), p>0.9999 (*E. praecox*), p =0.9181 (*E. tenella*) (Figure 4.11). Also, the background signal was very high for a non-phage assay. This assay was repeated with pre-blocking the periplasmic extracts for 2 hours and increasing the washing conditions from three washes (twice with PBST, containing 0.1% v/v) and once with PBS only) to four washes (three times with PBST containing 0.1% v/v and once with PBS only) after every incubation step, but background signals were still high and there was no significant difference between the test clone and MBP only control (data not shown). The data indicated that the MBP-scFv clone did not bind to oocysts.

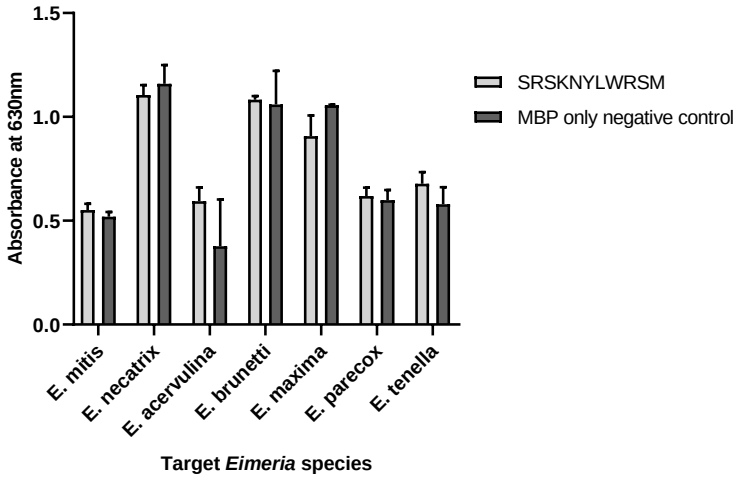


Figure 4:12: Binding of scFv-MBP clone SRSKNLYWRSM to individual *Eimeria* oocysts. Crude protein lysates were produced for SRSKNLYWRSM and the p5X only negative control. Whole oocysts 10^5 were put in duplicate sterile eppendorf tubes for both test clone and MBP crude protein lysate. Oocysts were spun down and resuspended in 100 µl of PBS. Crude protein lysate (800 µl) used to detect the immobilised oocysts and those remaining after the wash steps were detected using anti-MBP and goat anti-mouse-HRP antibodies. The TMB substrate was allowed a 10-minute incubation before absorbance values were taken at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference. Data represents the mean and standard deviation of two replicates.

4.2.6 Oocysts filtration immunoassay

A filtration phage ELISA was carried out to aid in washing oocysts in solution during ELISA wash steps. DHTPKWq clone and the anti-BSA control were assessed. At the first attempt, a filtration tube with pore size 0.45 µM was used to carry out the washes between incubation steps by transferring the oocysts from the eppendorf tubes to the filtration tubes, washing and centrifuging. This attempt was not successful because the smallest oocyst species size is ~ 10 µM and oocysts clogged the pores preventing the wash buffer from passing through. Even though there were significant differences between the clone DHTPKWq and anti-BSA control in some species, the background signals were still high (Figure 4.13). A different filter tube with a bigger pore size was then obtained 10 µm pore size. There were significant differences between DHTPKWq and anti-BSA at $p<0.0001$ when binding to five species (*E. necatrix, E. acervulina, E. brunetti, E. maxima and E. tenella*) and significant difference between DHTPKWq and anti-BSA at $p = 0.0003$ (*E. mitis*) and $p =0.0163$ (*E. praecox*) (Figure 4.14 [A]). When this assay was repeated, there was significant difference between DHTPKWq and anti-BSA at $p<0.0001$ when binding to five species *E. mitis*, *E. necatrix*, *E. brunetti*, *E. maxima* and *E. tenella*. There was no significant difference

between DHTPKWq and anti-BSA when binding to *E. acervulina* and *E. praecox* (Figure 4.14 [B]). This assay gave very similar results to the original spin down ELISA assay where this scFv bound reproducibly with to all species except *E. acervulina* and *E. praecox*, the former was recognised in 1 or 2 assays (as was the case here) and the latter was not recognised in 2 assays (the scFv bound in one of two assays here with a lower p value than for other species).

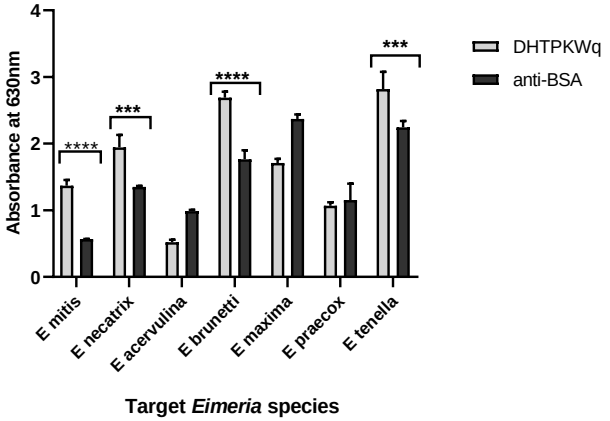


Figure 4:13: Binding of monoclonal scFv DHTPKWq to individual *Eimeria* oocysts using filtration tube to aid wash steps. Whole oocysts 10^5 were put in duplicate sterile eppendorf tubes for both test phage and anti-BSA control phage. Oocysts were spun down and resuspended in 100 µl of PBS. Monoclonal DHTPKWq and the anti-BSA negative control phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised oocysts and oocysts were transferred to filtration tubes (Costar spin-X centrifuge tube filters 0.45um) before wash steps and those remaining after the wash steps were detected using anti-M13- HRP antibody. The TMB substrate was allowed a 10-minute incubation before absorbance values were taken at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates.

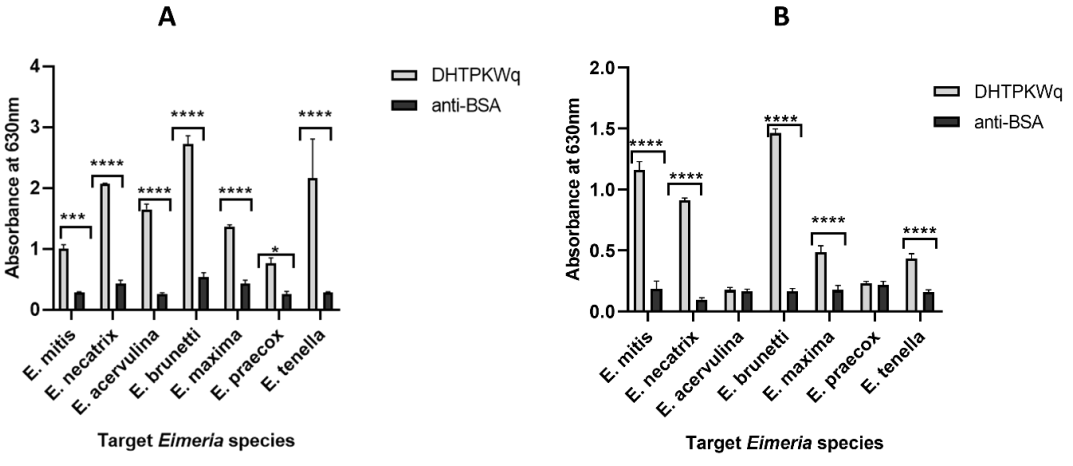


Figure 4:14: Binding of monoclonal scFv DHTPKWq to individual *Eimeria* oocysts using filtration tubes to aid wash steps. Whole oocysts 10^5 were put in duplicate sterile eppendorf tubes for both test phage and anti-BSA control phage. Oocysts were spun down and resuspended in 100 µl of PBS. Monoclonal DHTPKWq and the anti-BSA negative control phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised oocysts and oocysts were transferred to filtration tubes (MoBiTec Spin columns with filter 10 µm pore size, screw cap and bottom plug) before wash steps and those remaining after the wash steps were detected using anti-M13-HRP antibody. The TMB substrate was allowed a 10-minute incubation before absorbance values were taken at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates, (A) and (B) represent repeat assays.

The filtration assay was then performed with the STqHP and STQLP and both scFvs displayed significant binding to all seven species (Figure 4.15).

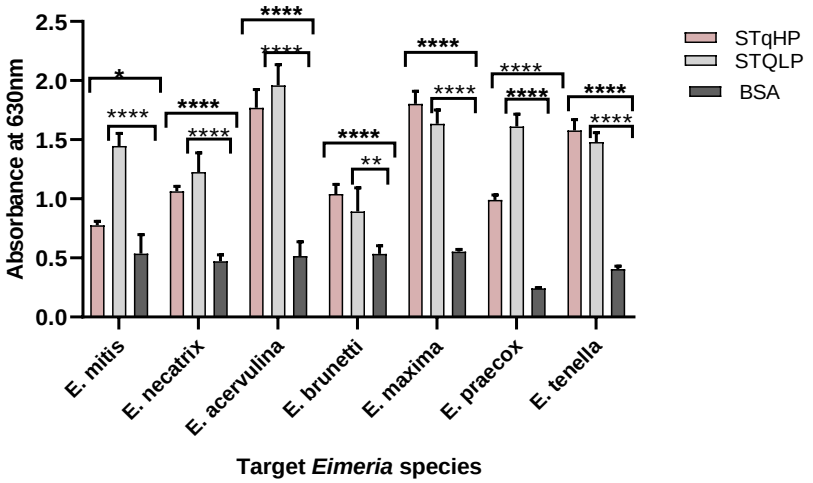


Figure 4:15: Binding of monoclonal scFvs STqHP and STQLP to individual *Eimeria* oocysts using filtration tubes to aid wash steps. Whole oocysts 10^5 were put in duplicate sterile eppendorf tubes for both test phage and anti-BSA control phage. Oocysts were spun down and resuspended in 100 µl of PBS. Monoclonal STqHP, STQLP and the anti-BSA negative control phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised oocysts. Oocysts were transferred to filtration tubes (MoBiTec Spin columns with filter 10 µm pore size, screw cap and bottom plug) before wash steps and those remaining after the wash steps were detected using anti-M13- HRP antibody. The TMB substrate was allowed a 10-minute incubation before absorbance values were taken at 630 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates.

Some improvement in the wash steps were noticed with this filter tube with reduced background signals. It was then used to test other clones. The filtration assays were performed for KDVGVVPK and HPMLGGLQGK that showed none or limited binding, respectively, with the previous spin down ELISA method. Here, there was no significant difference between binding of these scFvs and the anti-BSA control when binding to all seven species (Figure 4.16).

More clones were screened (data not shown) and while performing this assay it was noted that oocysts of *E. brunetti* and *E. tenella* were clogging the filtration tube pores at a centrifugation speed of 300 xg. At higher speeds, species with smaller oocysts such as *E. mitis* and *E. acervulina* were lost as they passed through the pores with the filtrate. This limited the practicality of the method. It was not clear why the filtration assays having worked successfully before started to be problematic, it may represent batch to batch variation in the filters as the problem emerged once a new pack of the filtration tubes were used.

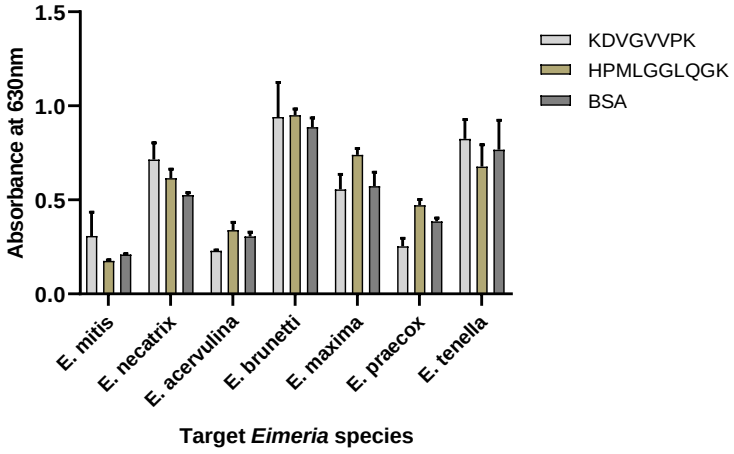


Figure 4:16: Binding of monoclonal scFvs KDVGVVPK and HPMLGGLQGK to individual *Eimeria* oocysts using filtration tubes to aid wash steps. Whole oocysts 10^5 were put in duplicate sterile eppendorf tubes for both test phage and anti-BSA control phage. Oocysts were spun down and resuspended in 100 µl of PBS. Monoclonal KDVGVVPK, HPMLGGLQGK and the anti-BSA negative control phage (400 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised oocysts and oocysts were transferred to filtration tubes (MoBiTec Spin columns with filter 10 µm pore size, screw cap and bottom plug) before wash steps and those remaining after the wash steps were detected using anti-M13- HRP antibody. The TMB substrate was allowed a 10-minute incubation before absorbance values were taken at 630 nm.

4.2.7 Optimisation of oocyst immobilisation and detection

4.2.7.1 Using buffers of different pH and different temperatures to immobilize whole oocysts

As previously stated in subsection 4.2.3, oocysts were not immobilised on Nunc MaxiSorp® plates in PBS pH 7.0. Here, using buffers of different pH conditions, different temperatures, and different plate types attempts were made to immobilize oocysts on plastic plates. Firstly, using Nunc MaxiSorp® plates, buffers were acetic acid at pH of 3.0 and 5.0, PBS at pH of 7.0 and carbonate buffer at pH 9.0 and oocysts were used at a concentration of 10^4 per well for each oocyst species. Monoclonal ELISA was performed after immobilising oocysts in the different conditions (refer to **chapter 2**, subsection 2.6.1) using confirmed positive clone SRSKNYLWRSM and produced background signals for all conditions (data not shown). It was not observed if the oocysts were immobilised under the microscope.

Next, using distinct assay plates, Polysorp®, Maleic Anhydride plate, Nunc™ Edge 96 well cell culture plate and Nunc MaxiSorp®, oocysts immobilisation was attempted using 10^4 per well oocysts from two *Eimeria* species. Different conditions were used for each plate; incubation at room temperature in four different pH buffers (the same as used above) and incubation in 37°C. The plates were viewed under an inverted microscope after incubating the oocysts overnight and it was confirmed that the oocysts were present in the plates. After washing the plates six times, the plates when viewed again under the inverted microscope and all the oocysts were lost; no oocysts were seen in any of the plates.

4.2.7.2 Immobilization of whole oocysts using polyclonal sera

Polyclonal sera raised against *E. tenella* in chickens was screened in an indirect ELISA against oocyst lysate of *E. tenella*, *E. maxima* and *E. necatrix* alongside a normal chicken serum control. It was confirmed that the *E. tenella* serum was binding to *E. tenella* oocyst lysate only (data not shown). This serum was then used in a sandwich ELISA incubating anti-*Eimeria tenella* polyclonal antibody (1:500) on Nunc MaxiSorp® plate overnight, washing the plate the next morning, blocking and incubating whole *E. tenella* oocysts (10^4) per well for an hour. Plate washing and bound SRSKNYLWRSM phage detection was carried out as described in **chapter 2**, subsection 2.3.5.1.2. Signals produced after 10 minutes of adding the substrate were background signals (data not shown).

4.2.7.3 Immobilization of whole oocysts using Nitrocellulose membrane/ Dot blots

Whole oocysts of all seven *Eimeria* species were spotted on nitrocellulose membranes. A BSA negative control as well as lysed oocysts of *E. tenella* as a positive control were also spotted. The membranes were incubated with a pool of sera from chickens challenged with each *Eimeria* species diluted 1:1000 and after wash steps incubated with anti-chicken IgG AP (1:2000) (refer to **chapter 2**, subsection 2.6.4). After incubation with antibodies, wash step and addition of colour substrate (BCIP®NBT), a dark purple colour was observed where the different species of oocysts were spotted on the membrane. The BSA negative control did not produce any dark purple colour change where BSA was dotted. There was also a slight dark purple colour change where the lysed oocysts positive control was spotted (Figure 4.17). This assay was carried out twice with similar results.

Following the faint signals obtained from the positive *E. tenella* oocyst lysate control, the immobilisation of whole oocysts was then compared to oocysts lysates on nitrocellulose membranes. 2.5 µl of 20 µg/ml protein derived from oocyst lysates were spotted on nitrocellulose membrane with a BSA negative control while 2.5 µl of whole oocysts (10^4 cells) were spotted on a different membrane alongside an oocyst lysate of *E. tenella* and BSA negative control and the assay was conducted with all other assay conditions remaining the same as described above. Whole oocysts were found to produce higher signals relative to the lysed oocysts under the same conditions (Figure 4.18). Whole oocysts were then used for further assays involving immobilisation on nitrocellulose membranes.

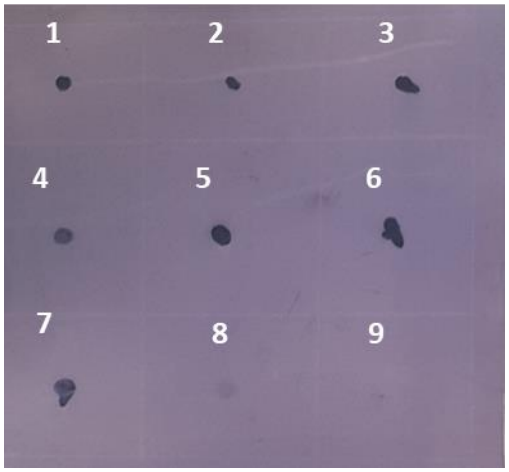


Figure 4:17: Dot blot assay with whole *Eimeria* oocysts. 2.5 µl of whole oocysts (10^4 cells on average) were spotted on nitrocellulose membrane. Numbers 1-7 are different species of whole oocysts (8) Oocyst lysate positive control and (9) BSA negative control. This was probed with a pool of polyclonal sera raised against each *Eimeria* species diluted 1:1000 and anti-chicken IgG AP (1:2000). After wash steps visualisation was performed with BCIP ® NBT substrate. (1) *E. mitis*, (2) *E. necatrix*, (3) *E. acervulina*, (4) *E. brunetti*, (5) *E. maxima*, (6) *E. praecox*, (7) *E. tenella*, (8)

Oocyst lysate positive control (9) BSA negative control.

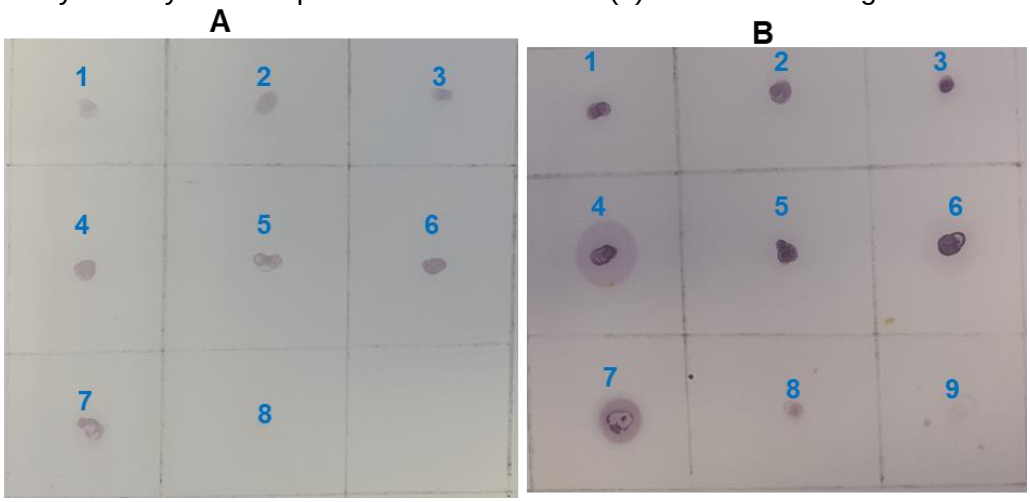


Figure 4:18: Repeat dot blot assay with whole *Eimeria* oocysts and oocyst lysates. 2.5 µl of oocyst lysate of the seven *Eimeria* species as well as a BSA negative control were dotted on a nitrocellulose membrane (A). Whole oocysts (10^4 cells on average) of the seven *Eimeria* species, oocyst lysate of *E. tenella* as well as BSA negative control were spotted on a different membrane (B). These were probed with a pool of anti-*Eimeria* species polyclonal sera diluted 1:1000 and anti-chicken IgG AP (1:2000). After wash steps visualisation was performed with BCIP ® NBT substrate. On both membrane A and B; (1) *E. mitis*, (2) *E. necatrix*, (3) *E. acervulina*, (4) *E. brunetti*, (5) *E. maxima*, (6) *E. praecox*, (7) *E. tenella*. On membrane A, (8) BSA negative control. On membrane B, (8) *E. tenella* oocyst lysate and (9) BSA negative control.

4.2.8 Optimisation of phage scFv dot blots

Since whole oocysts were readily immobilised on dot blots, it was thought to optimise phage screening on dot blots as that would be a high throughput assay compared to the spin down ELISAs that had been optimised previously. At the first attempt, phage (~10^{11} PFU/ml) was prepared for the clone designated SRSKNYLWRSM as well as anti-BSA control phage and they were used to screen all seven species of oocysts on colorimetric dot blots (5 mls per blot) using nitrocellulose membrane. The detection system was AP using rabbit anti-fd and anti-rabbit AP antibodies and BCIP ® NBT substrate. Washes following each antibody incubation was three times for 10 minutes each using TBST containing 0.1% (v/v) Tween 20. The blots had been blocked overnight with 3% (w/v) Marvel. Following adding of the substrate, the background signals were high and the test samples were not visible (data not shown).

4.2.8.1 With chemiluminiscent detection system

At the second attempt for optimising phage dot blots, a chemiluminiscent detection system was used to detect phage. The incubation, buffer and wash conditions were exactly as subsection 4.2.8. Signals produced after substrate was added were very high and the oocysts could not be detected (data not shown).

4.2.8.2 Validation of phage dot blots using lysozyme control

It was then thought to have another protein negative control screened with anti-BSA scFv phage on the dot blots alongside BSA to validate the assay by optimising conditions for phage-mediated detection. Anti-BSA scFv phage was screened against 200 µg/ml and 20 µg/ml concentrations of BSA as well as 200 µg/ml and 20 µg/ml of lysozyme (all using 2.5 µl spots). The detection system used was AP colorimetric. Washing, buffer and incubation conditions were the same as 4.2.8. Signals produced after adding the substrate were very high and BSA could not be detected (data not shown).

4.2.8.3 Optimisation of dot blots with control proteins, different phage concentrations, membrane and wash conditions

BSA and lysozyme were spotted as before on PVDF membrane and phage prepared from anti-BSA was used to detect the proteins. The phage was used in three different concentrations; 10^{11}, 10^{10} and 10^{9} /ml (5 mls of phage per blot). Two different wash conditions were employed in which one was the same buffer as described in 4.2.8 (TBST containing 0.1% (v/v) Tween 20) while the second wash condition used the addition of 1 M NaCl to enhance the wash stringency. Other washing steps and incubation conditions remained the same. Six different conditions were tested: A (10^{11} phage concentration + TBST), B (10^{10} phage concentration + TBST), C (10^{9} phage concentration + TBST), D (10^{11} phage concentration + TBST + 1 M NaCl), E (10^{10} phage concentration + TBST + 1 M NaCl) and F (10^{9} phage concentration + TBST + 1 M NaCl). Background signals were very high for the 10^{11} phage concentration (Figure 4.19). The 10^{10} phage concentration had less background and the test samples were visible, test samples were not very visible in the 10^{9} phage concentration but it produced the least background (Figure 4.19). Clearly BSA had higher signals than lysozyme in all conditions. The 1M NaCl wash did not seem to reduce the background signals. Generally, background signals produced were still too high for the assay to be valid.

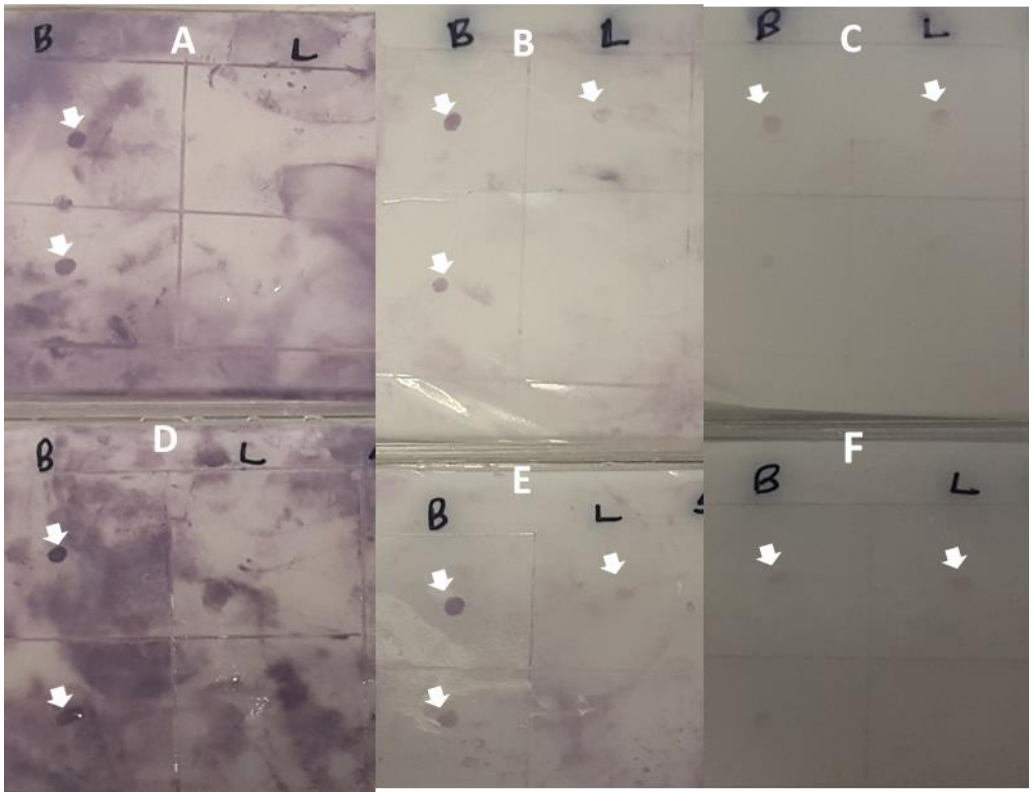


Figure 4:19: Phage dot blot assay with BSA and lysozyme. BSA (B) or lysozyme (L) were spotted onto PVDF membranes at 200 µg/ml (top panels) and 20 µg/ml (bottom panels). Six conditions were tested: A (10^{11} phage concentration + TBST), B (10^{10} phage concentration + TBST), C (10^{9} phage concentration + TBST), D (10^{11} phage concentration + TBST + 1 M NaCl), E (10^{10} phage concentration + TBST + 1 M NaCl) and F (10^{9} phage concentration + TBST + 1 M NaCl). These were probed with anti-BSA phage. After wash steps visualisation was performed with BCIP ® NBT substrate. White arrows indicate the signals where the test samples were dotted.

This assay was repeated with a higher concentration of Tween 20 in the wash buffer; 1% (v/v) Tween 20 was used and all other conditions of different phage concentration and 1 M NaCl remaining the same. This did not improve the assay (data not shown).

4.2.8.4 Optimisation using PEG precipitated phage

It was thought to compare signals and background noise produced using phage present in culture supernatant and PEG precipitated phage. The PEG precipitated and supernatant phage were used in three different concentrations; 10^{11}, 10^{10} and 10^{9} /ml (5 mls of phage per blot) just as was performed in 4.2.8.3. The wash, buffer and incubation conditions remained the same as described in 4.2.8. For blots A, B and C, PEG precipitated phage used at 10^{11}, 10^{10} and 10^{9} respectively and for blots D, E and F, phage present in culture supernatant was used. After addition of the

substrate, it was observed that the background signal seemed to have reduced in the assay as a whole. However, there was no difference between the phage present in culture supernatant and the PEG precipitated phage (Figure 4.20).

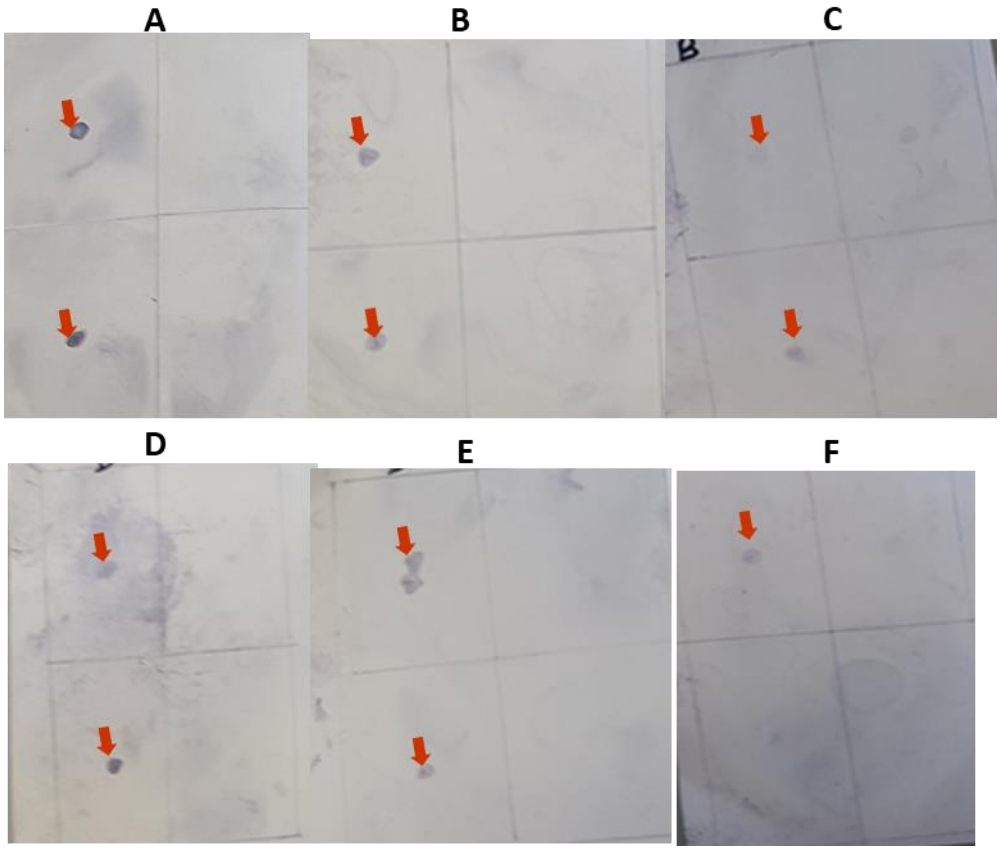


Figure 4:20: Phage dot blot assay with BSA and lysozyme using PEG precipitated phage and phage present in culture supernatant. BSA or lysozyme (200 µg/ml above and 20 µg/ml below) were spotted on a PVDF membranes. A (10^{11} PEG precipitated phage + TBST), B (10^{10} PEG precipitated phage + TBST), C (10^{9} PEG precipitated phage + TBST), D (10^{11} phage present in culture supernatant + TBST), E (10^{10} phage present in culture supernatant + TBST) and F (10^{9} phage present in culture supernatant + TBST). These were probed with anti-BSA phage. After wash steps visualisation was performed with BCIP ® NBT substrate. White arrows indicate the signals where the test samples were dotted.

4.2.8.5 Optimisation using the HRP detection system

A potentially more sensitive HRP detection system was then used. The incubation, blocking and wash conditions were the same as described in 4.2.8. The SRSKNYLWRM and anti-BSA control phage (~10^{10} PFU/ml) were screened against the seven species of *Eimeria* (Figure 4.21). Detection was carried out using anti-M13 HRP and signals obtained from the test samples were more specific than previously obtained with the optimisation methods described earlier using an AP detection system.

This assay was repeated and the background across the blots was much higher. Several attempts were made at changing wash conditions, wash buffer, membrane used, incubation steps, blocking conditions but the background signal remained high (data not shown).

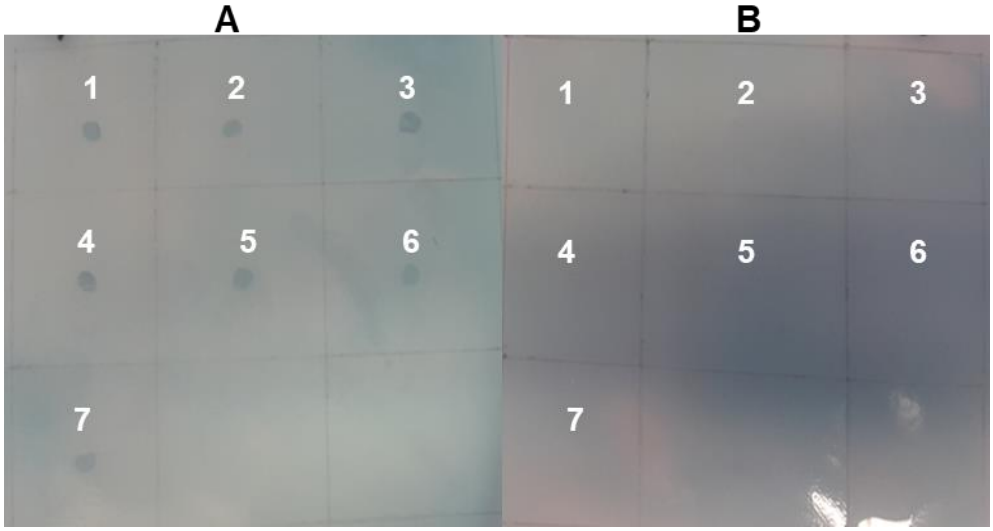


Figure 4:21: Dot blot analysis of oocysts species using phage produced from SRSKNYLWRM and anti-BSA control. Whole oocysts were dotted on duplicate nitrocellulose membrane; 1 (*E. mitis*), 2 (*E. necatrix*), 3 (*E. acervulina*), 4 (*E. brunetti*), 5 (*E. maxima*), 6 (*E. praecox*), 7 (*E. tenella*) and probed with SRSKNYLWRSM (A) and anti-BSA (B), before detection with anti-M13 HRP antibody and visualized with TMB membrane adapted substrate.

4.2.8.6 Optimisation of phage dot blots by improving blocking conditions and phage concentrations

Blocking conditions were altered to determine if that will improve the assay background. The study by (Tan and Tan, 2015) compared the sensitivities of phage dot blot assay for the detection of Hepatitis B surface antigen and used 10% milk blocker for the nitrocellulose membrane and the phage. The authors also used phage concentration at 10^{10}. In the dot blot assays performed in this study, phage concentrations of 10^{10} seemed to give the best signal/ background noise ratio hence this phage concentration was used along with increasing the blocking conditions from 3% to 10% milk. Phage was grown from five scFv clones that showed binding to *Eimeria* oocysts in spin down ELISA assays (SRSKNYLWRSM, DHTPKWq, SAqHP, STQLP and STqHP) alongside anti-BSA phage. They were used to probe blots dotted with whole oocysts of the seven species of *Eimeria* as well as a BSA negative control. The background signals were still high but faint signals from the blot probed with

SRSKNYLWRSM were visible for four species *E. mitis*, *E. necatrix*, *E. maxima* and *E. praecox* (Figure 4.22).

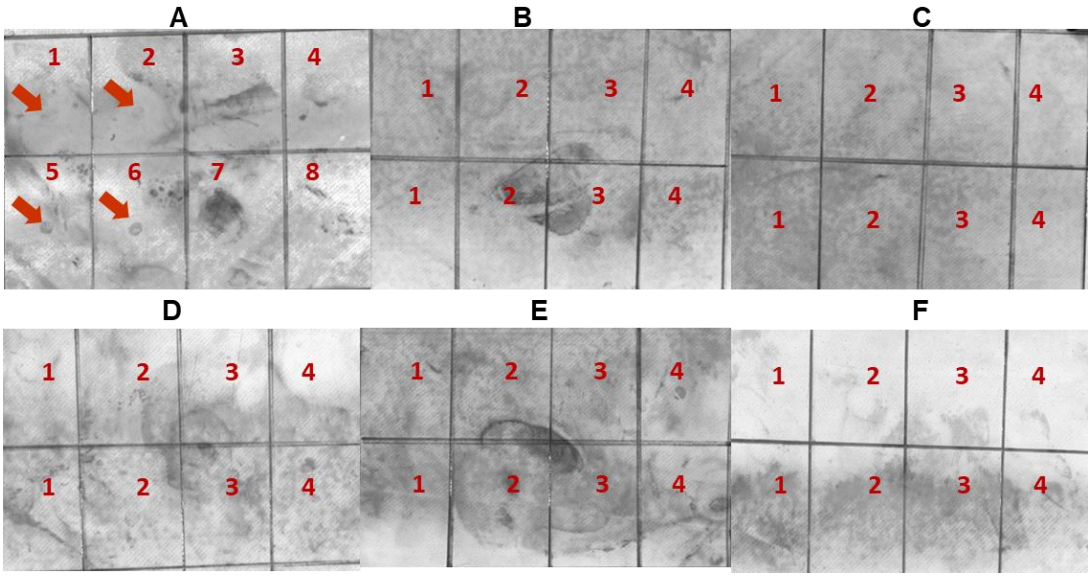


Figure 4:22: Dot blot analysis of whole oocysts species. Whole oocysts were dotted on nitrocellulose membrane; 1 (*E. mitis*), 2 (*E. necatrix*), 3 (*E. acervulina*), 4 (*E. brunetti*), 5 (*E. maxima*), 6 (*E. praecox*), 7 (*E. tenella*) and 8 (BSA control). Blots A, B, C, D, E and F were probed with phage preparations produced from clones designated SRSKNYLWRSM, DHTPKWq, SAqHP, STQLP, STqHP and anti-BSA respectively before detection with anti-M13 HRP antibody and visualized with TMB substrate. Red arrows indicate signals produced.

The assay was repeated exactly as described in 4.2.8.7 but with PVDF membrane and not nitrocellulose membrane. The signals produced were more noticeable especially for SRSKNYLWRSM clone and background signals appeared a little lower. SRSKNYLWRSM was clearly binding to all species of *Eimeria* while DHTPKWq and SAqHP binding to *E. mitis* and *E. brunetti* faintly. The relatively high background levels masked the clarity of the signals produced (Figure 4.23).

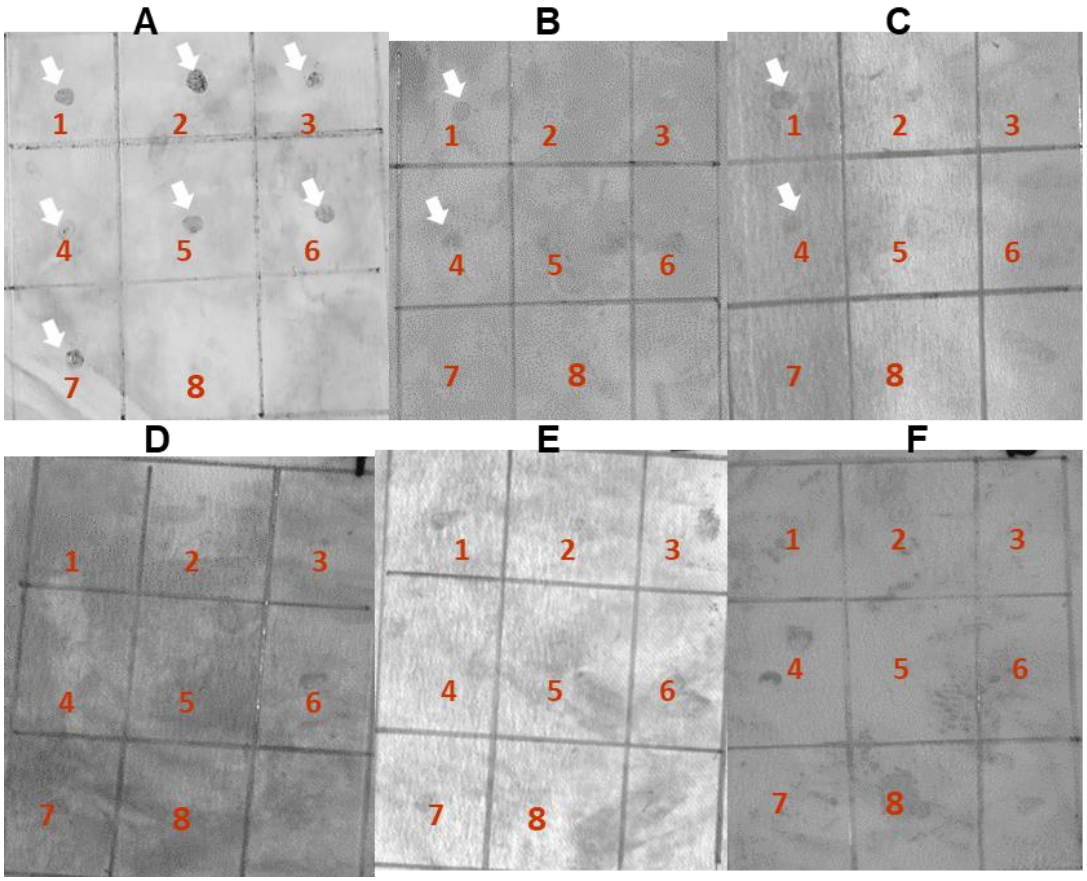


Figure 4:23: Repeat dot blot analysis of whole oocysts species. Whole oocysts were dotted on PVDF; 1 (*E. mitis*), 2 (*E. necatrix*), 3 (*E. acervulina*), 4 (*E. brunetti*), 5 (*E. maxima*), 6 (*E. praecox*), 7 (*E. tenella*) and 8 (BSA). Blots A, B, C, D, E and F were probed with phage preparations produced from clones designated SRSKNYLWRSM, DHTPKWq, SAqHP, STQLP, STqHP and anti-BSA respectively before detection with anti-M13 HRP antibody and visualized with TMB substrate. White arrows indicate signals produced.

The phage dot blot assays using the 10% blocking conditions and phage concentrations were repeated several times each time improving wash and incubation conditions but the relatively high background signals remained. It was then decided to develop an alternative assay even if not high throughput. Fluorescent microscopy was then employed to screen the scFvs that were binding to *Eimeria* oocysts. But in order to determine if the fluorescent detection system was functional, a fluorescent spin down assay was performed with the known positive scFv binder SRSKNYLYWRSM.

4.2.9 Phage fluorescent spin down ELISA Fluorescent microscopy

A spin down phage ELISA that detected the bound phage with fluorescence was carried out to determine if fluorescent microscopy might work. It was carried out with phage from a single clone SRSKNYLWRSM and anti-BSA control. Spin down ELISA was performed exactly as described in subsection 4.2.4 with the exception that binding was detected with rabbit anti-fd (1:2000) and anti-rabbit Alexafluor 488 (1:2000). Also, no substrate was added (refer to chapter 2, subsection 2.6.8.2). Relative fluorescent units (RFU) measured after washing off the secondary fluorescent tagged antibody showed that the clone SRSKNYLWRSM was significantly higher than anti-BSA control when binding to all species at $p<0.0001$ (Figure 4.24).

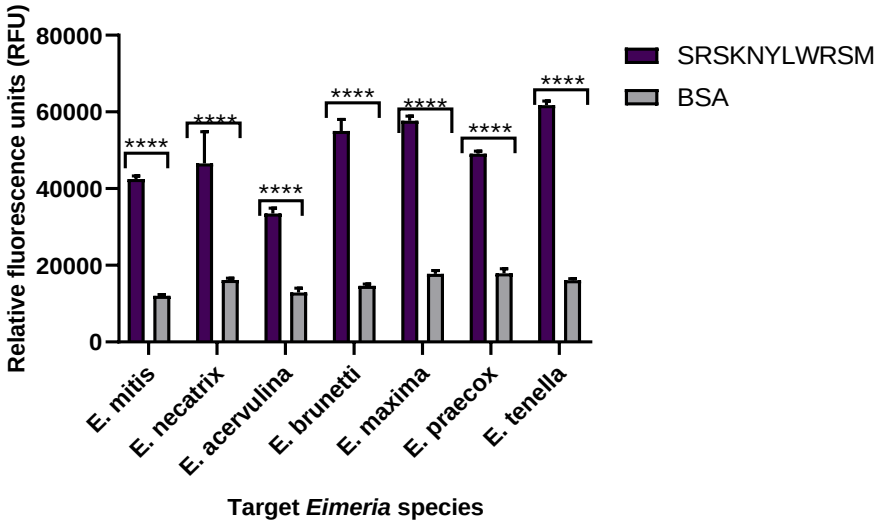


Figure 4:24: Fluorescent binding of monoclonal scFv SRSKNLYWRSM to individual *Eimeria* oocysts. Whole oocysts 10^5 from each of the seven species or BSA (20 µg/ml) were put in duplicate sterile eppendorf tubes. Oocyst were spun down and resuspended in 100 µl of PBS. Monoclonal scFv SRSKNLYWRSM and anti-BSA negative control phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the antigens and those remaining after the wash steps were detected with rabbit anti-fd and anti-rabbit Alexafluor 488 before fluorescence was read at 485 nm. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to determine any significant difference (p values <0.05) among the means of each species when compared to the anti-BSA control. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p≤ 0.0001 (****). Data represents the mean and standard deviation of two replicates.

4.2.10 Fluorescent microscopy analysis of scFv phage binding to oocysts

Next, fluorescent microscopy was used as an assay to determine binding of the isolated antibodies to oocysts and to determine if distinct binding patterns were observed for the scFvs. Even though fluorescent microscopy is not a high through put assay but once successfully validated, it will be an alternative to the assays that had been carried out in this chapter to allow further validation and characterisation of the binding of the antibodies.

2.2.10.1 Fluorescent microscopy

Phage prepared from SRSKNYLWRSM was used to screen the seven *Eimeria* species and the images clearly shows the scFv binding to all species of oocysts (Figure 4.25). Phage prepared from the clone designated SAqHP was found to bind to three of seven species of *Eimeria*. It bound to *E. mitis, E. necatrix* (weakly) and *E. tenella* (Figure 4.26). The screening of the seven *Eimeria* oocysts with phage prepared from the clone designated STqHP demonstrated its binding to *E. mitis*, *E. brunetti*, and *E. tenella* (Figure 4.27). The clone designated STQLP was found to bind to *E. mitis*, *E. maxima E. praecox* and *E. tenella* (Figure 4.28). Phage scFv binding of the clone designated DHTPKWq was found to bind *E. necatrix* and *E. acervulina* only (Figure 4.29).

A summary of the binding properties of the 5 scFvs in the spin down ELISA and the fluorescent microscopy assay are shown (Table 4.2).

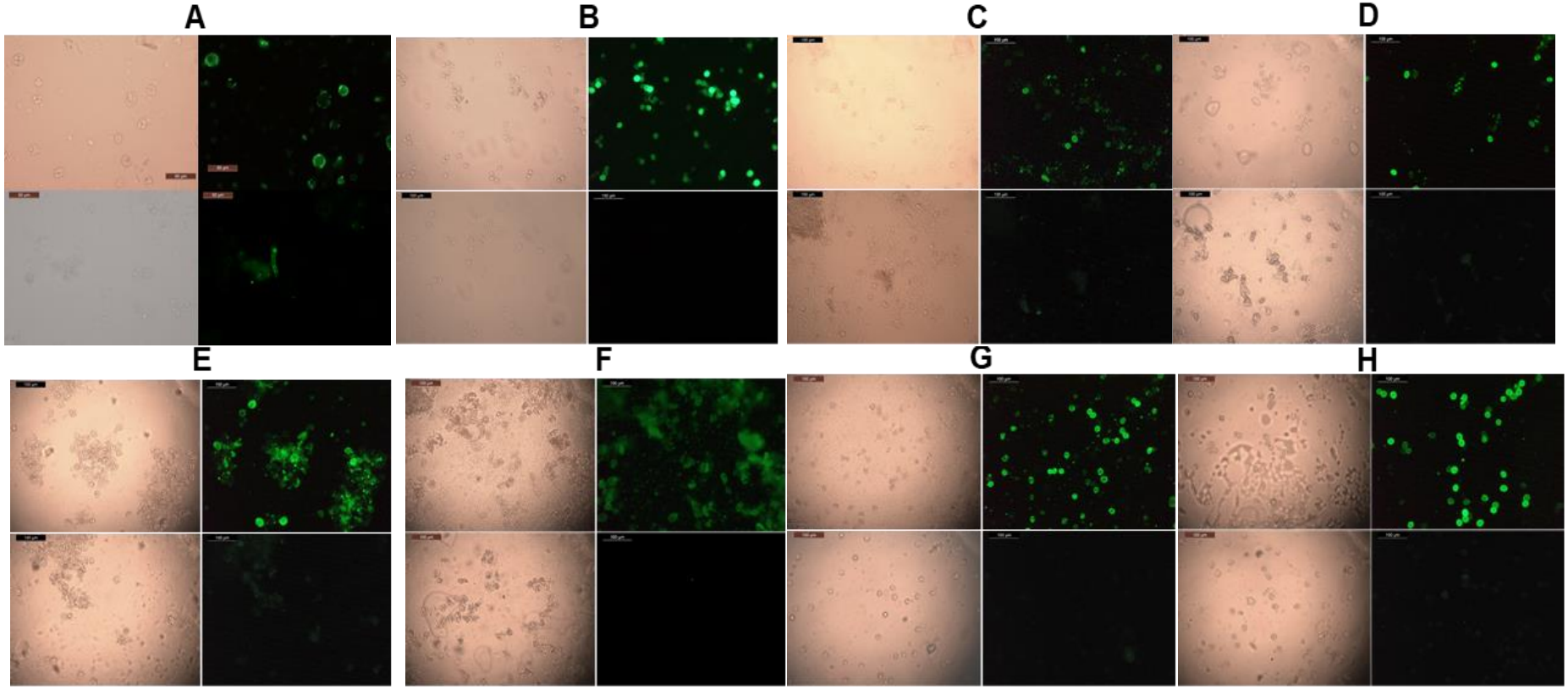


Figure 4:25: Fluorescent (right hand panels) and bright field (left hand panels) images of monoclonal phage SRSKNYLWRSM (above) and anti-BSA (below) binding to oocysts. Bound phage scFv were labelled with rabbit anti-phage polyclonal antibody and then Alexafluor 488 Goat anti-rabbit IgG. Oocysts were viewed and imaged using leica upright microscope. (A) Pool of all oocyst species (B) *Eimeria mitis* (C) *Eimeria necatrix* (D) *Eimeria acervulina* (E) *Eimeria brunetti* (F) *Eimeria maxima* (G) *Eimeria praecox* (H) *Eimeria tenella*. Scale bar for images is 50 µm.

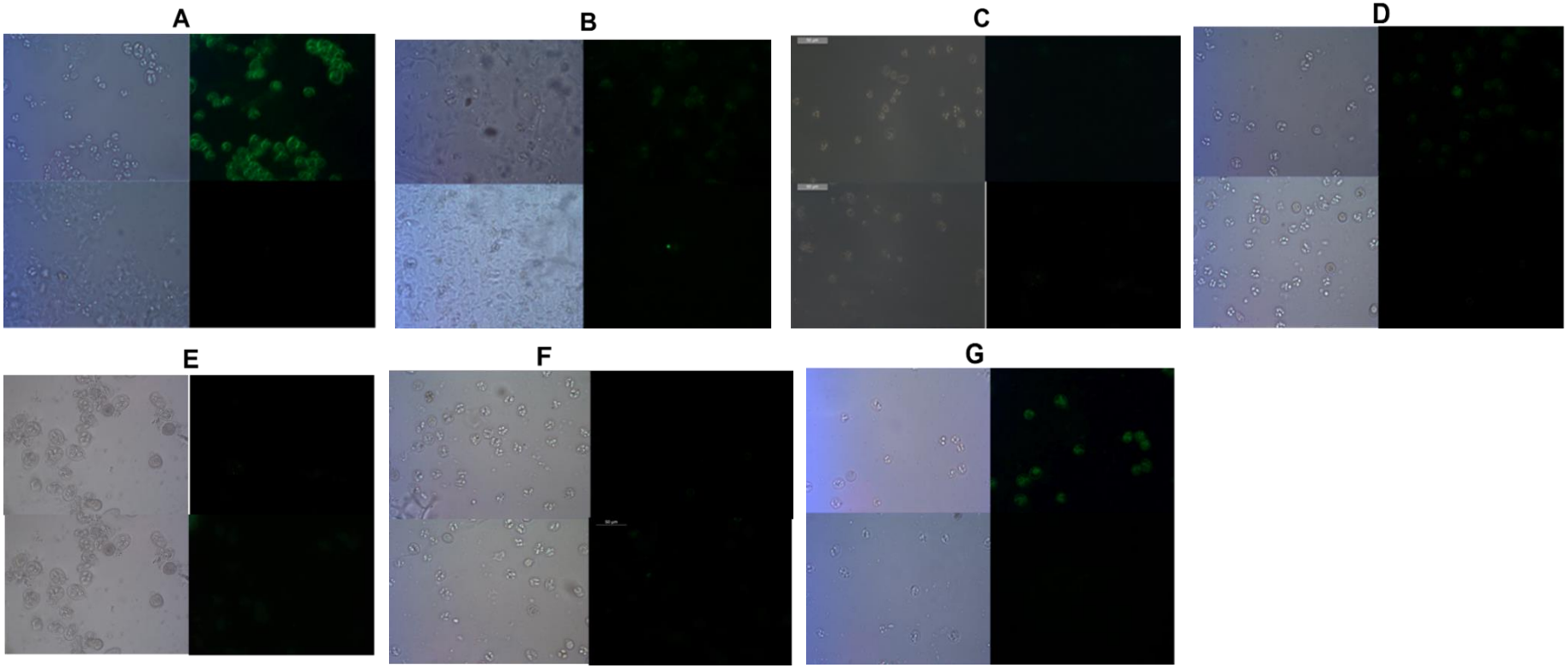


Figure 4:26: Fluorescent (right hand panels) and bright field (left hand panels) images of monoclonal phage SAqHP (above) and anti-BSA (below) binding to oocysts. Bound phage scFv were labelled with rabbit and phage polyclonal antibody and then Alexafluor 488 Goat anti-rabbit IgG. Oocysts were viewed and imaged using leica upright microscope. (A) *Eimeria mitis* (B) *Eimeria necatrix* (C) *Eimeria acervulina* (D) *Eimeria brunetti* (E) *Eimeria maxima* (F) *Eimeria praecox* (G) *Eimeria tenella*. Scale bar for images is 50 µm.

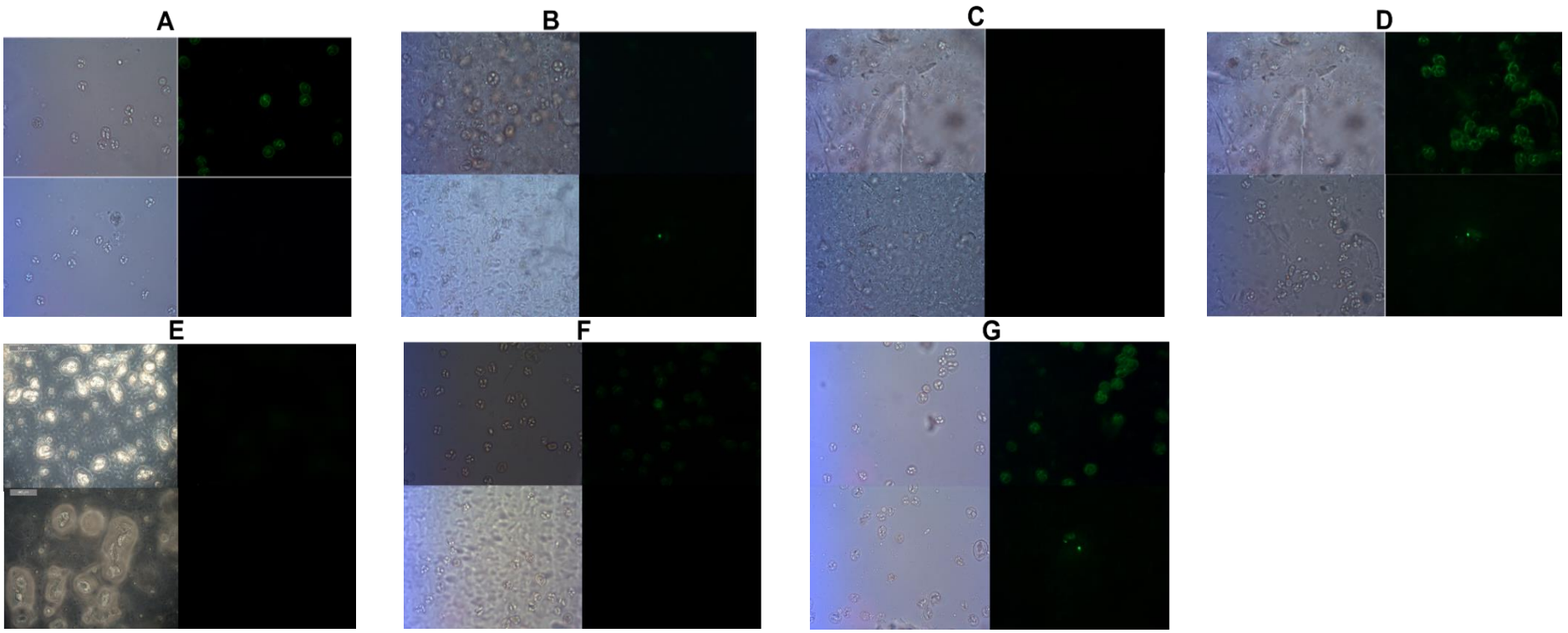


Figure 4:27: Fluorescent (right hand panels) and bright field (left hand panels) images of monoclonal phage STqHP (above) and anti-BSA (below) binding to oocysts. Bound phage scFv were labelled with rabbit and phage polyclonal antibody and then Alexafluor 488 Goat anti-rabbit IgG. Oocysts were viewed and imaged using leica upright microscope. (A) *Eimeria mitis* (B) *Eimeria necatrix* (C) *Eimeria acervulina* (D) *Eimeria brunetti* (E) *Eimeria maxima* (F) *Eimeria praecox* (G) *Eimeria tenella*. Scale bar for images is 50 µm.

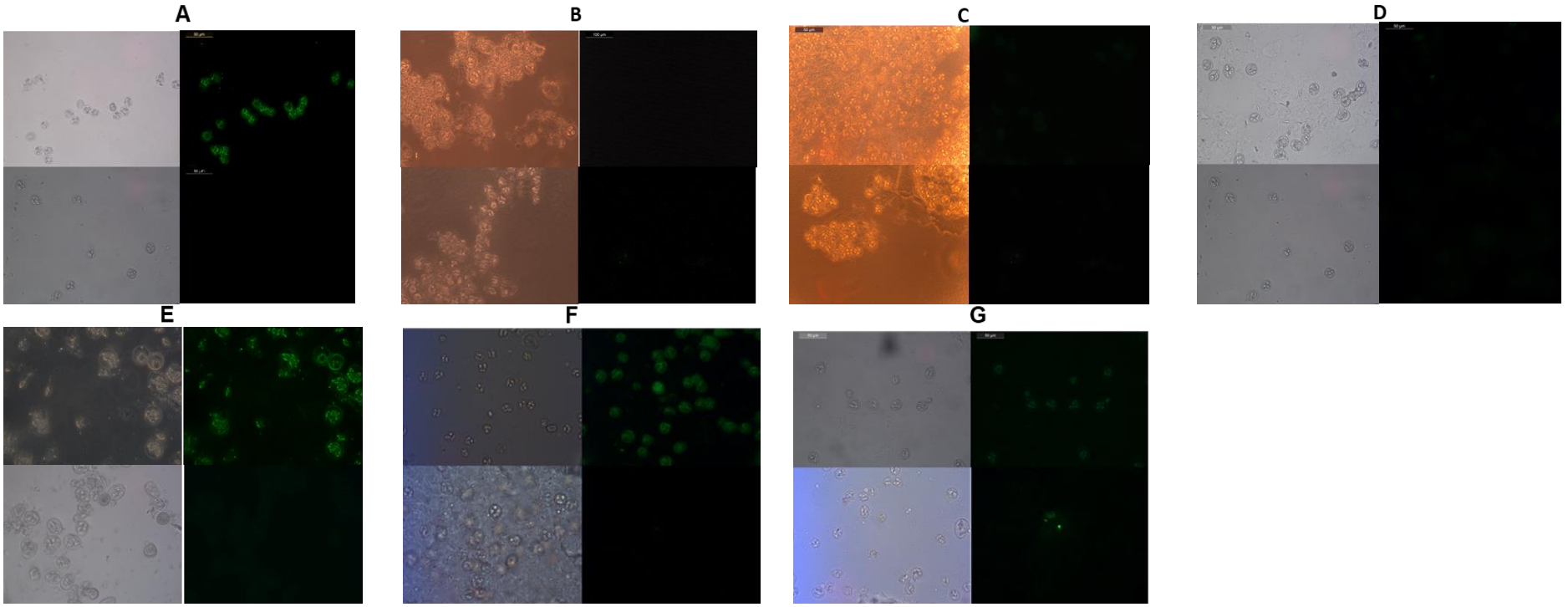


Figure 4:28: Fluorescent (right hand panels) and bright field (left hand panels) images of monoclonal phage STQLP (above) and anti-BSA (below) binding to oocysts. Bound phage scFv were labelled with rabbit and phage polyclonal antibody and then Alexafluor 488 Goat anti-rabbit IgG. Oocysts were viewed and imaged using leica upright microscope. (A) *Eimeria mitis* (B) *Eimeria necatrix* (C) *Eimeria acervulina* (D) *Eimeria brunetti* (E) *Eimeria maxima* (F) *Eimeria praecox* (G) *Eimeria tenella*. Scale bar for images is 50 µm.

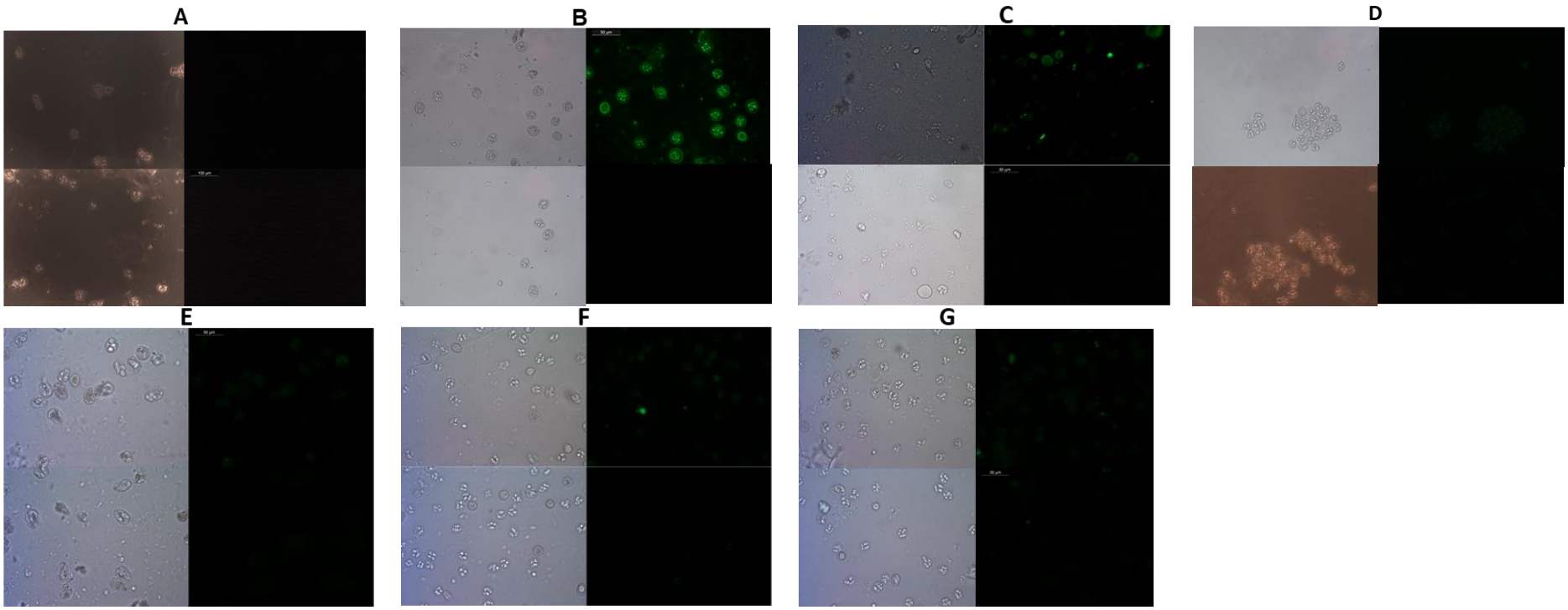


Figure 4:29: Fluorescent (right hand panels) and bright field (left hand panels) images of monoclonal phage DHTPKWq (above) and anti-BSA (below) binding to oocysts. Bound phage scFv were labelled with rabbit and phage polyclonal antibody and then Alexafluor 488 Goat anti-rabbit IgG. Oocysts were viewed and imaged using leica upright microscope. (A) *Eimeria mitis* (B) *Eimeria necatrix* (C) *Eimeria acervulina* (D) *Eimeria brunetti* (E) *Eimeria maxima* (F) *Eimeria praecox* (G) *Eimeria tenella*. Scale bar for images is 50 µm.

Table 4:2: Comparison of binding strengths and specificities of isolated scFvs in spin down ELISA assay (SDA) and fluorescent microscopy (FM)

Target species	**SRSKNLYWRSM**		**SAqHP**		**STqHP**		**STQLP**		**DHTPKWq**	
	SDA	**FM**	**SDA**	**FM**	**SDA**	**FM**	**SDA**	**FM**	**SDA**	**FM**
E. mitis	++++	++++	+++	++	+/-	++	+/-	+++	++	-
E. necatrix	++++	++++	-	+	+++	-	+++	-	+++	+++
E. acervulina	++++	++++	+/-	-	+/-	-	+++	-	+/-	+
E. brunetti	++++	++++	+/-	-	+++	++	+++	-	+++	-
E. maxima	++++	+++	+/-	-	++	-	+++	+++	++	-
E. praecox	++++	++++	+/-	-	+/-	-	+++	+++	-	-
E. tenella	++++	++++	+/-	+	+++	++	+++	+	++	-

++++ very strong binding
+++ strong binding
++ moderate binding
+ weak binding
+/- inconsistent binding across repeat SDA assays

Binding strength characterisation are based on the visual binding strength (fluorescent microscopy) and degree of significant difference between test scFv and negative control (spin down ELISA).

4.3 Discussion

In the previous chapter, traditional and next generation phage display were used to isolate 6 antibodies to *Eimeria* species that cause infections in poultry. These antibodies were screened and were found to be binding to *Eimeria* oocysts either selectively or pan-specifically (refer to **chapter 3**, tables 3.9 and 3.10). Here, different assays were used to test the isolated antibodies with the best results obtained with a spin down ELISA and a fluorescent microscopy assay, both of which are not particularly high throughput and target whole oocysts. The spin down ELISA sometimes produced variable results. This is most likely because of the loss of oocyst during repeated wash steps and limiting the wash steps led to high background signals. With this assay, it was observed that only the scFv SRSKNYLWRSM gave the same positive results no matter how many times it was screened because even in cases where oocysts were lost, signals in repeat assays were much higher in all assays compared to other scFvs, indicating a high affinity or binding to an antigen displayed at high levels on the oocyst surface (refer to **chapter 3**, figure 3.17 and table 3.9). Other scFvs that were possibly not as well expressed/displayed, possibly bound with lower affinity or bound to a rarer epitope on the oocysts and were more liable to produce variable results. This led to the assessment and or development of alternative assays for screening the scFvs. It was hoped that an assay that produced fewer variable results (refer to chapter 3, table 3.9) and was higher throughput would be developed. A phage ELISA to screen isolated antibodies was initially carried out with oocyst lysates using 96 well plates even though panning was done in solution against whole oocysts. Oocyst lysates were produced using a bead beater. No binding was observed in this assay which may be because the epitopes on the surface of the oocysts were destroyed in the process of lysing oocysts or membrane fragments (containing surface antigens) were not immobilising on the plates. Binding of whole oocysts on 96 well plates was not successful across a range of different immobilisation conditions and using distinct plates. Some studies have reported pre-treating *Cryptosporidium* oocysts before use in ELISAs. Certain conditions are employed in the treatment of oocysts such as the addition of sodium dodecyl sulfate or sodium taurochlorate at a final concentration of between 5-10% in addition to incubation at different temperatures. Temperatures of 37°C, 50°C, 60°C, 75°C and even boiling at 100°C for between 30 seconds to 20 minutes have been reported to be used in the treatment of oocysts. In the study by (Kramer *et al*., 2007), monoclonal mouse antibody specific to *Cryptosporidium* oocyst wall surface and a polyclonal rabbit antibody raised against the same antigen were tested as detection and capture molecules in an ELISA.

Oocysts were pre-treated and the effect of oocyst pre-treatment on antibody binding was assessed. Treatments were either control (untreated), boiled, freeze/thawed, or incubated with 5% bile or SDS at 37°C as five different treatments aimed at excysting oocyst or solubilising them. Excystation of oocysts has been frequently performed using bile treatments (Gold *et al*., 2001). The authors observed that treatment of oocysts with SDS or bile improved the signals produced as compared to the untreated controls but not as significantly as temperature treatments. At least a fivefold higher signal versus noise ratio was produced when boiled or freeze/ thawed treated oocysts was used compared to control untreated. The authors attributed the higher signal produced from pre-treatment of oocysts by boiling to not only excystation of oocysts but to the possibility of boiling releasing additional immunogenic proteins from the oocysts or denaturation /degradation of proteins making them more readily accessible for polyclonal antibody binding producing a higher signal. In the present study, panning was conducted against whole oocysts and so surface components without any pre-treatment, pre-treatment of oocysts was not a first approach when the ELISA was initially conducted. It was found that oocysts treated with Nonidet®40 after heating to 50°C or 90°C produced high concentrations of proteins. However, scFv phage ELISAs using Nonidet®40 treated oocysts did not produce positive results. This may not be attributed to the sensitivity of the assay or even the oocysts but likely because panning was carried out in solution with whole oocysts that were untreated. It is well known that heating antigens can change conformational characteristics or destroy surface epitopes (Tian *et al.,* 2018). For all five scFvs tested, none produced signals higher than background level signals against any lysate preparations.

An oocyst filtration assay was then employed to try and improve the reproducibility of the spin down assay by allowing more thorough washing whilst retaining whole oocysts. This was initially performed using a filter with a pore size of 0.4 µM. The smallest size of oocyst is 14 X 15.5 µm and it was found that the oocysts blocked the filters not allowing the wash buffer to pass through. Filtration tubes with pore sizes of 10 µM were then assessed, this was the largest filter pore size that was found and this worked well for the first batch of assays (refer to subsection 4.2.6 above) when screening three scFvs; DHTPKWq, STQLP and STqHP. Subsequently, the assay became problematic, the filtrate could not pass through at the recommended centrifugation speed of 300 xg. At higher speeds the oocysts of *E. mitis* and *E. acervulina* were passing through the filter which is because those species have the smallest sized oocysts, *E. mitis* (14 X 15.5 µm) and *E. acervulina* (14 X 18 µm). The assay became technically challenging and not very practicable.

The antibodies that were seen to be binding to species of *Eimeria* oocysts were successfully cloned from the Griffin vector into pMAL-p5X vector which was aimed at producing scFv as MBP-scFv fusion protein. The aim was that soluble antibody may produce less background binding than when it is fused to the phage particles. Sub cloning into pMAL-p5X also allows high level expression and purification of the scFv-MBP fusion by affinity purification compared to the relatively low level of scFv display on phage (Duong-Ly and Gabelli, 2015). A periplasmic extraction method was employed to produce soluble scFvs (Arbabi-Ghahroudi *et al.,* 2005). This method uses the periplasmic environment that favours disulphide bond formation which aids the proper folding of the scFvs (Bardwell *et al.,* 1991). A single scFv clone was assessed before applying it to the other antibody clones and it was determined that the scFv was expressed from the pMAL vector but after purification the protein quantity was low, indicating it is likely being expressed at low levels and/or the MBP purification step is not efficient for these scFv clones. The spin down ELISAs was carried out to determine whether the scFv in crude periplasmic extract could bind oocysts. The assay produced very high background and signals produced with MBP only were similar those produced by the MBP-scFv test samples. This may be due to the low levels of scFv expression.

As an alternative to immobilising oocyst on plastic plates, the optimisation of oocyst immobilisation on nitrocellulose or PDVF membranes was carried out. Dot blot assays are straight forward and high throughput assays. The need for small quantities of oocysts for this assay makes it an even more attractive assay as it may prove to be sensitive and save cost and resources (Tian *et al.,* 2017). Oocysts were successfully immobilised on membranes but the challenge encountered was that phage particles produce high background signals on membranes. (Tan and Tan, 2015) carried out phage dot blots using nitrocellulose membranes pre-blocking the membranes with 10% (w/v) milk. Here, the use of this blocker did not reduce the background signals sufficiently enough for the assay to be valid. The addition of NaCl in the wash buffer was not successful in reducing background signals. NaCl is generally known to increase the ionic strength of the buffer reducing non-specific binding.

Fluorescent microscopy was performed to enable the analysis of the selected scFvs. The assay could detect binding of phage scFv to whole oocysts and showed that some of the scFvs were selectively binding to certain species of oocysts and could potentially be used to discriminate between species. One scFv (SRSKNLYWRSM) was pan-specific clearly binding to all species and producing strong signals. ScFvs SAqHP bound to *E. mitis*, *E. necatrix* and *E. tenella*, STqHP bound to *E. mitis*, *E. brunetti* and *E. tenella*, STQLP bound to *E. mitis, E. maxima*, *E. praecox* and *E. tenella* and

DHTPKWq bound to *E. necatrix* and *E. acervulina* (refer to table 4.2). The pattern of scFv binding in the fluorescent microscopy assay produced 5 distinct patterns, these were unique for *E. necatrix*, *E. acervulina* or *E. brunetti* or were shared by *E. mitis/E. tenella* or *E. maxima/E. praecox*. These properties could be further characterised, in particular looking at reproducibility between assays, to develop a diagnostic assay. However, as no scFv was specific for a single species this was not pursued.

Fluorescent microscopy has been successfully used to detect oocysts and other stages of parasitic development (Rose *et al.,* 1989). (Abi-Ghanem *et al*., 2008) in their study on phage display selection and characterization of scFv recombinant antibodies against *E. tenella* sporozoites, they used immunofluorescence to validate the binding of the selected scFv after screening with ELISA. Therefore, this assay is valid and can be applied to screen scFvs against parasites selected from analysis of phage sub libraries before purification and further characterisation of antibodies. This however, may not be a method of diagnosis favoured in comparison to traditional methods. Diagnosing coccidiosis in flocks without the need for differentiating infections is straightforward, species-level diagnosis is the major problem and a diagnostic must be an improvement over what is already available before it can gain widespread use.

The spin down assay on occasions gave different binding specificities compared to fluorescent microscopy. On the whole, the FM gave fewer positive results than the SDA – the differential detection of distinct species in FM may therefore reflect differences in epitope presentation on the surface of the different species and some fall below the limits of detection of the assay. This is hard to interpret in the SDA as it is only semi-quantitative.

Phage derived antibodies can be applied in various diagnostic platforms that enable rapid and accurate identification to target antigen most of which either use antigen or antibody capture on solid supports. Lateral flow dip stick assay which is based on capillary action occurring on a membrane migrating molecules along the membrane which causes a reaction with the target antigen (Beadle *et al.,* 1994).

Phage-displayed recombinant antibodies have been used in microfluidic platforms. Microfluidics platforms require small sample volume and produce fast and precise results (Nahavandi *et al.,* 2014). Microfluidic technology has evolved to include on chip immunoassay, on chip flow cytometry, on chip detection and imaging (Lee *et al.,* 2010, Meagher *et al.,* 2008). Other assays that recombinant antibodies have been applied to include; nanoparticles, silica, polystyrene and latex agglutination test (LAT) (Baptista *et al.,* 2008, Wilson, 2008), and DNA integrated assays (Omar *et al.,* 2014).

In this study, two assays were developed for the detection of antibodies to *Eimeria* infections in poultry. These assays are however not high throughput and one of the

aims of the study is to isolate antibodies that are suitable for the development of a high throughput assay that can potentially be applied in the field. Poultry coccidiosis is a flock problem hence applying a diagnostic on a representative sample gives an idea of the general flock health. An assay that can be applied in field conditions using oocysts extracted from chicken faeces with minimum flock handling time and cross contamination is ideal. The study therefore looked at alternative panning strategies to isolate antibodies against oocyst components that were readily immobilised on ELISA plates.

5. Isolation of single domain antibodies (V_HH) against chicken *Eimeria* oocysts homogenates using Next generation phage display

5.1 Introduction

The present chapter aimed to isolate antibodies against oocyst homogenates of species of *Eimeria* in poultry using NGPD. The approach used was the application of phage display using V_HH camelid library which was based on a pSD3 bacteriophage display vector (Li *et al.,* 1999) expressing pIII-V_HH fusions. The diversity of the V_HH is introduced in the CDR3 and it was comprised of three sub-libraries containing different lengths of CDR3 sequences – 12mers, 16mers and 21mers, and had a diversity of ~$1x10^9$ (Dr Alison Gray, personal communication). This library was paired with a helper phage; exphage to improve display efficiency (Baek *et al.,* 2002).

V_HH antibodies, also known as nanobodies are single-domain antibody fragments first described by (Hamers-Casterman *et al*., 1993) which are naturally produced by camelids (Harmsen and De Haard, 2007, Bever *et al.,* 2016). V_HH antibodies are versatile tools for research, diagnostics, and therapeutics and they offer several advantages for biotechnological applications over multidomain antibodies such as scFv and Fabs (Woods, 2019, Harmsen and De Haard, 2007). The multidomain nature of scFv makes its genetic manipulation more complex compared to V_HH (Woods, 2019). The V_HH have superior expression in recombinant forms because they are formed by a single polypeptide that has high solubility and stability and they are well suited for fusion protein display and selection systems such as phage display (Harmsen and De Haard, 2007, Vanlandschoot *et al.,* 2011, Monegal *et al.,* 2009). Another advantage of the V_HH is that due to their small size at between 12-15 kDa and longer CDR3 regions, they are able to access hidden epitopes and clefts such as enzyme active sites more effectively than conventional antibodies (Lauwereys *et al.,* 1998). V_HHs have a prolate shape and possess convex paratopes which can interact with antigen epitopes not accessible to conventional monoclonal antibodies, scFvs and Fabs (Steeland *et al., 2016,* Flajnik *et al.,* 2011).

The approach used in the previous chapter was the application of phage display panning of the Griffin scFv library against whole *Eimeria* oocyst. In terms of applying NGS analysis to identify binders, the approach was limited by the need to rescue specific clones from polyclonal phagemid sub-libraries. This process was very inefficient due to the variability of the flanking regions of the identified CDR3s, hence only a small number of scFvs were isolated. Also, difficulties were encountered in sub cloning isolated scFvs into an expression vector and making soluble antibodies (detailed in chapter 4). Phage derived antibody candidates are normally screened as soluble antibodies to ensure that the candidate's binding properties rely solely on the antibody fragment. Another limitation encountered in the previous approach was

developing a high throughput assay. Isolated antibodies were screened using spin down ELISA which sometimes gave variable results. Extensive attempts at immobilising whole oocysts to solid surfaces suitable for high throughput assay formats were unsuccessful. The approach used in the present chapter sought to potentially improve the assay format by using lysed oocysts that are readily immobilised in 96 well plates thereby circumventing the difficulties encountered in the immobilisation of target in the previous chapter. The V_HH phage format used in the current study was aimed at eliminating the need to rescue clones from polyclonal phagemid libraries as was performed in **chapter 3**. The use of a single scaffold V_HH with diversity only in the CDR3 allows the direct synthesis of identified CDR3 clones directly into any expression vector rather than their rescue from polyclonal phage pools which is laborious and not very efficient.

5.2 Results

5.2.1 V_HH phage display; panning for binders to chicken *Eimeria* oocysts

5.2.1.1 Quantification of oocysts

The second batch of *Eimeria* oocysts obtained for this research were quantified and the number of oocysts for each of the seven species were between 1.1 X 10^7 and 3.3 X 10^8 in 50 ml (Table 5.1).

Table 5:1: Concentrations of oocysts in *Eimeria* samples counted with a haemocytometer

Species	Concentration per ml	Total oocyst number (in 50 ml)
Eimeria mitis	1.5 X 10^6	7.5 X 10^7
Eimeria necatrix	6.0 X 10^5	3.0 X 10^7
Eimeria acervulina	4.5 X 10^6	2.2 X 10^8
Eimeria brunetti	1.3 X 10^6	6.3 X 10^7
Eimeria maxima	1.4 X 10^6	6.8 X 10^7
Eimeria praecox	2.3 X 10^5	1.1 X 10^7
Eimeria tenella	6.7 X 10^6	3.3 X 10^8

5.2.1.2 Antigen preparation: Sonication and solubilisation of oocysts

Oocysts homogenates were produced for each of the *Eimeria* species and the protein content was quantified using the Bradford assay (figure 5.1)

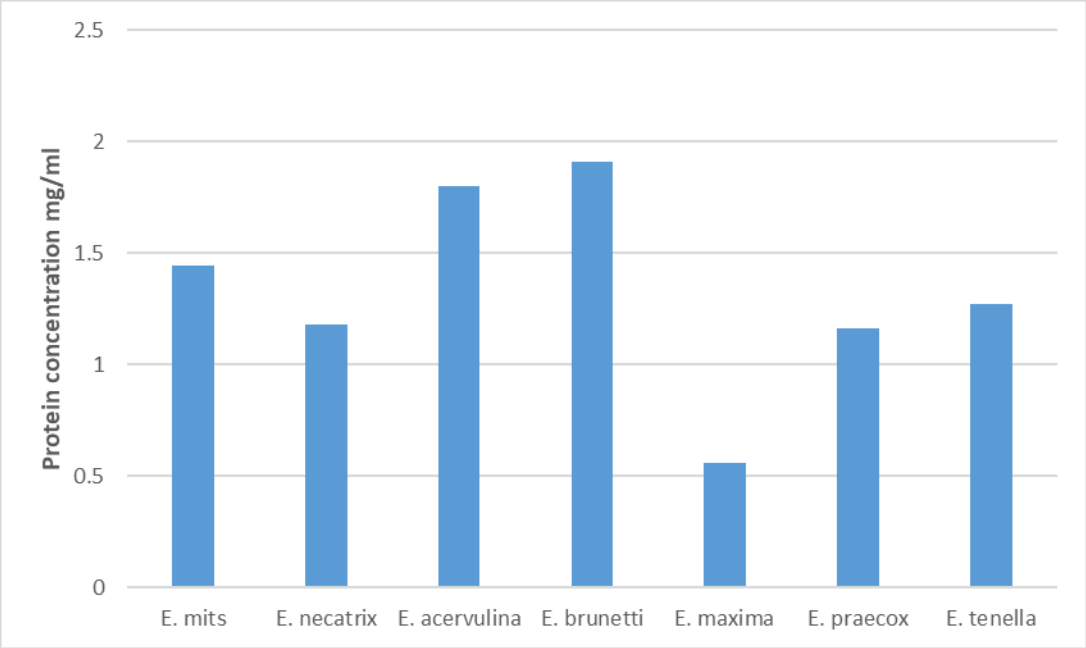


Figure 5:1: Protein concentration of the sonicated and solubilised oocysts of individual *Eimeria* species. These were derived from a Bradford protein assay.

5.2.1.3 Preparation and titre of phage

The V_HH phage library prepared for panning experiments had a titre of 10^{12} PFU/ml after PEG precipitation. Supernatant phage was used for the subsequent rounds of panning as well as the screens and titres were around 10^{11} PFU/ml (Figure 5.2). These tires are as expected when using exphage as the helper phage (Baek *et al.,* 2002).

5.2.1.4 Panning Rounds

Using the camelid V_HH library, phage selection was carried out against all seven species of oocysts using homogenates produced using sonication. The first round of panning was without subtraction while there was a pre-panning subtractive step in round two panning. In the third round of panning, there was a pre-panning subtractive step as well as post-panning subtraction of phage. Subtraction was performed against oocyst homogenates of all the other six species before incubating with the species the phage was raised for; this was carried out for all seven species. Each panning round glycerol stock was grown to produce phage with input titres ~ 10^{11} PFU/ml. Phage screening was carried out for phage isolated against each species of *Eimeria* versus the other six species using round 3 output phage. Titres produced from eluted phage after each round of panning was in the range of 2.8 $X10^5$- 4.1 X 10^7 PFU/ml (Figure 5.2). Eluted phage titres decreased with progressive rounds of panning for all *Eimeria* target species which may reflect the increased use of subtraction steps. Screening of round 3 panning phage against all *Eimeria* species was carried out to generate positive and negative replicate samples (6 of each) to be compared during the NGS analysis (refer to materials and methods subsection 2.7.8 for details).

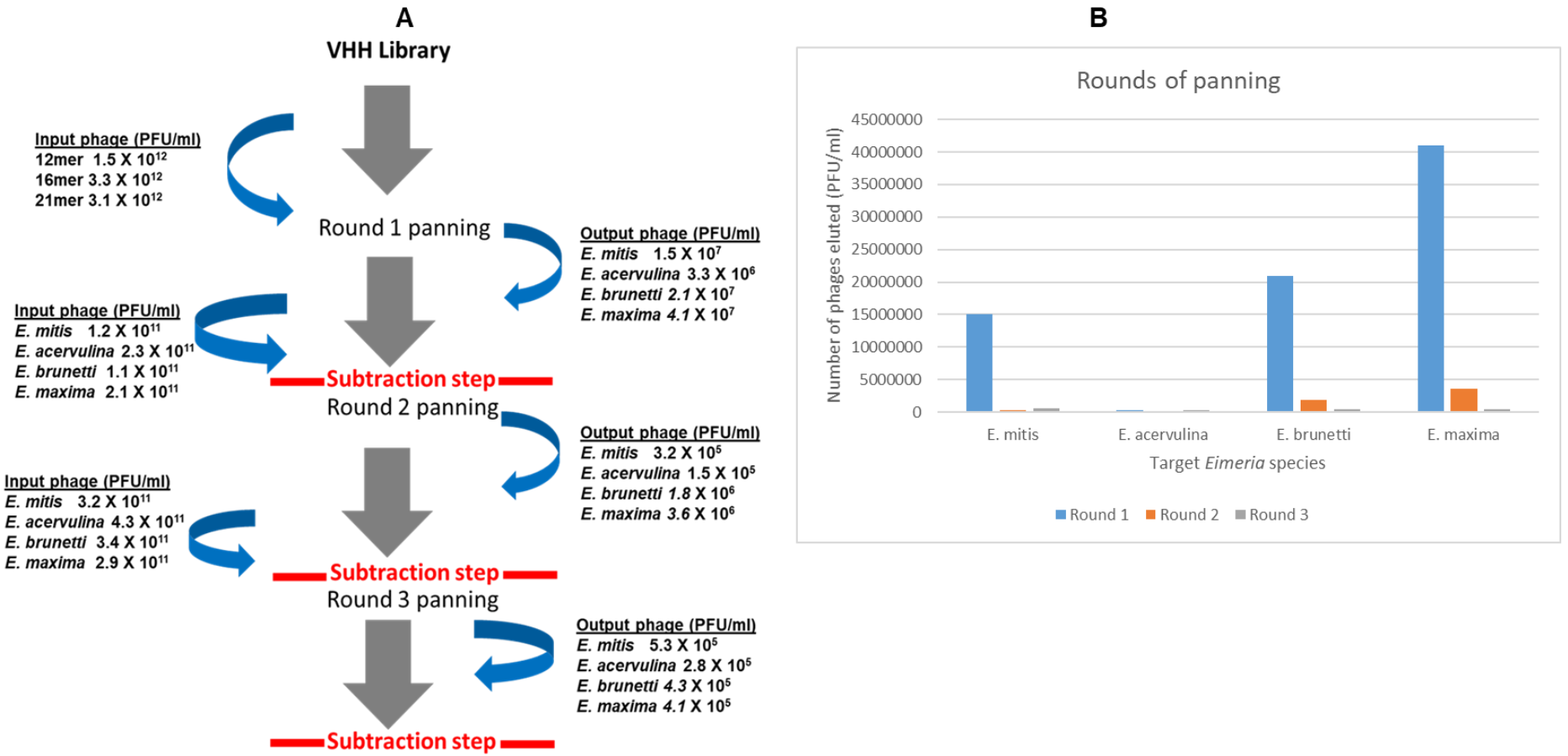


Figure 5:2: Schematic representation of the panning (A) strategy used with the V_HH library and number of eluted phage after rounds of panning (B). Three rounds of panning were performed for all seven *Eimeria* target antigens with no subtraction step in the first panning round, a pre-panning subtractive step in the 2nd round of panning and a pre-panning subtractive step as well as a post-panning subtraction in the 3rd panning round. Representative input and output phage titres are shown at each step (A) as well as graphical representation of eluted phage after each panning round for selected targets (B) (PFU/ml).

5.*2.1.5 Preparation of DNA for Ion Proton Sequencing*

V_HH phage library was used in panning experiments to select for antibodies against oocyst homogenates for the seven *Eimeria* species and DNA was extracted from phage from the round 3 panning. Eighty-four samples which comprise of phage bound to each species being screened against its target *Eimeria* antigen in 6 sets of duplicate wells (positive samples) and versus the duplicate wells of each of the other six species (negative controls). The duplicates were pooled such that for each species selection, 12 samples were generated (6 positive samples and 6 negative controls) totalling 84 samples. DNA was extracted from the bacterial stocks of all samples for use as template for Heavy chain CDR3 (HCDR3) amplifications. The samples were quantified using the Nanodrop and they were between 7.03-69.5 ng/µl. The phagemid DNA was used as template for the amplification of ~350 bp amplicons spanning the HCDR3 region. Two rounds of PCR were performed (Figure 2.2 in materials and methods). Representative samples are shown for round 1 (Figure 5.3) and round 2 PCR (Figure 5.4). Extracts were labelled E# for species and 1-6# for positive replicates of phage panning selections. The 84 samples were tagged with barcodes 1-85 (barcode 42 was excluded as previously stated because it has been shown not to be specific). *Eimeria mitis* (positives 1-6) were tagged with barcodes 1-6, *E. mitis* phage versus the other six species were tagged with barcodes 7-12. *Eimeria necatrix* (positives 1-6) were tagged with barcodes 13-18 (excluding 16), *E. necatrix* phage versus the other six species were tagged with barcodes 19-24, *E. acervulina* (positives 1-6) were tagged with barcodes 25-30 while *E. acervulina* phage versus the other six species were tagged with barcodes 31-36. *Eimeria brunetti* (positives 1-6) were tagged with barcodes 37-43 and *E. brunetti* phage versus the other six species were tagged with barcodes 44-49, *E. maxima* (positives 1-6) were tagged with barcodes 50-54 while *E. maxima* phage versus the other six species were tagged with barcodes 56-61. *E. praecox* (positives 1-6) were tagged with barcodes 62-67 while *E. praecox* phage versus the other six species were tagged with barcodes 68-73, *E. tenella* (positives 1-6) were tagged with barcodes 74-77 while *E. tenella* phage versus the other six species were tagged with barcodes 80-85. The water control was tagged with barcode 86. Sample labelling is summarised in Table 5.2. The PCR products from round 2 were then quantified by fluorometry using Qubit and an equal contribution by concentration from each sample was used to produce a pooled sample. The amplicon was electrophoresed on 2% (w/v) agarose gel (Figure 5.5) and band purified, and the sample was further purified using Agencourt®AMPure®XP PCR purification kit. Purification using Agencourt®AMPure®XP PCR purification kit was carried out twice after which the amplicon was re-quantified by Fluorometry using

Qubit, the final concentration was 17.3 ng/µl and the sample was sent off for NGS sequencing according to the specifications of University of Pennsylvania.

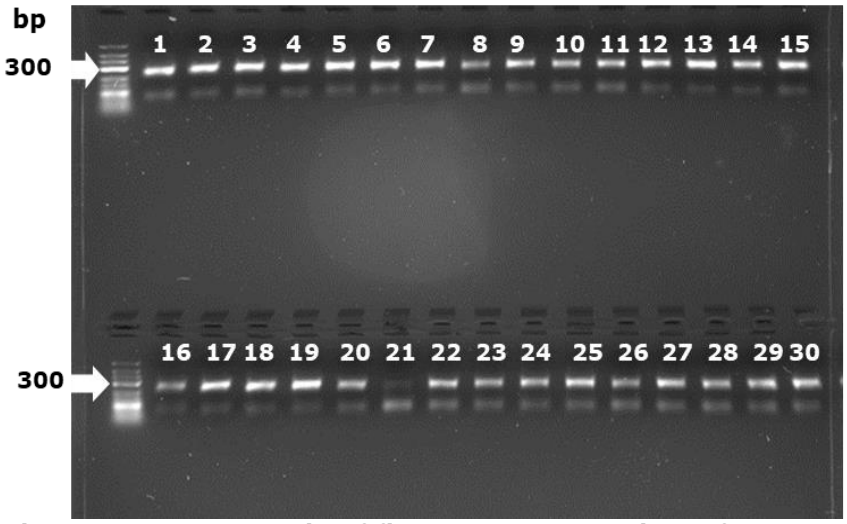


Figure 5:3: An example of first step PCR products for NGPD. 10 µl of amplified PCR products were mixed with 2 µl of DNA loading dye and run on a on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Samples labelled 1-6 represent target species *E. mitis* positive screening replicates, samples 7-12 represent screening of the target species *E. mitis* versus the other six. Samples labelled 13-18 represent target species *E. necatrix* positive screening replicates while samples labelled 19-24 represent screening of target species *E. necatrix* versus the other six and samples labelled 25-30 represent target species *E. acervulina* positive screening replicates. Molecular markers are indicated in bp. The round 1 PCR products were then purified and used as a template for round 2 PCR.

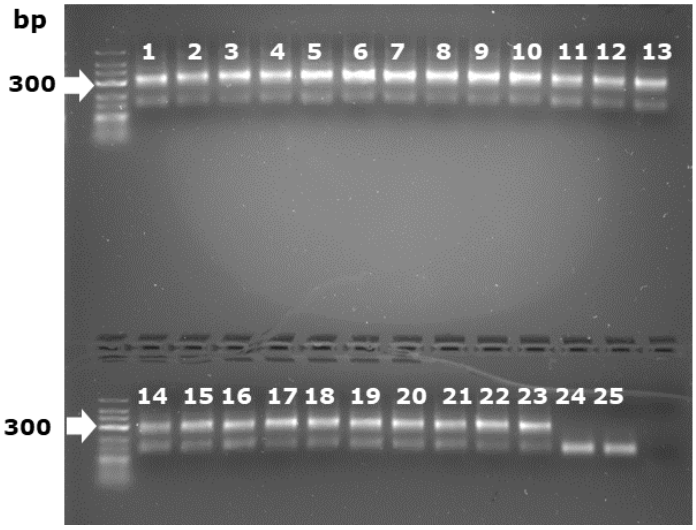


Figure 5:4: An example of second step PCR products for NGPD: 10 µl of amplified PCR products were mixed with 2 µl of DNA loading dye and run on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the

gel and samples were loaded using 6x loading buffer (R0611). Samples labelled 1-6 represent target species *E. mitis* positive screening replicates, samples 7-12 represent screening of the target species *E. mitis* versus the other six. Samples labelled 13-18 represent target species *E. necatrix* positive screening replicates while samples labelled 19-23 represent screening of target species *E. necatrix* versus the other five (the comparison of the target species with the sixth species is not shown) and samples labelled 24 and 25 represent water control from the 1st and 2nd step PCR reactions. Molecular markers are indicated in bp. The step 2 PCR products were then purified and pooled together in equal concentrations and band prepared before purification and sending for Ion torrent sequencing.

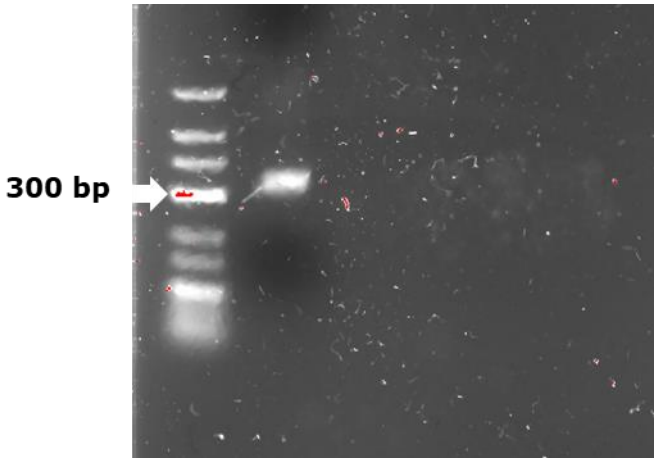


Figure 5:5: Final pooled PCR amplicon that was sent for Ion torrent sequencing. After the 2nd step PCR amplification, the PCR products were quantified by Qubit 2.0 Fluorometer and the DNA from all 84 barcoded samples were pooled in an equal ratio so that the reads will be equalised during NGS. The sample was purified using Agencourt®AMPure®XP and then analysed on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). This was extracted, purified, and was the template for Ion torrent sequencing.

Table 5:2: Summary of samples prepared for next generation sequencing for panning

Samples summary[1]	**Sample No. Range**[2]	**Barcode No. Range**[3]
E. mitis positive replicates 1-6	1 to 6	1 to 6
E. mitis versus *E. necatrix*	7	7
E. mitis versus *E. acervulina*	8	8
E. mitis versus *E. brunetti*	9	9
E. mitis versus *E. maxima*	10	10
E. mitis versus *E. praecox*	11	11
E. mitis versus *E. tenella*	12	12
E. necatrix positive replicates 1-5	13 to 18 (- #16)	13 to 18 (- #16)
E. necatrix versus *E. mitis*	19	19
E. necatrix versus *E. acervulina*	20	20
E. necatrix versus *E. brunetti*	21	21
E. necatrix versus *E. maxima*	22	22
E. necatrix versus *E. praecox*	23	23
E. necatrix versus *E. tenella*	24	24
E. acervulina positive replicates 1-6	25 to 30	25 to 30
E. acervulina versus *E. mitis*	31	31
E. acervulina versus *E. necatrix*	32	32
E. acervulina versus *E. brunetti*	33	33
E. acervulina versus *E. maxima*	34	34
E. acervulina versus *praecox*	35	35
E. acervulina versus *E. praecox*	36	36
E. brunetti positive replicates 1-6	37 to 42	37 to 43 (- #42)
E. brunetti versus *E. mitis*	43	44
E. brunetti versus *E. necatrix*	44	45
E. brunetti versus *E. acervulina*	45	46
E. brunetti versus *E. maxima*	46	47
E. brunetti versus *E. praecox*	47	48
E. brunetti versus *E. tenella*	48	49
E. maxima positive replicates 1-6	49 to 54	50
E. maxima versus *E. mitis*	55	51 to 56
E. maxima versus *E. necatrix*	56	57
E. maxima versus *E. acervulina*	57	58

E. maxima versus *E. brunetti*	58	59
E. maxima versus *E. praecox*	59	60
E. maxima versus *E. tenella*	60	61 to 66
E. praecox positive replicates 1-6	61 to 66	67
E. praecox versus *E. mitis*	67	68
E. praecox verus *E. necatrix*	68	69
E. praecox versus *E. acervulina*	69	70
E. praecox versus *E. brunetti*	70	71
E. praecox versus *E. maxima*	71	72
E. praecox versus *E. tenella*	72	73 to 78
E. tenella positive replicates 1-6	73 to 78	79
E. tenella versus *E. mitis*	79	80
E. tenella versus *E. necatrix*	80	81
E. tenella versus *E. acervulina*	81	82
E. tenella versus *E. brunetti*	82	83
E. tenella versus *E. maxima*	83	84
E. tenella versus *E. praecox*	84	85

[1] Sample descriptions for screening rounds (positive replicates and negative controls). In each species versus another species, the first species is the target species panned against in the three panning rounds while the second species is that used in the screening of phage (negative controls). Positive replicates are screened against the same target species they were raised against.

[2] Sample numbers assigned to each sample type. BC 16 was not used because despite repeat second stage PCRs, no product was obtained.

[3] Barcode number assigned to each sample.

5.2.2 Conventional screening of V_HH phage binders

5.2.2.1 V_HH polyclonal phage ELISA

The binding properties of the polyclonal phage was assessed using oocyst homogenates as antigen with phage grown from the initial V_HH library; 12mer, 16mer, 21mer combined (10^{11}-10^{12} PFU/ml) and all of the three panning rounds for each of the seven *Eimeria* species target, as well as an anti-BSA monoclonal scFv positive control (used a phage antibody specific for BSA). The assay used rabbit anti-fd and goat anti-rabbit AP antibodies with a pNPP substrate. Absorbance signals produced with a no antigen control gave a background level after 1 hour of adding substrate of ~0.1-0.17 while the anti-BSA positive control produced an absorbance signal of 0.38 (Figure 5.6). None of the polyclonal phage samples from panning produced a signal above background.

The signal produced by the anti-BSA positive control was considered positive at >2X of the background signal, but the signals were not as high as those that had been produced in previous assays hence the polyclonal ELISA was repeated using a more sensitive detection system. The assay was repeated with an HRP detection system leaving all other variables the same and absorbance signals produced were compared to the no antigen control signal of ~0.1-0.2; the anti-BSA positive control produced a signal of ~ 4.3 after 15 minutes of adding the TMB substrate (Figure 5.7). Again, none of the polyclonal phage samples from panning produced a signal above background.

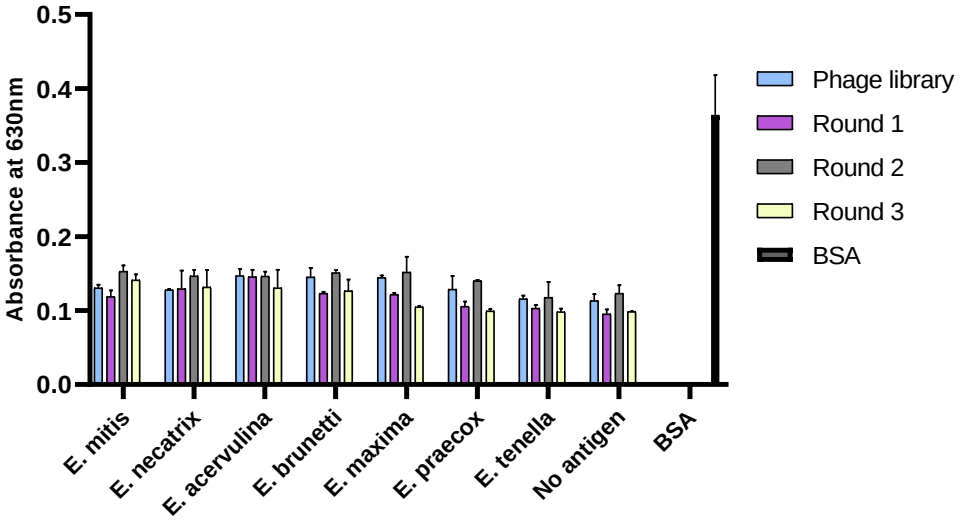


Figure 5:6: Polyclonal phage ELISA for *Eimeria* oocyst homogenates. V_HH phage were produced for each panning round when targeting each of the seven *Eimeria* species and camelid V_HH library. scFv anti-BSA positive control phage was also produced. Oocyst homogenate or BSA was immobilised overnight in 100 µl aliquots at 50 µg/ml on a Nunc MaxiSorp® plate. Polyclonal V_HH or monoclonal scFv

phage were used to detect the immobilised oocyst homogenates or BSA and those remaining after the wash steps were detected by a rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed a 1-hour incubation in the well before absorbance was read at 405 nm.

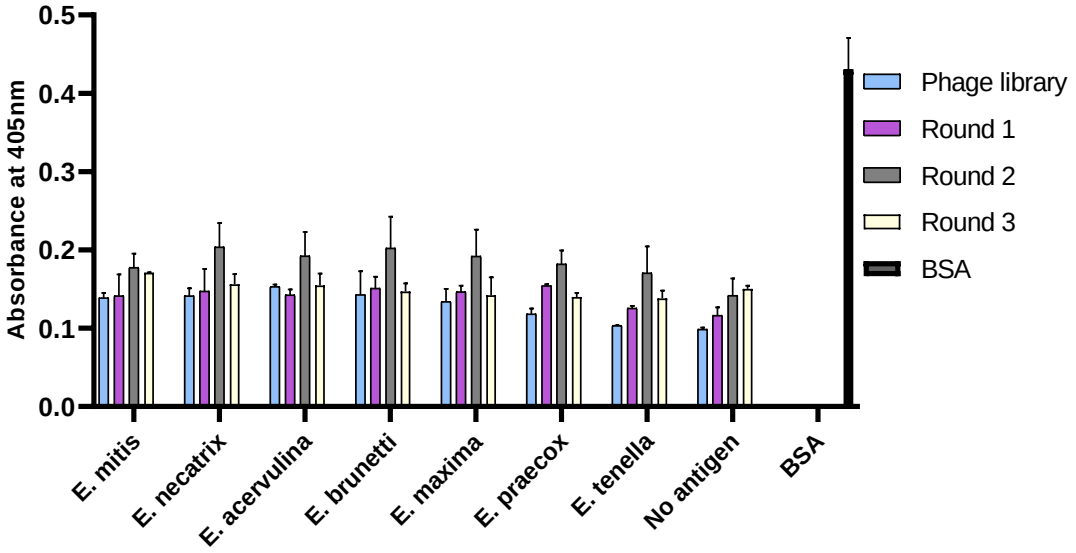


Figure 5:7: Repeat polyclonal phage ELISA for *Eimeria* oocyst homogenates. X axis represents the target *Eimeria* species used in panning. V_HH phage were produced for each panning round when targeting each of the seven *Eimeria* species and camelid V_HH library. scFv anti-BSA positive control phage was also produced. Oocyst homogenate or BSA was immobilised overnight in 100 µl aliquots at 50 µg/ml on a Nunc MaxiSorp® plate. Polyclonal V_HH or monoclonal scFv phage were used to detect the immobilised oocyst homogenates or BSA and those remaining after the wash steps were detected by an anti-M13-HRP antibody. The TMB substrate was allowed 15 minutes incubation in the well before absorbance was read at 630 nm.

The signal produced by the positive control in the repeat ELISA was still low hence the polyclonal ELISA was repeated using another batch of reagents and the HRP detection system. This repeat ELISA also failed to improve the assay as signals produced were still background level and the anti-BSA positive control was still only 2X background (data not shown). This suggested that the anti-BSA clone may not be functioning as it ideally should.

The assay was repeated with a different positive control phage (anti-elastin phage) using an AP detection system and signals produced including the no antigen control gave a background signal after 1 hour of adding substrate of ~0.2-0.3 while the positive control produced an absorbance signal of 1.07 (Figure 5.8). None of the polyclonal phage samples from panning gave any signal above background. This

assay was repeated again using a HRP detection system and signals produced by polyclonal phage were again comparable to the no antigen control at ~0.2-0.3 after 15 minutes of adding substrate; the positive control produced a signal of 0.95 after 15 minutes of incubating the TMB substrate in the wells (data not shown). These assays suggested that the polyclonal phage was not binding to the oocyst homogenates given that the positive control repeatedly produced strong signals using the different detection systems.

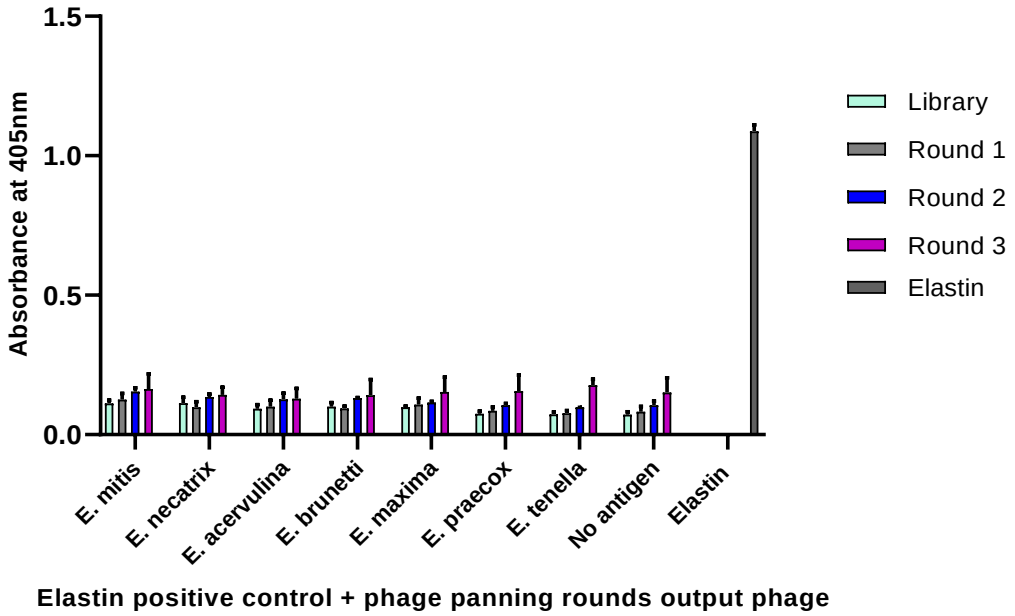


Figure 5:8: Repeat polyclonal phage ELISA for *Eimeria* oocyst homogenates. V_HH phage were produced for each panning round when targeting each of the seven *Eimeria* species, camelid V_HH library as well as anti-elastin positive control. Oocyst homogenate or elastin was immobilised overnight in 100 µl aliquots at 50 µg/ml on a Nunc MaxiSorp® plate. Polyclonal V_HH phage as well as monoclonal anti-elastin phage were used to detect the immobilised oocyst homogenates or elastin and those remaining after the wash steps were detected by a rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation in the well before absorbance was read at 405 nm

5.2.2.2 Colony picking

As previously carried out in chapter 3, individual colony picking was employed alongside polyclonal ELISA in addition to the NGS method to identify positive V_HH binders to individual *Eimeria* species.

5.2.2.3 V_HH monoclonal phage ELISA; initial approach

Following the failure of the polyclonal ELISAs to produce any signals. Monoclonal ELISAs were carried out for all species. From the round 3 panning glycerol stocks for each species, 10 single colonies were picked for each species; totalling 70 clones for the seven species of *Eimeria*. Monoclonal phage preparations were made for each individually picked clone and the individual V_HH binding properties were assessed by phage ELISA using oocyst homogenates as antigen and a rabbit anti-fd and goat anti-rabbit AP antibodies with a pNPP substrate for detection of binding. Absorbance signals were compared against a monoclonal anti-elastin phage-V_HH positive control and signals produced were background signals after 1 hour of adding substrate at ~0.2-0.3. The positive control produced an absorbance signal of 1.12 (Figure 5.9).

5.2.2.4 V_HH monoclonal phage ELISA; second approach

Due to the initial ELISA approach not producing any positive clones, as it was just 10 clones initially selected for each of the panning experiments, it was thought to increase the number of individually picked clones to improve the chances of isolating binders. Another set of 96 single colonies for each of the *Eimeria* species were picked from the round 3 panning glycerol stocks totalling 672 clones for the seven species of *Eimeria*. Monoclonal phage preparations were made for each individually picked clone and the individual V_HH binding properties were assessed by phage ELISA as before with the anti-elastin phage-V_HH positive control and a no antigen background control. Seven clones produced signals over 2X background (data not shown). All seven clones were at the edge of the ELISA plates hence positive signals may have been as a result of edge effects. Phage were re-prepared for these clones and re-tested and signals produced for all clones were comparable to background signals at between 0.2-0.3 while the positive control produced a strong signal at 1.1 (data not shown). Despite this extensive screening of random clones, no positive binders were identified from any of the seven panning experiments.

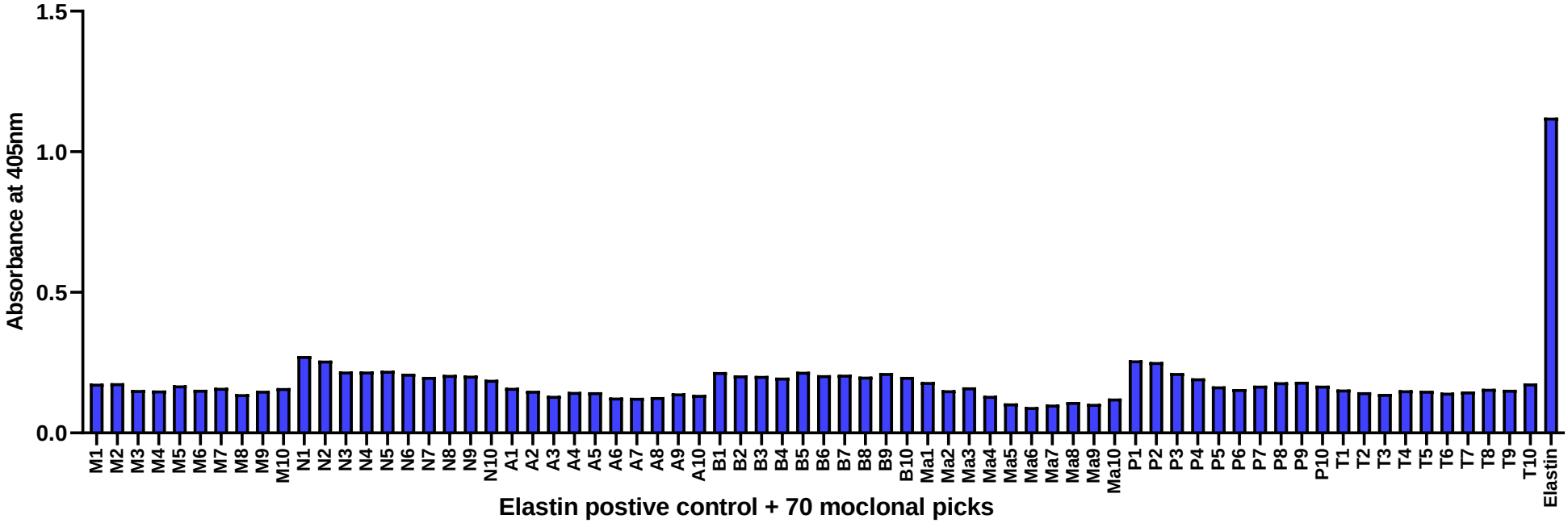


Figure 5:9: The binding of monoclonal phage antibodies to *Eimeria* oocyst homogenate. Clones labelled M1-10 represent *E. mitis* 1-10, clones labelled N1-10 represent *E. necatrix* 1-10, clones labelled A1-10 represent *E. acervulina* 1-10, clones labelled B1-10 represent *E. brunetti*, clones Ma1-10 represent *E. maxima* 1-10, clones labelled P1-10 represent *E. praecox* 1-10, clones T1-10 represent *E. tenella* 1-10. V_HH phage were produced from 70 randomly picked colonies from round 3 of panning; 10 for each *Eimeria* species after panning against oocyst homogenates. Oocyst homogenates or elastin was immobilised overnight in 100 µl aliquots at 50 µg/ml on Nunc MaxiSorp® plate. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) for each individually picked colony were used to detect immobilised oocyst homogenates while anti-elastin phage was used to detect elastin. Bound phage were detected by rabbit anti-fd, goat anti-rabbit AP antibody and pNPP substrate. After 1 hour, absorbance values were taken at 405 nm.

5.2.3 Next generation phage display

5.2.3.1 Data analysis and V_HH rescue

Polyclonal phage ELISA as well as colony pick ELISAs did not reveal any binders to the oocyst homogenates. The recovered panning phage titre levels reduced through progressive panning rounds and this may indicate that the subtractive steps removed a lot of the binders that are not species specific but may well be high affinity binders. Species specific binders may be rare as they may bind to rare epitopes or may be low affinity binders. NGS analysis was applied to highlight species specific binders present at low levels.

The NGS data was received in a compressed FASTQ file format from the University of Pennsylvania and the data was de-compressed and de-multiplexed (refer to materials and methods **chapter 2** subsection 2.7.8).

Analysis of NGS data was performed to identify and quantify enriched V_HHs for each species (Figure 5.10). The positive replicate of each of the seven *Eimeria* species were individually compared against the pool of the negative screening samples and Z scores, which reflect the frequency and ratio of binding, were generated for each CDR3 sequence. Subsequently, CDR3 sequences that had a Z score of above 3 in at least 4 out of the 6 positive replicates were selected (Figure 5.11). *Eimeria mitis* had no CDR3 that was present in all six positive replicates, 5 CDR3s that were present in five positive replicates and 13 CDR3s that were present in four positive replicates. Five positive replicates were sequenced for *E. necatrix* having obtained no PCR product in one of the positive replicates and 7 CDR3s were present in all five positive replicates and 28 CDR3s were present in four out of five of the positive replicates. *Eimeria acervulina* had no CDR3s present in all six positive replicates, 8 CDR3s present in five positive replicates and 26 CDR3s present in four positive replicates. *Eimeria brunetti* had no CDR3 present in all six of the positive replicates, 4 CDR3s present in five positive replicates and 41 CDR3s present in four positive replicates. *Eimeria maxima* was the only species that had CDR3s in all six positive replicates with 23 CDR3s fitting that criteria, 95 CDR3s were seen in 5 positive replicates, and 65 CDR3s seen in 4 replicates. *Eimeria praecox* had no CDR3 present in all six or five of the six positive replicates. There were only 3 CDR3s present in four of the six positive replicates hence some of the final selected CDR3s for this species were represented in 3 out of six positive replicates. *Eimeria tenella* had no CDR3 present in all six of the positive replicates and 7 CDR3s were seen in 5 positive replicates while 5 CDR3 were seen in four of the six positive replicates (a representative stacked graph is presents in Figure 5.12).

As a final check, the CDR3s selected were then searched for in the files to see if it appeared enriched in any other barcodes having Z score of 3 and above in any sample (for panning against a distinct *Eimeria* species) and if it appeared as being enriched against more than one species, it was not selected. The top 5 CDR3 sequences were then selected for each species (Table 5.3).

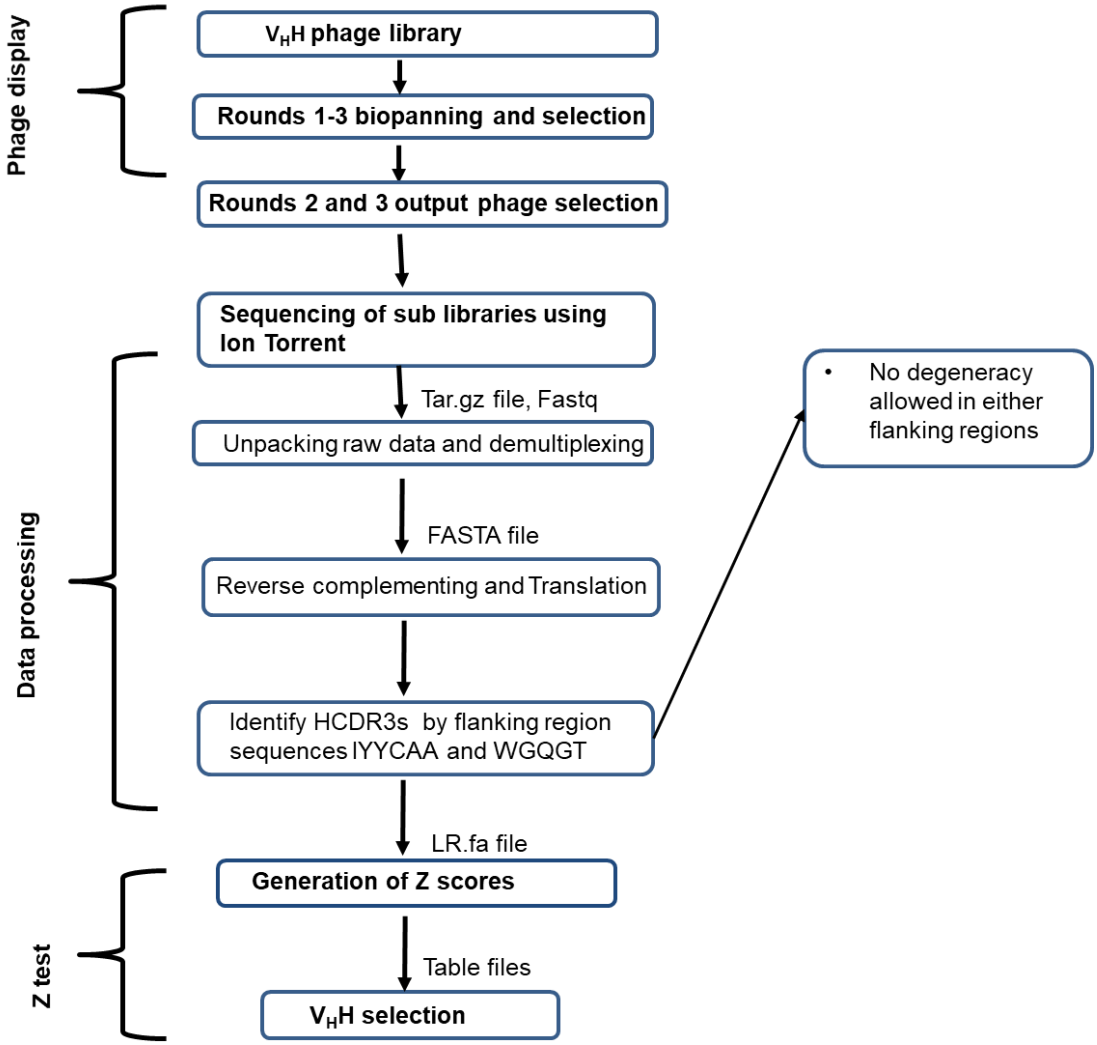


Figure 5:10: Schematic representation of phage display panning followed by analysis of NGS data. Each step is indicated on the left, with data file types indicated between each stage of analysis. Panning experiments were performed over three selection rounds. Round 3 output phage were screened against the species they were raised against (positive controls) and other species (negative controls). Round 3 screening sub libraries were sequenced by Ion Torrent sequencing and raw data was received as a tar.gz library file and extracted as FASTQ files. Sequence reads were separated according to barcodes by demultiplexing to form FASTA files. They were translated and reverse complemented. CDR3 sequences were identified by the flanking motifs IYYCAA and WGQGT with no degeneracy allowed. A two proportion Z-test was performed to produce Z-scores for each CDR3 (see Figure 5.11). CDR3 sequences were then selected for their enrichment in multiple positive replicates in screening rounds indicating specificity to only the target *Eimeria* species.

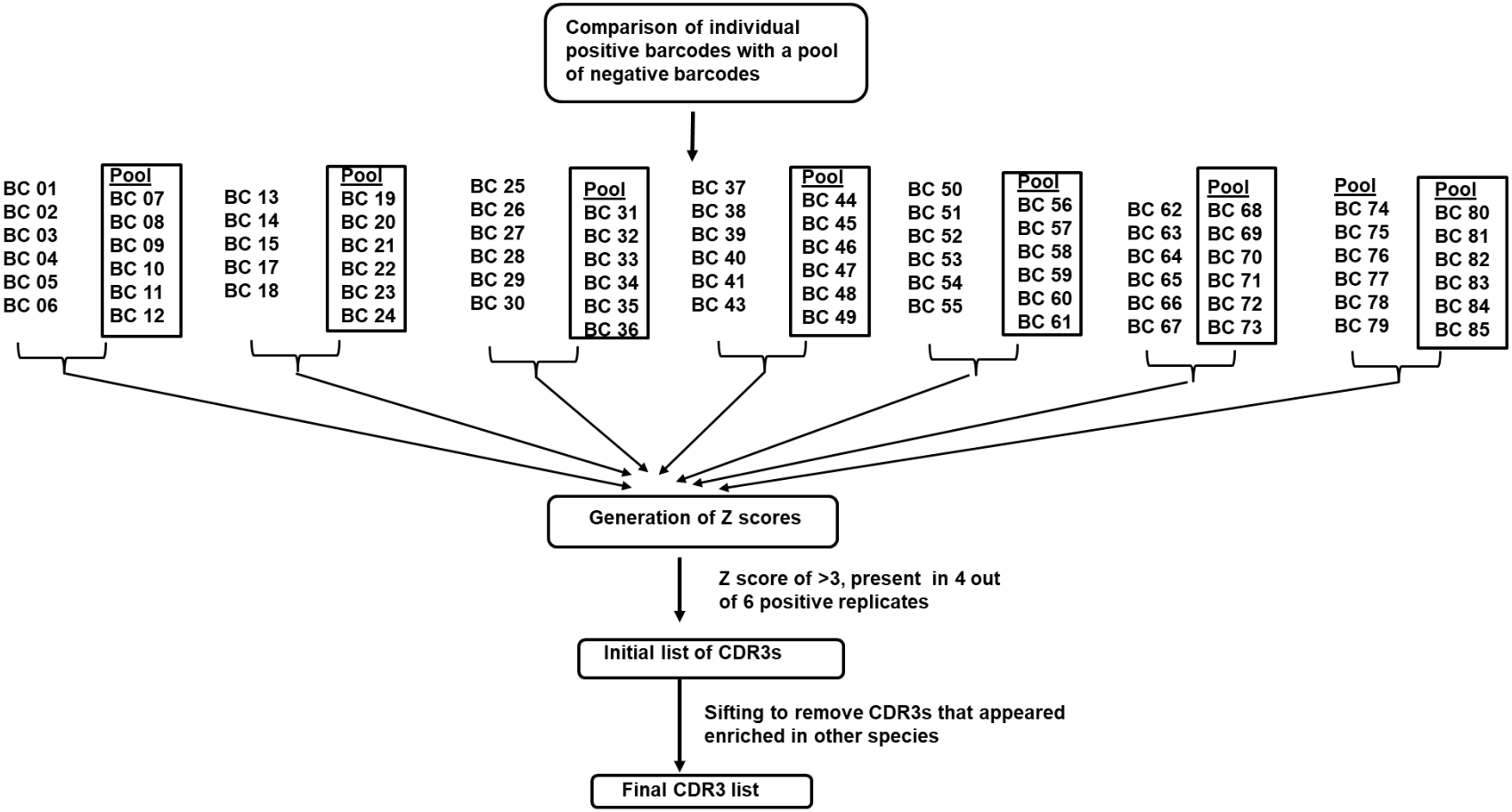


Figure 5:11: Workflow of strategy employed for the analysis of NGS data. Individual positive barcode replicates were compared against the pool of negative barcodes for the other 6 species and Z scores were generated. Subsequent analysis was carried out to select for CDR3s that had a Z score of above 3 and were present in at least 4 out of 6 positive replicates and an initial list of CDR3s was produced. For each species, the list of CDR3s was then sifted to remove CDR3s that appeared enriched in other species to maximise the chances of isolating species-specific CDR3s.

Table 5:3: A heat map matrix generated using conditional formatting in Microsoft Excel for selected V_HH from panning against oocyst homogenates of the seven *Eimeria* species. The selected V_HHs are represented according to the V_HH CDR3 sequence, species specificity, frequency of a Z score >3 in positive replicates, Z scores in the positive replicates and highest Z score seen in the positive replicates.

CDR3[1]	Specificity[2]	Frequency in positive barcode replicates[3]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
DqRSPNSLCAqC	*E. mitis*	5 of 6	9	15.16	3.72	3.68	9.64	-2.85
TSTGTLQSSRPPKHSH	*E. mitis*	4 of 6	7.95	4.74	5.52	11.31	-3.56	-2.23
YqLTFAHTPAAFVYVFRSVAS	*E. mitis*	5 of 6	4.04	7.78	11.89	20.6	31.83	-8.82
AKSSPQPMTVSLACqP	*E. mitis*	4 of 6	12.27	79.61	24.14	50.32	-1.59	-68.41
AqSHAqCRLSNKELKR	*E. mitis*	5 of 6	3.26	4.72	9.15	10.61	4.58	-3.61
WaqYSKPPSTYVYYLGKRYLI	*E. necatrix*	5 of 5	45.55	41.28	35.25	13.57	42.59	
YLRSqCEqHNFLLDALNVEDA	*E. necatrix*	4 of 5	5.01	-1.56	8.72	19.4	31.32	
KAALqMqHWHSQKREL	*E. necatrix*	4 of 5	3.17	4.45	3.03	4.12	-0.9	
RqPVERQRMTVATAPRKTqRV	*E. necatrix*	5 of 5	5.89	34.67	25.29	50.29	22.19	
CFGQCEHSqYTRGMTGRGRHS	*E. necatrix*	5 of 5	34.19	24.01	20.46	7.2	28.97	
YqLTFCSYACCVLFMFFRSVAS	*E. acervulina*	5 of 6	44.92	19.34	6.51	20.28	36.71	-1.27
PSASYAGMqRRWKQNL	*E. acervulina*	5 of 6	51.8	0.56	18.48	13.95	36.9	20.33
IHWqREKqLTTqSRTVPAHNM	*E. acervulina*	5 of 6	4.33	8.38	8.41	4.76	4.96	2.84
MVVWKqHSSLAKTRCY	*E. acervulina*	4 of 6	6.2	13.79	11.38	7.88	0.47	-1.72
PHLKFSCFpCFCqHHGS	*E. acervulina*	5 of 6	3.47	4.45	4.93	6.12	6.83	1.17
YNRLqRTGNATT	*E. brunetti*	5 of 6	15.41	7.04	8.06	33.92	8.14	-12.36
HsqLCKVPCVTRPRNY	*E. brunetti*	4 of 6	14.6	51.55	5.58	8.51	-7.22	-18.04
lcqLLLHPLCAL	*E. brunetti*	4 of 6	23.38	41.99	21.86	6.05	1.22	-15.3
NyqPNWNPqKTS	*E. brunetti*	4 of 6	24.78	31.29	16.69	4.8	0.51	-2.97
IMGPMSTANKMNTMPQ	*E. brunetti*	4 of 6	0.89	26.57	10.46	-12.21	4.39	3.89
YHYNRLYNSqSSAVEV	*E. maxima*	6 of 6	3	3.49	3.75	6.94	7.82	5.6
VPYLIRHTPPDSTqYV	*E. maxima*	6 of 6	4.28	3.06	3.6	6.97	8.47	6.45
YADqYASKSFKL	*E. maxima*	5 of 6	2.28	5.71	7.09	8.27	4.36	8.36
LCLNRSqLTVNQ	*E. maxima*	5 of 6	127.88	18.22	33.13	23.47	28.45	-10.47
DLNYPRTqHRRKQKNE	*E. maxima*	5 of 6	6.02	17.4	19.8	22.04	20.2	0.17
KVTMPGKNQAqLSIAR	*E. praecox*	4 of 6	4.98	3.52	3.46	4.23	-5.91	-0.03
TRWPTNYLHSFDLGPq	*E. praecox*	3 of 6	6.17	0.74	3.1	4.49	-0.08	-2.3
qWLVESRRAKRRPMPG	*E. praecox*	3 of 6	3.52	4.34	5.14	-1	1.26	-1
RqPVERQRMTVATATRKTqRV	*E. praecox*	3 of 6	3.17	3.18	4.88	2.18	-3.16	-3.16
WHSWRqLQYAPAHIKG, ASSns	*E. praecox*	4 of 6	3.52	8.99	28.4	27.02	-1.32	-23.62
VnqMHYTIRDLV	*E. tenella*	4 of 6	6.09	5.7	5.09	4.52	-0.09	-4.1
qLqqDNPLSVPF	*E. tenella*	4 of 6	32.25	35.37	33.27	8.67	-33.37	-16.44
TSHFTQLYRYLF (3)	*E. tenella*	4 of 6	3.57	3.31	4.34	5.8	-2.86	-2.69
TRCqAPNFNKPLQRAR	*E. tenella*	4 of 6	3.65	4.4	5.02	8.44	-14.38	-13.06
CPPNRMPRHDATVDSD	*E. tenella*	4 of 6	24.66	23.94	18.99	3.87	-1.37	-38.72

[1] CDR3s of selected V_HH

[2] The species the V_HH were raised against

[3] The number of positive screening replicates the V_HH appeared in that had a Z score of >3

[4] Z scores in the positive replicate barcodes. (Blue-red > 10)

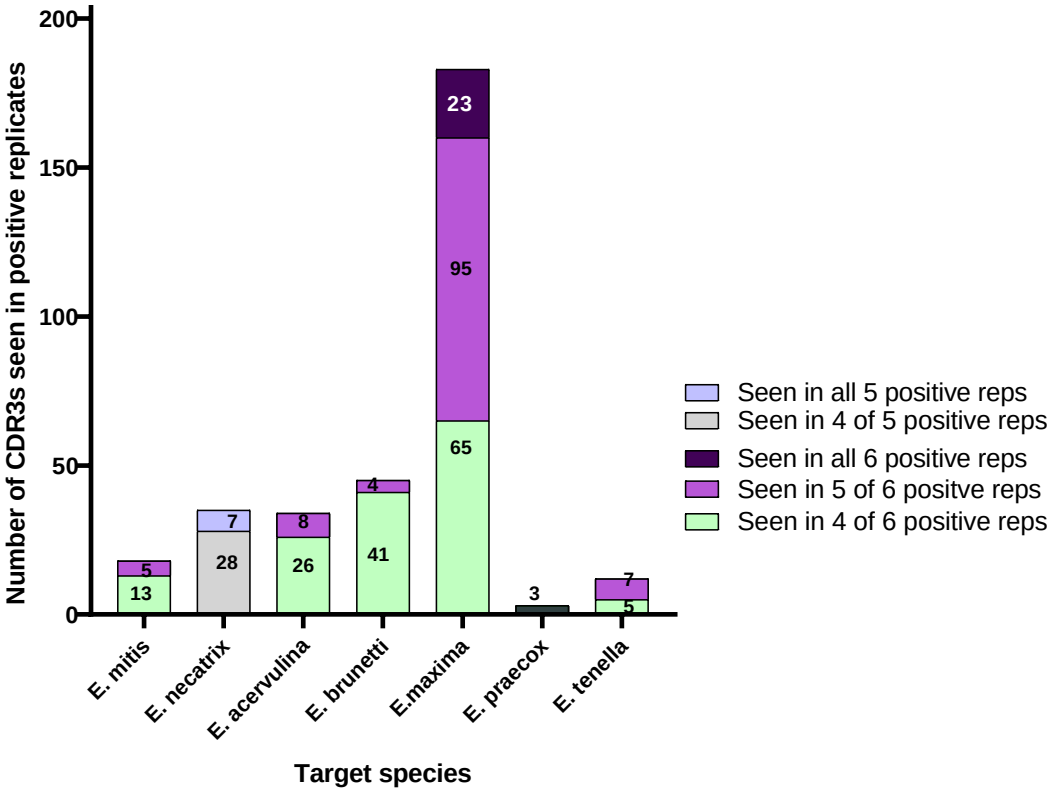


Figure 5:12: The numbers of CDR3s seen in 4 (*E. necatrix*), 5 or 6 positive replicate samples. Following screening of each phage with the species it was targeting as well as the other six negative controls, samples were sequenced by Ion Torrent sequencing and data analysed using a two proportion Z-test. CDR3s were selected based on those that were seen having a Z score of above 3 and present in at least 4 of the positive replicate samples (except for *E. praecox* that 3 CDR3s were selected that were seen in 3 of 6 positive replicates, not represented here).

5.2.3.1 Data analysis and V_HH synthesis

The V_HH library has the advantage of having the same antibody scaffold sequences in the framework and HCDR1 and HCDR2 regions which is not so with the scFv library previously used in this study. Randomisation was only in the CDR3 hence this allowed the direct synthesis of selected clones into the phagemid vector containing a template V_HH without needing to rescue clones of low abundance from polyclonal phage pools. The selected V_HHs were synthesized using the anti-beta-lactamase V_HH clone in pSD3 as the template.

Amino acid sequences for CDR3 selected from the NGS data were converted to their optimal nucleotide codons for *E. coli* expression and phosphorylated primers were designed to synthesise the optimal V_HH sequence by each covering half of the CDR3 sequence and 18 nucleotides into the adjacent framework region of the template V_HH. The forward primer thus covered half the CDR3 and the framework region 4, and the reverse primer covered the other half of the CDR3 and the framework region 3, amplifying in the other direction (reference Table 2.7 Chapter 2).

5.2.3.2 V_HH clone synthesis by inverse PCR – Sanger sequencing of resulting clones

Inverse PCR reactions were carried out using a Q5 DNA polymerase for the specific synthesis of the V_HH clones. Of the thirty-five V_HHs selected to be synthesized, specific products were obtained for 34. Representative samples of inverse PCR product size are shown (Figure 5.13). The gel fragments from the 34 clones were gel purified and DNA quantified using fluorometry and they were within the range of 53.5-111.1 ng/µl. These were ligated overnight, microdialysed and transformed into bacteria. Two colonies each from the 34 clones were grown and miniprepped and sent for Sanger sequencing to confirm the sequences. All 34 rescue sequences are represented in Figure 5.14 and had the correct target CDR3 sequence.

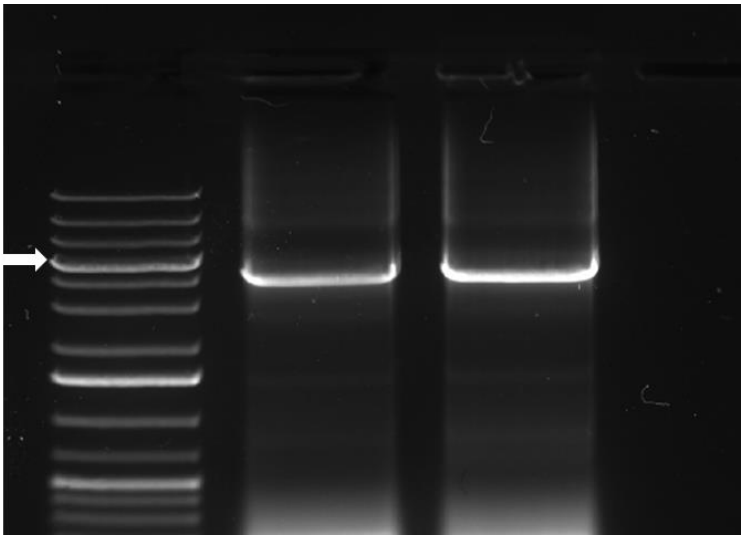


Figure 5:13: An example of inverse PCR products for V_HH rescues. The entire reaction (50 µl) PCR products were resolved on a 2% (w/v) agarose gel with a KB+ ladder (SM1333). Products were expected around 5kb (Indicated by white arrow next to the ladder). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Inverse PCR products with a correct band at ~5kb were extracted from the agarose gel and purified.

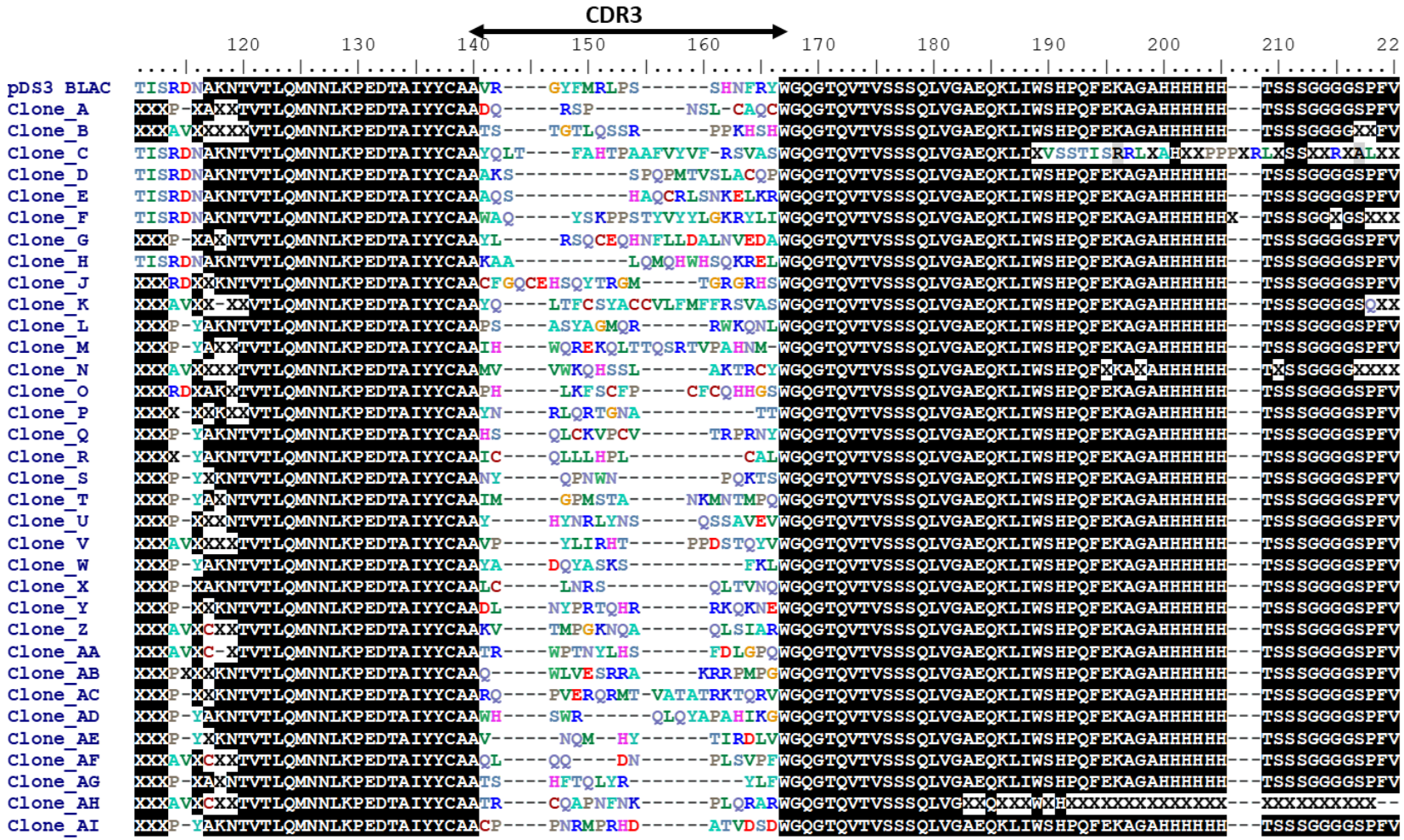


Figure 5:14: Sanger sequences of V_HH synthesised clones. Following bacterial transformation of V_HH clones, two colonies were miniprepped and 5 µl of 100 ng/µl DNA was sent for Sanger sequencing with appropriate primers. Sequences received were aligned using ExPASy alignment tool. All 34 clones contained the correct V_HH CDR3 sequence previously identified through NGS analysis.

5.2.4 Screening of isolated clones

From *in silico* analysis of NGS data, binders with potential specificity to all seven species of *Eimeria* were identified, these were successfully synthesised and confirmed via Sanger sequencing. The binding properties of all 34 clones were then assessed via ELISA to identify actual binders and their specificity to target species.

All 34 clones raised against the seven *Eimeria* species that were synthesized successfully were screened, with some assays repeated and, in all cases, signals remained at background levels (data not shown), no positive binder was identified. Representative data is shown here, individual phage preparations from three clones; AKSSPQPMTVSLACqP (raised against *E. mitis*) AqSHAqCRLSNKELKR (raised against *E. mitis*) WaqYSKPPSTYVYYLGKRYLI (raised against *E. necatrix*) were prepared as well as monoclonal V_HH, anti-elastin phage and then screened on Nunc Maxisorp® plates using a rabbit anti-fd and goat anti-rabbit AP antibodies. Absorbance signals produced were comparable to no antigen control after 1 hour of adding pNPP substrate (~0.1-0.2) while the positive control (phage antibody specific for elastin) produced an absorbance signal of 0.76 (Figure 5.15). Clones YHYNRLYNSqSSAVEV (*E. maxima*), YADqYASKSFKL (*E. maxima*) and LCLNRSqLTVNQ (*E. maxima*) were screened in a similar manner as described above and signals produced including no antigen control remained at background levels while the positive control phage was at ~ 1.4 (Figure 5.16). Clones PHLKFSCFPCFCQHHGS (*E. acervulina*), YNRLQRTGNATT (*E. brunetti*) and HSQLCKVPCVTRPRNY (*E. brunetti*) were also screened and signals produced were also comparable to the no antigen control while the positive control phage produced at least 4X background signals (Figure 5.17).

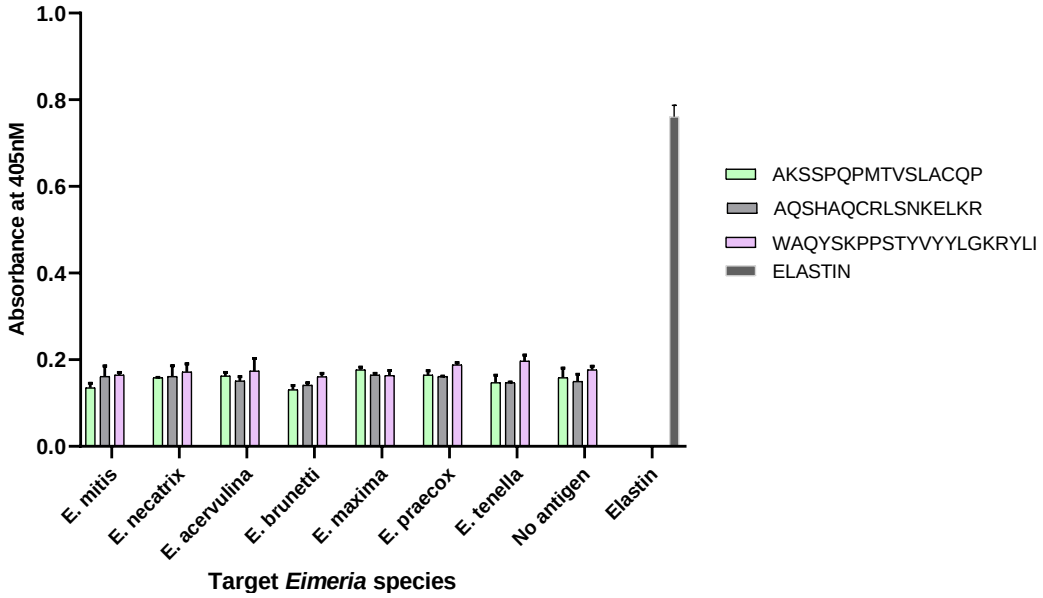


Figure 5:15: Monoclonal V_HH phage ELISA for *Eimeria* oocyst homogenates. V_HH phage were produced for each test clone and the anti-elastin positive control. Oocysts homogenate or elastin was immobilised overnight in 100 µl aliquots at 50 µg/ml on a Nunc MaxiSorp® plate. Monoclonal V_HH phage were used to detect the immobilised antigens and those remaining after the wash steps were detected by a rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation in the well before absorbance was read at 405 nm

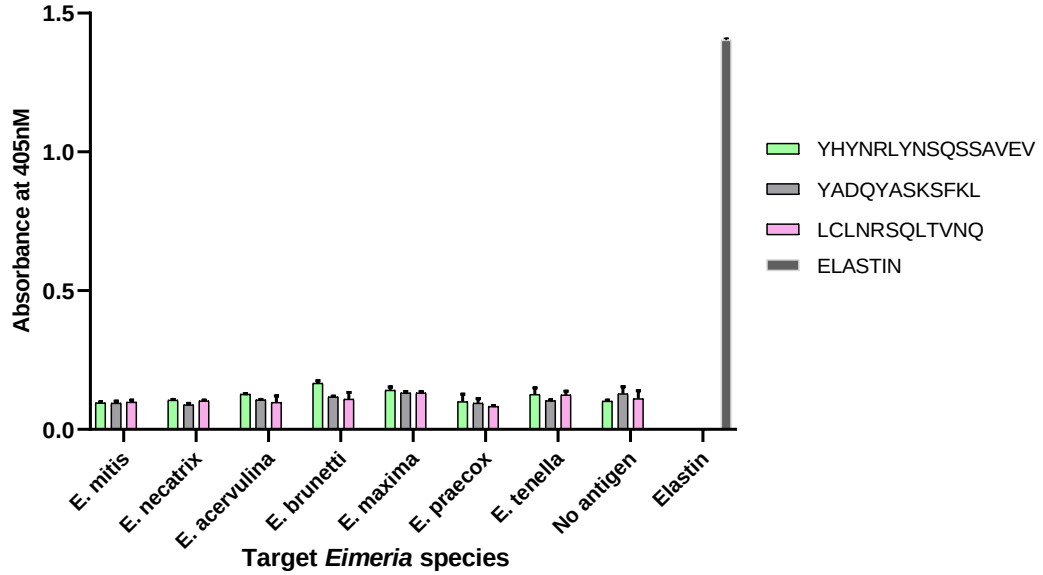


Figure 5:16: Monoclonal phage ELISA for *Eimeria* oocyst homogenates. V_HH phage were produced for each test clone and the anti-elastin positive control. Oocysts homogenate or elastin was immobilised overnight in 100 µl aliquots at 50 µg/ml on a Nunc MaxiSorp® plate. Monoclonal V_HH phage were used to detect the immobilised antigens and those remaining after the wash steps were detected by a rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation in the well before absorbance was read at 405 nm

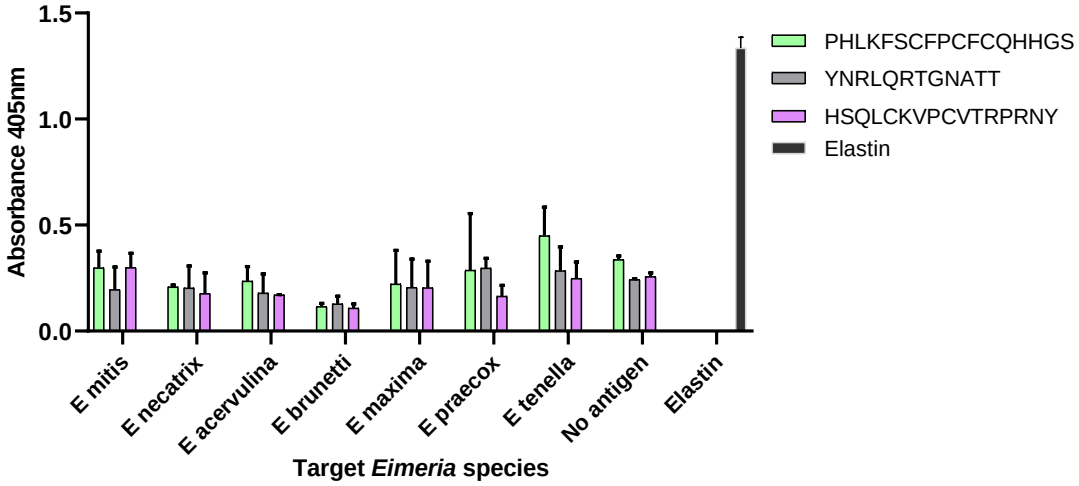


Figure 5:17: Monoclonal phage ELISA for *Eimeria* oocyst homogenates. V_HH phage were produced for each test clone and the anti-elastin positive control. Oocysts homogenate or elastin was immobilised overnight in 100 µl aliquots at 50 µg/ml on a Nunc MaxiSorp® plate. Monoclonal V_HH phage were used to detect the immobilised antigens and those remaining after the wash steps were detected by a rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation in the well before absorbance was read at 405 nm

5.3 Discussion

This chapter set out to identify and isolate V_HH single domain antibodies specific to each of the seven species of *Eimeria.* The approach targeted oocyst homogenates, and coupled conventional phage display and NGPD for screening antibody binding. Chapter 3 reported the effective use of these screening approaches to isolate antibodies to whole *Eimeria* oocysts and chapter 4 detailed the development of a diagnostic assays for the isolated antibodies.

The present chapter had the potential to improve on the assay formats, a spin down ELISA or fluorescent microscopy of whole oocysts, by using homogenised oocysts as the target in panning. The homogenates, because of their high protein contents were easily immobilized on 96 well plates and therefore represent a high throughout assay format. This study previously described many failed attempts at immobilizing whole oocysts onto solid supports to allow a high throughout assay format (**chapters 3 and 4)**.

Prior to panning, oocysts of the seven species were sonicated and total protein yields of the crude extract were quantified using Bradford's protein assay before they were diluted. A V_HH camelid library was then panned against six replicate wells of homogenised oocysts using 5 µg per well.

This chapter looked to improve upon the NGPD strategy conducted in **chapter 3** to isolate species-specific antibodies. The number of panning rounds were reduced from five panning rounds to three exploiting the potentially higher diversity of clones at round 3 for colony picking and NGS analysis. This was because in the first panning approach in **chapter 3**, there was a 90% clonal dominance by the 5th round of panning using the scFv library. Several studies have shown that even with one or two rounds of panning, phage fusions which are highly specific can be identified and isolated. (AC't Hoen *et al*., 2012) demonstrated that after just 1 round of panning, high affinity peptide binders can be identified.

Similarly, in the study by (Rentero Rebollo *et al*., 2014) a rare target binder peptide was identified after just one round of selection. (Miyazaki *et al*., 2015) using an immune V_HH library also isolated specific binders to target just after one panning round. Importantly, with the reduced number of panning rounds, the bias towards parasitic phage that have just growth advantages and not necessarily any binding activity is reduced. In this approach, unlike **chapter 3**, output bacterial phage pools each targeting one of the seven *Eimeria* species were grown individually and not pooled together before further rounds of panning which was also intended to maximise the isolation and identification of species-specific candidates. Subtraction

steps were also included in the panning strategy used in this chapter and included a pre-panning subtraction in the 2nd panning round and both a pre-panning and post-panning subtraction of phage in the 3rd panning round. Subtraction was performed against the six non-target species before incubating the phage with the target species the phage was isolated against. This was aimed at isolating truly species-specific antibodies. Subtractive panning has been used to eliminate the isolation of phage antibodies that are not specific to a particular target by allowing the interaction of the phage to non-targets. Such interactions with non-targets reduces the unspecific phage enriching for specific phage particles. (Zhang *et al*., 2014), using a subtractive panning strategy, isolated specific scFvs that bound to oesophageal cancer cells after using normal human oesophageal epithelial cells as a non-target control to subtract over three rounds of selection. (Sasso *et al*., 2015) using an scFv library against Claudin-1, a protein involved in hepatitis C virus infection expressed on hepatocytes, isolated specific binders after two negative selection steps before incubating the non-binding supernatant to positive samples.

Screening rounds using round 3 output phage included positive samples produced by binding to each phage sub-library versus the *Eimeria* species it was raised against (six repeats), and negative samples where the same phage sub-library was bound to the other six *Eimeria* species. Screening rounds were meant to aid the direct comparison of negative samples and positive samples during NGS analysis of data enabling the identification of clones that appeared to be species-specific binders. The approach combined stringent subtractive steps both during panning and during *in silico* clone selection which were employed in this approach to remove cross-reactive binders and also those that were binding to blocker or plastic support.

Polyclonal phage ELISA was conducted using the original V_HH library as a negative control and the amplified phage of the panning rounds as positive control. Polyclonal phage ELISA did not demonstrate any binding activity to oocyst homogenates in experiments targeting any nof the 7 *Eimeria* species. Initial monoclonal phage ELISA also did not demonstrate any binding activity to the target even when 96 colonies were picked for each target. Relatively few clones had 2X background signals but they were those that were close to the edges of each plate with one exception, which could be attributed to edge effect. Repeat analysis of the clones did not reveal any binding activity to oocyst homogenates. Colony picking has been proven to be an effective way of isolating enriched binders within a sub-library and most phage display studies report succesfully identifying binders using colony picks (Ravn et al., 2010b, Yang et al., 2017). (Miyazaki *et al*., 2015) using an immunised V_HH library, isolated 13 antigen specific binders through colony picking. In the previous chapter, scFv

binders were isolated using colony picks even with just 10 picks per target species. One of the limitations of colony picking is that realistically, only a few hundred colonies are picked out of which many will be duplicates of the same clone; this was observed in the previous chapter with scFvs. In this study, the stringent subtraction steps that were conducted may have led to a few phage remaining after subtraction steps thereby making it unlikely for actual binders to be isolated for each target despite picking 96 clones per target.

The analysis involved the sequencing of sub-libraries from the screening rounds. The use of Z scores was employed to rank CDR3s in terms of their specific enrichment against the target species of *Eimeria.*

Previously within this study when using an scFv library, the bioinformatics analysis compared growth and selection phases as well as comparison of each species versus the other six. This led to the isolation of binders to *Eimeria* oocysts but they bound to multiple species. The bioinformatics analysis in the present chapter was more geared towards finding species-specific clones by directly analysing the positive replicates in each species to the pool of the negative samples generating Z scores (Zhang *et al.,* 2011) . The analysis was aimed at finding binders that were truly enriched since the comparison was against a single replicate of positive samples versus the entire pool of negative samples which meant that samples that were sufficiently enriched in one replicate versus an entire pool representing binding to all 6 non-target species were isolated.

In the present chapter, CDR3s were selected based on those that had a Z score of above 3 and had appeared in at least 4 positive replicates which aimed at ensuring they were true binders to the target *Eimeria* species. In addition to selecting CDR3s that appeared in at least 4 positive replicates and had Z score of >3, the stringency of the search was also increased by searching data sets for each CDR3 selected and once it is seen as being enriched in other species, suggesting it was not species-specific, it was removed. Except for *E. maxima* and *E. tenella,* in all other species none of the CDR3s had a Z score of above 3 and were present in all positive replicates. Even so, some CDR3s present in all positive replicates were found enriched in other species and therefore not selected.

The V_HH library used in this study was randomised in the CDR3 region whilst the sequences in the surrounding areas were conserved. This allowed the rapid synthesis of candidates that were selected from the bioinformatics directly into the phagemid. Theoretically, any target clone identified using this system could also be easily directly synthesized into any expression vector that had a V_HH clone present within it. However, (Ravn et al., 2010b) in their study isolated six scFvs in phage fusion format,

bacterial supernatants and periplasmic extracts. They found out that all six scFvs bound to their target antigens as phage fusions but only the candidates obtained from colony picking bound to their target as soluble antibodies. Here therefore, the target clones were screened in the same phage display format as used in the panning to maximise the chances of identifying species-specific binders. In this study, 34 of 35 candidates identified for further characterisation were successfully synthesized and confirmed via Sanger sequencing. This is a 97% success rate and is a huge improvement from the strategy used in **chapter 3** for scFv rescue of target clones of low abundance from polyclonal phage pools where other non-target clones can interfere with amplification. For scFvs, most of the candidates were not rescued as intended despite repeated attempts. All 34 V_HH's that were synthesised failed to produce positive ELISA signals against oocyst homogenate. This may be that clones selected were not true binders and the subtractive steps and subtractive *in silico* steps may be too stringent to allow the selection of binders. It could also be that those identified may be random events and true binders may have had much higher and more consistent Z scores. The need to use alternative selection process to identify binders may be another possibility.

It could also be that the selected candidates may bind but with very low affinity and/or to low abundance epitopes and the sequencing selection is more sensitive method for detecting binding events than the ELISA.

The bioinformatics strategy in the present chapter may have been improved by sequencing each of the panning rounds for input and output phage in addition to the screening round. This would have afforded the opportunity to perform analysis of the selection and growth phases as was performed in chapter 3 with the scFv, this analysis would have revealed enrichment patterns across panning rounds. The strategy may have also been improved by sequencing the base library and comparing it to the selection rounds to determine enrichment of clones. (Miyazaki *et al*., 2015) compared the frequencies of sequences before and after panning an immune V_HH library against a transmembrane protein IZUMO1, and found the sequences that had increased frequencies after panning showed binding activity to the target.

Alternative assays could have been used to screen candidate V_HHs that may have been more sensitive to detect even low affinity binders to the target, or binders to low abundant epitopes. It has been reported that high sensitivity screening methods of selected phage-displayed antibody clones can increase the number of antigen binding clones that can be isolated (Emanuel *et al.,* 2000). Various assays have been used to functionally test recombinant antibodies aside from conventional ELISA based methods some of which include microfluidics (Hung *et al.,* 2016), microarray

(Pavlickova *et al.,* 2004), fluorescent bead-based (Luminex) assays (Shin *et al.,* 2016) surface plasmon resonance (SPR) (Lebani *et al.,* 2017) (as mentioned in **chapter 4**).

A few NGPD studies have coupled ELISA with surface plasmon resonance (SPR) for the functional screening of isolated antibodies. (Turner *et al*., 2016) after sequencing an immune V_HH library of staphylococcal enterotoxin B, interrogated the library using sequences of known SEB binding V_HH antibodies from a library obtained through phage panning from a previous study and identified additional V_HH candidates. These V_HH candidates were tested by surface plasmon resonance (SPR) and were shown to be comparable to V_HHs obtained through conventional biopanning methods having similar affinities and melting temperatures. In the study by (Miyazaki *et al*., 2015), ELISA and SPR were employed to screen V_HH candidates panned against $IZUMO1_{PFF}$; a transmembrane protein. Twenty-one V_HH single clones were screened via ELISA and 11 were found to be specific to the target and two which were most abundant were shown in a BIAcore 2000 to have moderate binding affinities. (Ngubane *et al*., 2013) employed the use of SPR to test whether an identified highly enriched peptide against *Mycobacterium tuberculosis* (M. tb) bound to its ligand when not displayed by carrier phage. The peptide was synthesised with biotinylation modification, it was immobilized on a streptavidin sensor chip and tested against whole cell lysates from M. tb demonstrating that the peptide bound to the whole cell lysates.

While in these studies both assays have been used to complement each other and the sensitivities of both assays were not directly compared against each other, a study by (Lofgren *et al*., 2007) compared the sensitivities of ELISA and SPR with a monoclonal antibody; panitumumab and found that the ELISA was more sensitive for the detection of higher affinity mAbs. The SPR assay was more sensitive for detection of lower affinity mAbs. This assay may be used in future studies to test the binding affinities of candidate V_HHs to crude lysates of *Eimeria* oocyst.

It has been reported that when using a subtractive panning strategy, high affinity pan-reactive antibodies would be depleted in negative selection while moderate to low affinity pan-reactive antibodies may be inadvertently isolated during positive selection (Lebani, 2015).

Here, using homogenates meant that the targets are not well defined as crude lysates will contain a variety of different proteins, lipids and carbohydrates any any differences between species may be in relatively low abundance.

The bioinformatics approach used in this study was not successful in isolating binders to oocyst homogenates. It may be that the crude oocyst homogenates do not present

antigens that are species-specific and relatively abundant and so present appropriate targets for the phage display panning. The next chapter aimed to take a more defined approach by conducting NGPD against distinct oocyst wall peptides that have species-specific sequences and can be presented as panning targets in relatively high concentrations.

6. Isolation of single domain Antibodies (V_HH) against distinct peptide regions of the 56 kDa gametocytes antigen (Gam56) in *Eimeria* species of poultry.

6.1 Introduction

This chapter aimed to isolate antibodies to distinct peptide regions of the 56 kDa gametocyte antigen (Gam56) in *Eimeria* species of poultry. The target, Gam56 is a protein produced during the gametocyte stage of coccidiosis infection and is located in the wall-forming bodies of the microgametocyte where it plays an active role in oocyst wall biogenesis. The protein is located in the inner wall of the oocysts.

In the life cycle of *Eimeria*, upon fertilization of the macrogametes and microgametes to form the oocysts, Gam56 along with another protein Gam82 are present in the wall forming bodies of the macrogametocytes and are released. They are then broken down into amino acids and cross-linked oxidatively via their tyrosine residues to form the dehydrated, hardened, impervious and environmentally resistant oocyst wall (Xu *et al.,* 2013, Sharman *et al.,* 2010).

The same highly selective method of phage display was used to approach that was used in the previous chapter was applied here to target Gam56 peptides. As in the previous chapter, the present chapter used the V_HH camelid library based on a pSD3 bacteriophage display vector (Li *et al.,* 1999) expressing pIII-V_HH fusions. This was performed to isolate binders to unique regions of the Gam56 present on the oocysts of individual *Eimeria* species. In **chapter 3**, using the same highly selective methods of phage display coupled with NGPD, antibodies were isolated against whole oocysts by conducting phage panning in five panning rounds with no subtraction. Antibodies isolated either bound to all species of oocysts or a few species but the approach was limited by the possible assay format when using whole oocysts. This was phage spin down ELISA or phage fluorescent microscopy. It was sought to improve the assay format and the specificity of isolated antibodies in the next chapter by panning with stringent subtraction steps using oocyst homogenates which had the potentially to improve the assay to a higher throughput format and the subtraction steps had the potential of aiding in the selection of species-specific antibodies. Following the inability to isolate species-specific antibodies by that approach. This chapter sought to improve the target by using well defined and distinct regions of the 56 kDa gametocyte antigen for all species of oocysts which maybe in high abundance compared to the epitopes that may be present in oocyst homogenates. The target has been well described in the literature and it is one of two major proteins that are involved in oocyst wall formation (Sharman *et al.,* 2010). It would be diufficult to apply the final assay format in the field as the target antigen is not on the outer oocyst wall but the assay could target oocyst homogenates on ELISAs, denaturing western or dot blots.

6.2 Results

6.2.1 V_HH phage display; panning for binders to distinct peptide regions of *Eimeria* oocyst wall

6.2.1.1 Target selection

The target; 56 kDa gametocyte antigen was selected for this study because it is one of two immunodominant gametocyte antigens of *Eimeria* species involved in the formation of the oocyst wall. It has also been biochemically characterised, the functional genomics and its role in oocyst wall formation are well described and understood (Belli *et al.,* 2002b, Belli *et al.,* 2002a). Also, in the gene databases, the protein sequences of the Gam56 gametocyte is more complete than the other major protein involved in oocyst wall formation which is Gam82. The Gam82 gametocyte antigen is missing sequences for *E. praecox* hence was not used as a target here. In addition, sufficient regions of diversity; 2 to 6 completely unique amino acid differences were found in the sequences of the Gam56 gametocyte antigen within limited regions of the protein, hence could be used as peptide targets for antibody production.

6.2.1.2 Peptide design and synthesis

Amino acid sequences of the 56 kDa gametocyte antigen of each of the seven *Eimeria* species were aligned and compared to each other and areas of at 2 to 6 unique amino acid residues were identified and synthesized. For all species except *E. necatrix* and *E. tenella*, this was the case in the region between amino acids residues 18 to 34 (Figure 6.1). This peptide from all 7 species was synthesised as a target for panning. *Eimeria necatrix* and *E. tenella* had only one amino acid change in this region between each other and all individual residues were present in at least one of the other five species. Therefore, for these two species, peptides were also synthesized from an alternative region between the 411 to 424 amino acid residues. This region contained 6 amino acid difference between the two species (Figure 6.1). Peptides used as targets for antibody isolation are listed with their accession numbers (Table 6.1), they were synthesised with biotinylation of the N terminal and amidation of the C terminal.

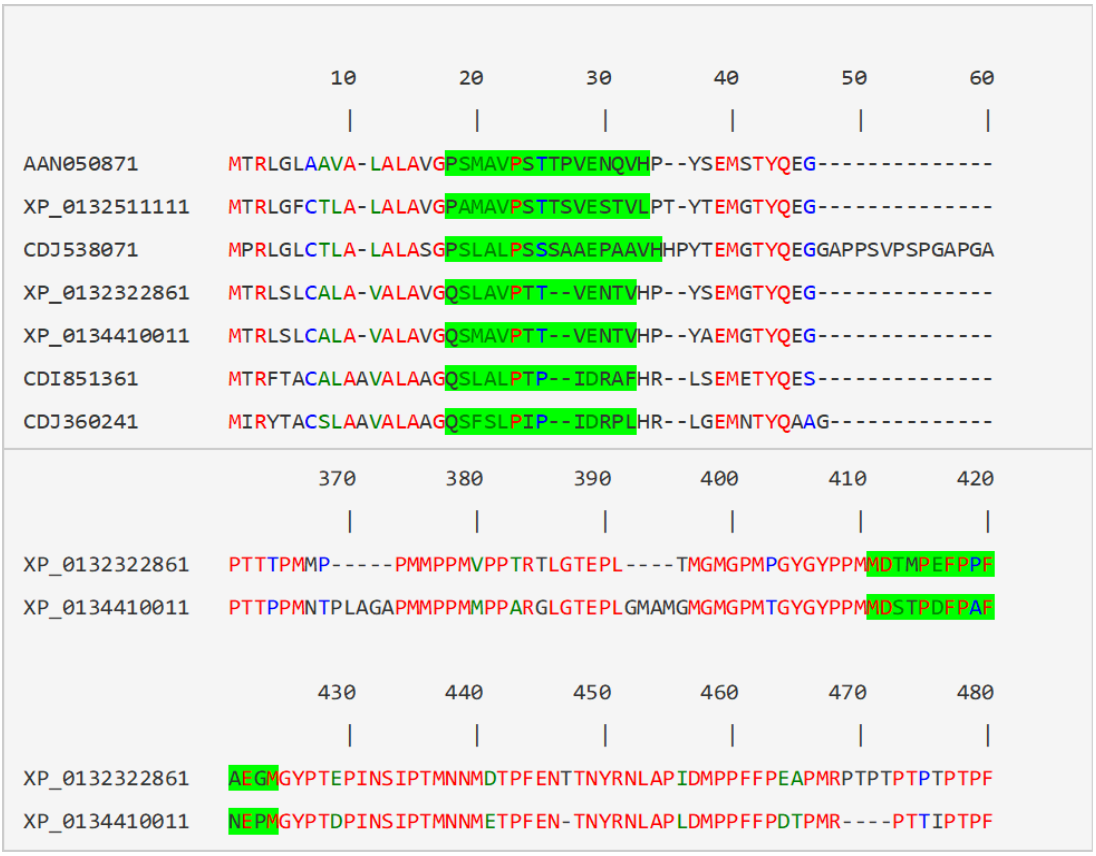


Figure 6:1: Amino acid sequences of regions within the 56 kDa gametocyte antigen of the different *Eimeria* species. PSMAVPSTTPVENQVH (*E. maxima*), PAMAVPSTTSVESTVL (*E. acervulina*), PSLALPSSSAAEPAAVH (*E. brunetti*), QSLAVPTTVENTV (*E. tenella*), QSMAVPTTVENTV (*E. necatrix*), QSLALPTPIDRAF (*E. praecox*), QSFSLPIPIDRPL (*E. mitis*), MDTMPEFPPFAEGM (*E. tenella*), MDSTPDFPAFNEPM (*E. necatrix*). Areas of high variability are shown between residues 18 to 34. The 56 kDa gametocyte antigen for *Eimeria necatrix* and *Eimeria tenella* only had a single amino acid difference in the region 18 to 34 amino acids. As an alternative approach for these species, the 56 kDa gametocyte antigen was also targeted in regions 411 to 424 that contained 6 amino acid difference. Areas highlighted green represent the peptides synthesised.

Table 6:1: Accession numbers and peptide sequences for the target peptides from the 56 kDa gametocyte protein of *Eimeria*

Accession number	Species	Selected peptide sequence
CDJ36024.1	*Eimeria mitis*	QSFSLPIPIDRPL
XP_013441001.1	*Eimeria necatrix*[1]	QSMAVPTTVENTV
XP_013441001.1	*Eimeria necatrix* [2]	MDSTPDFPAFNEPM
XP_013251111.1	*Eimeria acervulina*	PAMAVPSTTSVESTVL
CDJ53807.1	*Eimeria brunetti*	PSLALPSSSAAEPAAVH
AAN05087.1	*Eimeria maxima*	PSMAVPSTTPVENQVH
CDI85136.1	*Eimeria praecox*	QSLALPTPIDRAF
XP_013232286.1	*Eimeria tenella*[1]	QSLAVPTTVENTV
XP_013232286.1	*Eimeria tenella* [2]	MDTMPEFPPFAEGM

6.2.1.3 Preparation and titre of phage

The V_HH phage prepared for panning experiments propagated as 12mer, 16mer and 21mer when titred had values of 10^{12} PFU/ml after PEG precipitation. Supernatant phage was used for the other rounds of panning as well as the screens (as was conducted in **chapter 5)** and titres were around 10^{11} PFU/ml. These tires are as expected when using exphage as the helper phage (Baek *et al.,* 2002).

6.2.1.4 Panning rounds

The panning strategy using the camelid V_HH library for peptide antigens were the same as that described for **chapter 5** with the exception that immobilisation of antigen was performed on streptavidin coated plates. Phage selection was performed against 9 distinct peptides; seven of which are related peptides while the other two were related to each other but different from the other seven. As was conducted in the previous chapter **(chapter 5**), the panning strategy involved three rounds of panning and the steps involved are summarised in Figure 6.2. A pre-panning subtraction step was performed in the second panning round and a pre-panning and post-panning subtraction of phage were performed in the 3rd panning round. Subtraction in this chapter was performed by subtracting phage against related peptides from the other *Eimeria* species (either 6 different versions for region 18-34 or a single alternative for region 411-424) before incubating the phage with the target peptide. Eluted phage titre values decreased with progressive rounds of panning for all targets and values were 2.3×10^3 to 1.2×10^5 (Figure 6.2).

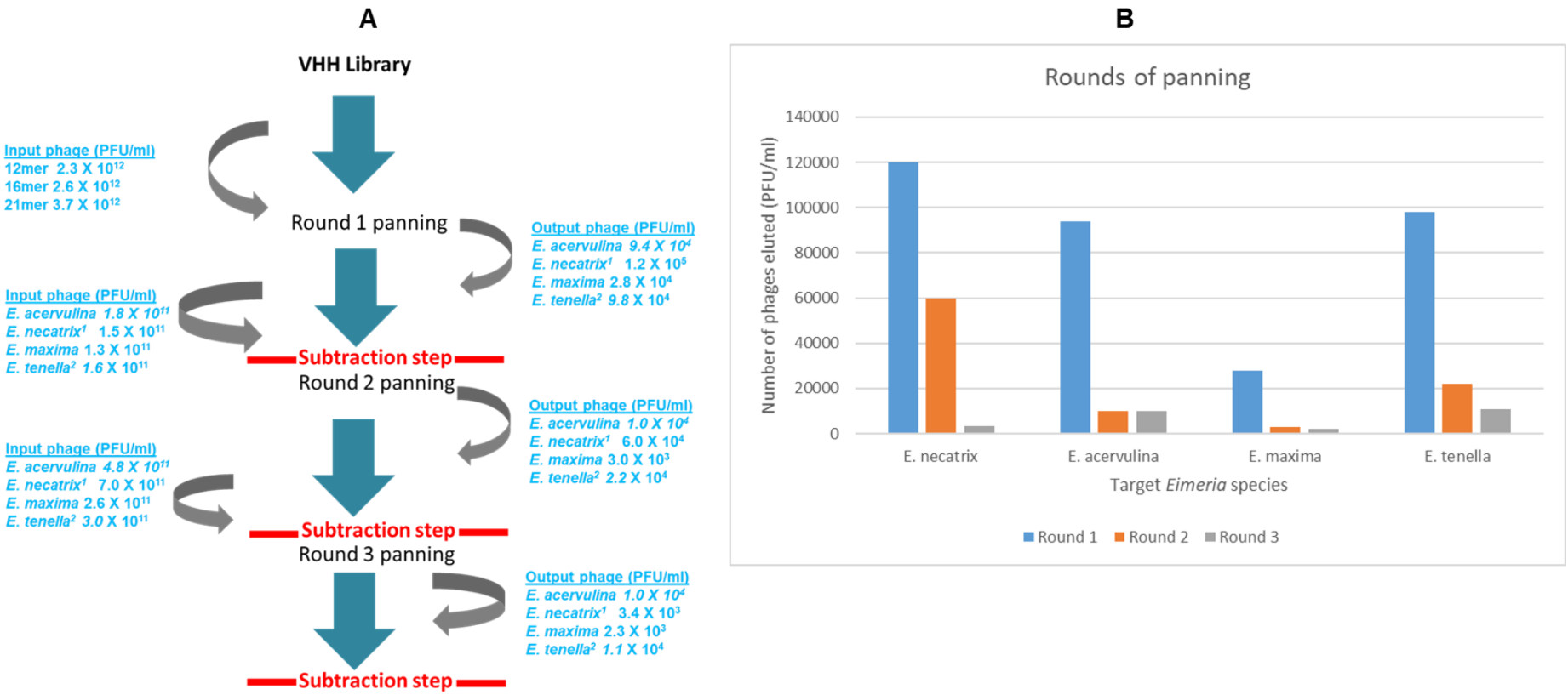


Figure 6:2: Schematic representation of the panning strategy (A) used with the V_HH library against synthetic peptides and number of eluted phage after rounds of panning (B). Three rounds of panning were performed for nine *Eimeria* target peptide antigens with no subtraction step in the first panning round, a pre-panning subtractive step in the 2nd round of panning and a pre-panning subtractive step as well as a post-panning subtraction in the 3rd panning round. Representative input and output phage titres are shown at each step (A) as well as graphical representation of eluted phage after each panning round for selected panning experiments (B) (PFU/ml).

6.2.1.5 Preparation for Ion proton sequencing: Phage reinfection and DNA extractions

V_HH phage library was used for panning experiments to select for antibodies against distinct oocysts wall peptides of *Eimeria* species. Screening rounds were conducted from the round 3 bacterial stock against 9 distinct peptides and a total of 108 samples were generated from each peptide having 6 replicate positive samples and 6 replicate negative controls (refer to chapter 2, figure 2.9). DNA was extracted from the bacterial stocks of all samples for use as template for HCDR3 amplifications. The step 1 and 2 PCR reactions produced ~300 bp and ~350bp amplicons respectively. The PCR products were checked on agarose gels and representative samples are shown for step 1 (Figure 6.3) and step 2 (Figure 6. 4). The 108 samples were tagged with barcodes 1-96 and barcode 1-13 (Table 6.2; barcode 42 was excluded). The samples were quantified using fluorometry (data not shown) and equal concentrations of each sample were pooled together within two different NGS runs; 95 barcoded samples in one run (excluding barcode 42) and 13 samples in the second run. The first pool comprising of 95 samples had a total 9500 ng with equal contribution from each sample while the second pool had 1300 ng equal contribution from each sample. The samples were further purified twice using Agencourt®AMPure®XP PCR purification kit and the two pools had a final concentration of 47 ng/µl and 12 ng/µl, respectively. A 200 ng aliquot of each of the pooled DNA samples were sent for Ion Torrent sequencing at the University of Pennsylvania.

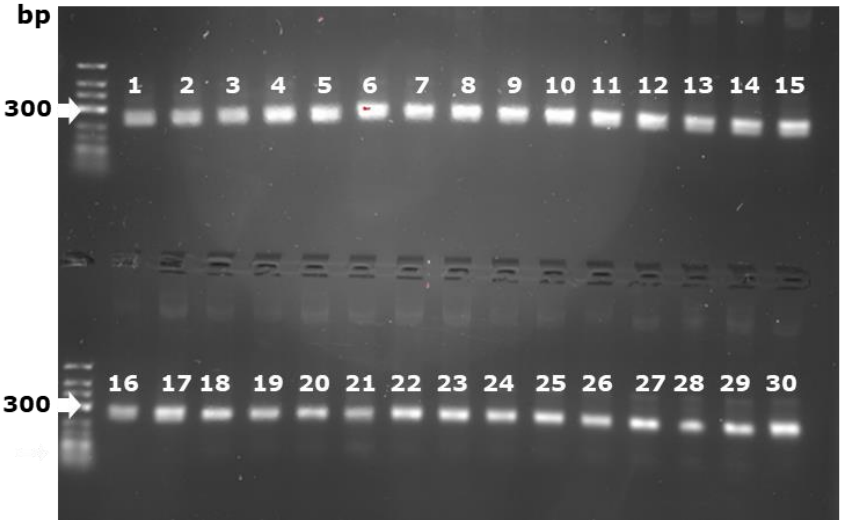


Figure 6:3: An example of the first step PCR products for NGPD. 10 µl of amplified PCR products were mixed with 2 µl of DNA loading dye and run on a on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Samples labelled as 1-6 represent the target peptide QSFSLPIPIDRPL six positive screening

replicates, samples 7-12 represent screening of binders to the target peptide QSFSLPIPIDRPL versus the other six related peptides as negative controls; QSMAVPTTVENTV, PAMAVPSTTSVESTVL, PSLALPSSSAAEPAAVH, PSMAVPSTTPVENQVH, QSLALPTPIDRAF and QSLAVPTTVENTV. Samples 13-18 represent target peptide QSMAVPTTVENTV positive replicates while samples 19-24 represent screening of target peptide QSMAVPTTVENTV against the other 6 related peptides; QSFSLPIPIDRPL, PAMAVPSTTSVESTVL, PSLALPSSSAAEPAAVH, PSMAVPSTTPVENQVH, QSLALPTPIDRAF and QSLAVPTTVENTV and samples 25-30 represent target peptide PAMAVPSTTSVESTVL positive replicates. Molecular markers are indicated in bp. The round 1 PCR products were then purified and used as a template for round 2 PCR.

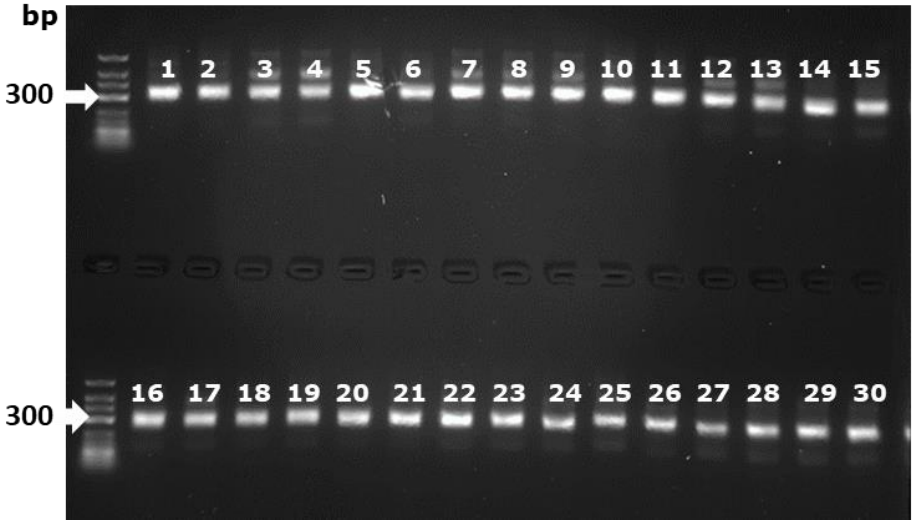


Figure 6:4: An example of second step PCR products for NGPD. 10 µl of amplified PCR products were mixed with 2 µl of DNA loading dye and run on a on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). Samples were labelled as 1-6 representing the target peptide QSFSLPIPIDRPL six positive screening replicates, samples 7-12 represent screening of the target peptide QSFSLPIPIDRPL versus the other six related peptides as negative controls; QSMAVPTTVENTV, PAMAVPSTTSVESTVL, PSLALPSSSAAEPAAVH, PSMAVPSTTPVENQVH, QSLALPTPIDRAF and QSLAVPTTVENTV. Samples 13-18 represent target peptide QSMAVPTTVENTV positive replicates while samples 19-24 represent screening of target peptide QSMAVPTTVENTV against the other 6 related peptides; QSFSLPIPIDRPL, PAMAVPSTTSVESTVL, PSLALPSSSAAEPAAVH, PSMAVPSTTPVENQVH, QSLALPTPIDRAF and QSLAVPTTVENTV and samples 25-30 represent target peptide PAMAVPSTTSVESTVL positive replicates. Molecular markers are indicated in bp. The round 2 PCR products were then purified and pooled in equal concentrations before gel prepping the PCR amplicons for Ion torrent sequencing.

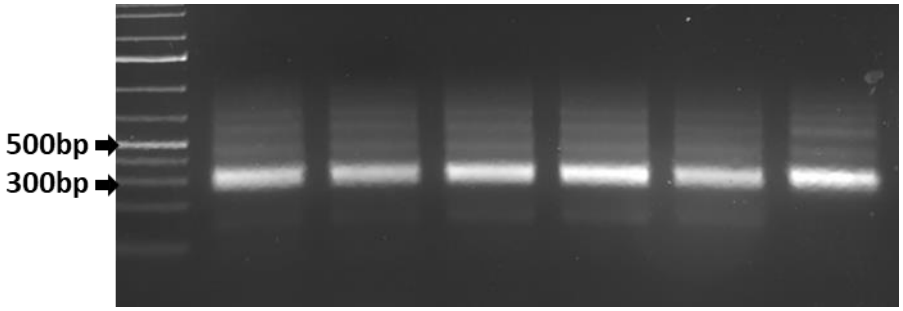


Figure 6:5: Pooled PCR amplicon from phagemid DNA . After the 2nd step PCR amplification, the PCR products were quantified by Qubit 2.0 Fluorometer and the DNA from all 108 barcoded samples were pooled in an equal ratio in 2 separate NGS runs so that the reads will be equalised during NGS. The sample was purified using Agencourt®AMPure®XP and then analysed on a 2% (w/v) Agarose Gel using LR Gene Ladder (SM1193). Nancy (01494) was used to stain the gel and samples were loaded using 6x loading buffer (R0611). This was extracted, purified, and was the template for Ion torrent sequencing

Table 6:2: Summary of samples prepared for next generation sequencing for nine *Eimeria* peptides

Samples summary[1]			
Target peptide	**Peptides that target peptides were screened against**	**Sample No. Range[2]**	**Barcode No. Range[3]**
QSFSLPIPIDRPL positive replicates 1-6	QSFSLPIPIDRPL	1 to 6	1 to 6
QSFSLPIPIDRPL	QSMAVPTTVENTV	7	7
QSFSLPIPIDRPL	PAMAVPSTTSVESTVL	8	8
QSFSLPIPIDRPL	PSLALPSSSAAEPAAVH	9	9
QSFSLPIPIDRPL	PSMAVPSTTPVENQVH	10	10
QSFSLPIPIDRPL	QSLALPTPIDRAF	11	11
QSFSLPIPIDRPL	QSLAVPTTVENTV	12	12
QSMAVPTTVENTV positive replicates 1-6	QSMAVPTTVENTV	13 to 18	13 to 18
QSMAVPTTVENTV	QSFSLPIPIDRPL	19	19
QSMAVPTTVENTV	PAMAVPSTTSVESTVL	20	20
QSMAVPTTVENTV	PSLALPSSSAAEPAAVH	21	21
QSMAVPTTVENTV	PSMAVPSTTPVENQVH	22	22
QSMAVPTTVENTV	QSLALPTPIDRAF	23	23
QSMAVPTTVENTV	QSLAVPTTVENTV	24	24
PAMAVPSTTSVESTVL positive replicates 1-6	PAMAVPSTTSVESTVL	25 to 30	25 to 30
PAMAVPSTTSVESTVL	QSFSLPIPIDRPL	31	31
PAMAVPSTTSVESTVL	QSMAVPTTVENTV	32	32
PAMAVPSTTSVESTVL	PSLALPSSSAAEPAAVH	33	33
PAMAVPSTTSVESTVL	PSMAVPSTTPVENQVH	34	34
PAMAVPSTTSVESTVL	QSLALPTPIDRAF	35	35
PAMAVPSTTSVESTVL	QSLAVPTTVENTV	36	36
PSLALPSSSAAEPAAVH positive replicates 1-6	PSLALPSSSAAEPAAVH	37 to 42	37 to 43 (-#42)
PSLALPSSSAAEPAAVH	QSFSLPIPIDRPL	43	44
PSLALPSSSAAEPAAVH	QSMAVPTTVENTV	44	45
PSLALPSSSAAEPAAVH	PAMAVPSTTSVESTVL	45	46
PSLALPSSSAAEPAAVH	PSMAVPSTTPVENQVH	46	47
PSLALPSSSAAEPAAVH	QSLALPTPIDRAF	47	48
PSLALPSSSAAEPAAVH	QSLAVPTTVENTV	48	49
PSMAVPSTTPVENQVH positive replicates 1-6	PSMAVPSTTPVENQVH	49 to 54	50 to 55
PSMAVPSTTPVENQVH	QSFSLPIPIDRPL	55	56
PSMAVPSTTPVENQVH	QSMAVPTTVENTV	56	57
PSMAVPSTTPVENQVH	PAMAVPSTTSVESTVL	57	58

PSMAVPSTTPVENQVH	PSLALPSSSAAEPAAVH	58	59
PSMAVPSTTPVENQVH	QSLALPTPIDRAF	59	60
PSMAVPSTTPVENQVH	QSLAVPTTVENTV	60	61
QSLALPTPIDRAF positive replicates 1-6	QSLALPTPIDRAF	61 to 66	62 to 67
QSLALPTPIDRAF	QSFSLPIPIDRPL	67	68
QSLALPTPIDRAF	QSMAVPTTVENTV	68	69
QSLALPTPIDRAF	PAMAVPSTTSVESTVL	69	70
QSLALPTPIDRAF	PSLALPSSSAAEPAAVH	70	71
QSLALPTPIDRAF	PSMAVPSTTPVENQVH	71	72
QSLALPTPIDRAF	QSLALPTPIDRAF	72	73
QSLAVPTTVENTV positive replicates 1-6	QSLAVPTTVENTV	73 to 78	74 to 79
QSLAVPTTVENTV	QSFSLPIPIDRPL	79	80
QSLAVPTTVENTV	QSMAVPTTVENTV	80	81
QSLAVPTTVENTV	PAMAVPSTTSVESTVL	81	82
QSLAVPTTVENTV	PSLALPSSSAAEPAAVH	82	83
QSLAVPTTVENTV	PSMAVPSTTPVENQVH	83	84
QSLAVPTTVENTV	QSLALPTPIDRAF	84	85
MDSTPDFPAFNEPM positive replicates 1-6	MDSTPDFPAFNEPM	85- 90	86 to 91
MDSTPDFPAFNEPM negative replicates 1-6	MDTMPEFPPFAEGM	91- 96	92 to 96 and 1
MDTMPEFPPFAEGM positive replicates 1-6	MDTMPEFPPFAEGM	97-102	2 to 7
MDTMPEFPPFAEGM negative replicates 1-6	MDSTPDFPAFNEPM	108	8 to 13

[1] The screening approach. Each peptide is highlighted with a different colour code for ease of identification. The first column represents the target peptides while the second column represents the peptides in which the target peptides were screened against to generate positive or negative control samples. Positive replicates are screened against the same target peptides they were raised against. The seven related peptides were screened amongst themselves while the two related peptides were screened against each other.

[2] Sample numbers assigned to each sample type.

[3] Barcode number assigned to each sample. Blue highlighted samples were included in the first NGS run of 96 samples while green highlighted samples were included in the second NGS run.

6.2.2 Conventional screening of V_HH phage binders

6.2.2.1 Colony picking

Individual colony picking was employed alongside polyclonal ELISA in comparison to the NGS method to help identify positive V_HH binders to individual distinct peptide of *Eimeria* species.

6.2.2.2 Polyclonal phage ELISA

The binding properties of the polyclonal phage was assessed using all target peptides as antigen with phage grown from the initial V_HH library; 12mer, 16mer, 21mer combined (10^{11}-10^{12} PFU/ml) and all three panning rounds for each of the nine peptide targets as well as a monoclonal V_HH, anti-elastin positive control (phage antibody specific for elastin). Target peptides or elastin was immobilised on 96 well Pierce™ Streptavidin coated plates or Nunc Maxisorp® plate respectively. Phage binding was detected using a rabbit anti-fd and goat anti-rabbit AP antibodies. ELISA signals for binding to the 9 target peptides and also a no antigen control 1 hour after adding the pNPP substrate remained at background level for all nine peptides as compared to the no antigen control, at ~0.2-0.3. The positive control screened on Nunc Maxisorp® plates produced an absorbance signal of 1.15 (Figure 6 .6).

The assay was repeated and substrate was incubated in the wells overnight to determine if the signals produced will be higher. When the absorbance was read the next day at 405nm, the signals produced were very high at around ~1-2 and the anti-elastin positive control produced an absorbance signal of ~3.5 (Figure 6 .7). Even though the signal produced by the positive control was 1.5-2X higher than the test samples, the background signals produced were too high to be a valid assay and this assay was repeated.

The polyclonal phage ELISA was repeated with a different detection system; anti-M13-HRP antibody in an attempt to increase sensitivity. The TMB substrate was added and allowed 10 minutes incubation with the well before the absorbance values were taken at 630 nm and signals produced by all peptides were background signals of ~0.2-0.3 while the positive control phage produced signals of ~1.1 (data not shown).

The polyclonal ELISAs performed on Streptavidin coated plates using both detection systems suggested that binders were not enriched giving that there was no increase in the signals produced from the phage library through the subsequent rounds of panning.

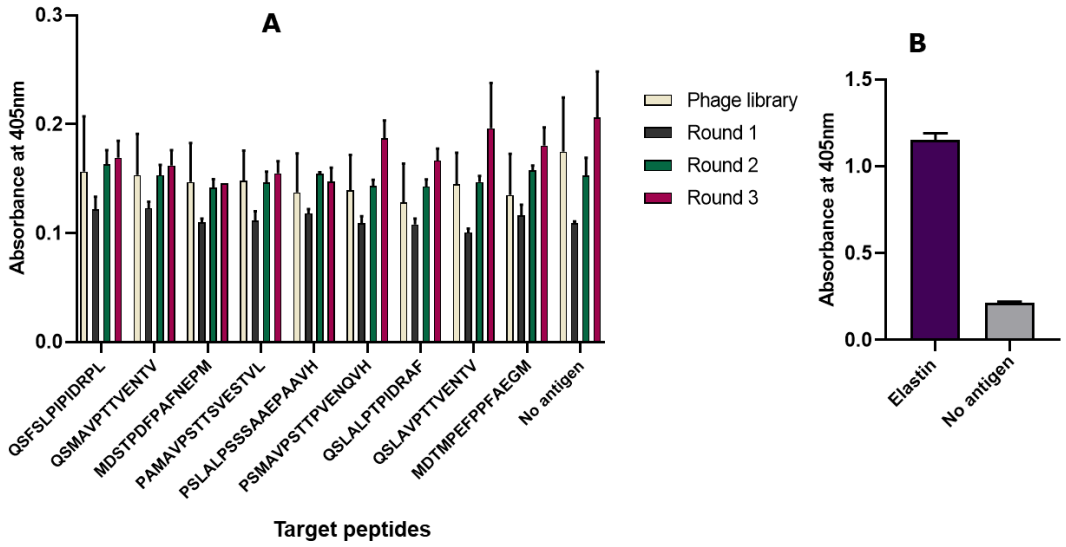


Figure 6:6: Repeat polyclonal phage ELISA for distinct *Eimeria* species specific peptides from Gam56 protein. X axis represents the target peptides used in panning. V_HH phage were produced for each panning round when targeting each of the nine peptides along with the camelid V_HH library and the anti-elastin positive control. Peptides (10 µg/ml, A) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and a Nunc MaxiSorp® plate, respectively. Polyclonal V_HH phage were used to detect the immobilised antigens and those remaining after the wash steps were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation before absorbance was read at 405 nm. (A) represents polyclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

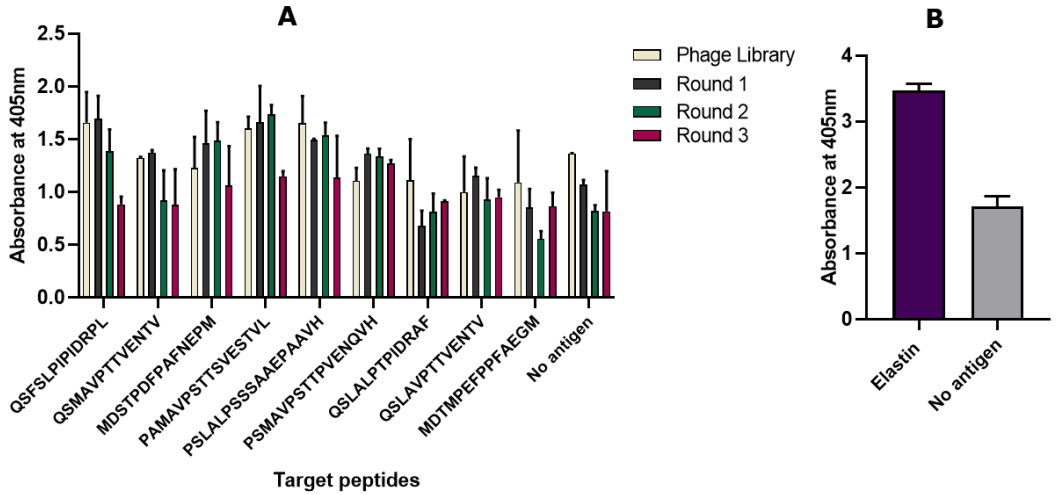


Figure 6:7: Polyclonal phage ELISA for the detection of distinct *Eimeria* species-specific peptides from Gam56 protein. V_HH phage were produced for each panning round when targeting each of the nine peptides along with the camelid V_HH library and the anti-elastin positive control. Peptides (10 µg/ml, A) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and a Nunc MaxiSorp® plate, respectively. Polyclonal V_HH phage were used to detect the immobilised antigens and those remaining after the wash steps were detected

with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed an overnight incubation before absorbance was read at 405 nm. (A) represents polyclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

6.2.1.3 Monoclonal phage ELISA

Ten single colonies were picked for each peptide from the round 3 panning glycerol stocks, totalling 90 clones for the nine distinct peptides. Monoclonal phage preparations were made for each individually picked clone as well as the anti-elastin positive control and the individual V_HH binding properties were assessed by phage ELISA using a rabbit anti-fd and goat anti-rabbit AP antibodies. Absorbance signals were compared against a monoclonal anti-elastin phage-V_HH positive control and signals produced in certain clones; MA 5 (QSMAVPTTVENTV clone 5), ST 9 (MDSTPDFPAFNEPM clone 9), PA 9 (PAMAVPSTTSVESTVL clone 9), LA 8 (PSLALPSSSAAEPAAVH clone 8), SM 2 (PSMAVPSTTPVENQVH clone 2), QS 7 (QSLALPTPIDRAF clone 7), LV 4 (QSLAVPTTVENTV clone 4) and TM 10 (MDTMPEFPPFAEGM clone 10) were >2x background signals after 1 hour of adding the substrate at 0.7, 0.90, 0.81. 0.9, 1.15, 0.86, 0.97 and 0.90 respectively while the positive control produced an absorbance signal of 1.85 (Figure 6.8). This assay was conducted in parallel with an HRP detection system and the background signals were higher for the HRP detection system (data not shown).

The monoclonal phage ELISA was repeated for the clones that produced >2X background signals and targeted distinct *Eimeria* species; MA 5, ST 9, PA 9, LA 8, SM 2, QS 7, LV 4 and TM 10. The clones were re-grown, and fresh phage were produced (10^{11} PFU/ml) and assays again included the anti-elastin positive control. Each phage preparation was tested in triplicates against all 9 peptides as well as the no antigen control. The anti-elastin positive control produced a signal of 1.49 (4X background) (Figure 6.9). All of the clones produced near background level signals of between ~0.2-0.4 when comparing the binding to the target peptides with binding to the 8 control peptides and/or no antigen control (Figure 6.9). Clone QS 7 produced relatively high binding to a single peptide, QSFSLPIPIDRPL. However, QS 7 was produced against a different peptide; QSLALPTPIDRAF to the one the positive signal was produced with. All the other clones were excluded from further screening while QS 7 was re-grown and fresh phage produced (10^{11} PFU/ml) including the anti-elastin positive control. The phage preparation was tested in triplicates against all 9 peptides as well as elastin. Anti-elastin phage was tested against elastin as well as all 9

peptides. Apart from the positive control, all signals produced were at background levels (data not shown). The analysis of the randomly picked clones were not pursued further.

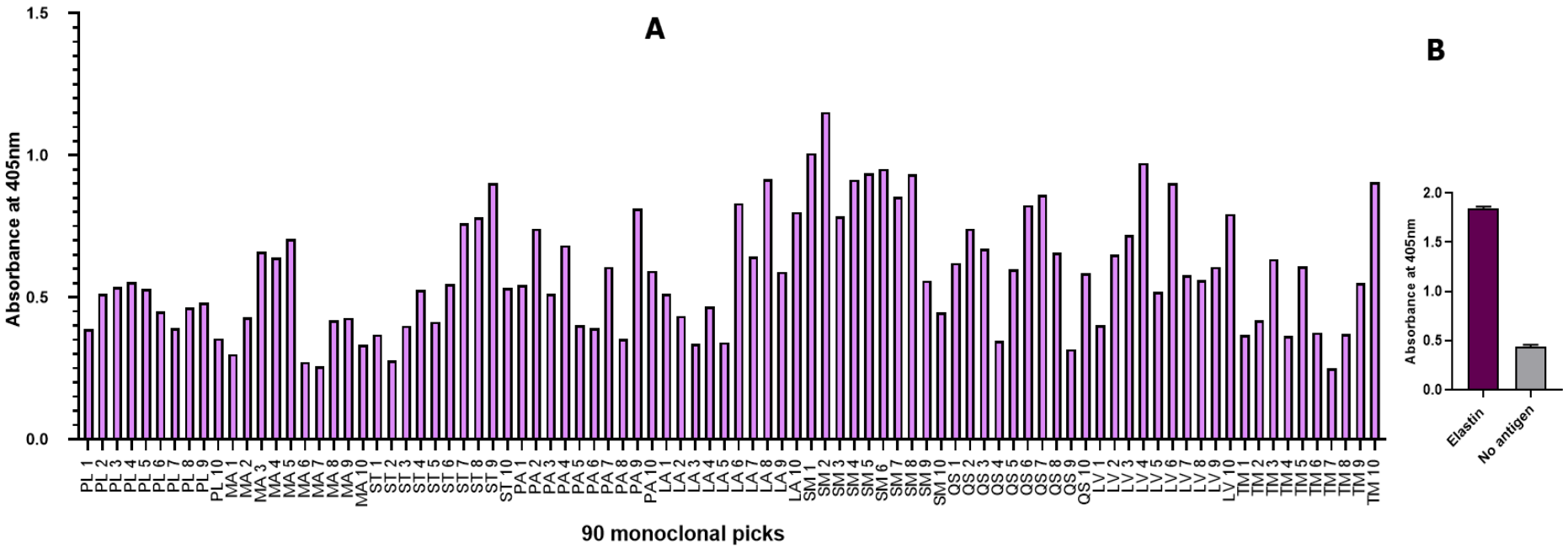


Figure 6:8: The binding of monoclonal phage antibodies to distinct *Eimeria* species-specific peptides from Gam56 protein . V_HH phage were produced from 90 randomly picked colonies from round 3 of panning; 10 from each target peptide after 3 rounds of panning. Clones labelled PL 1-10 represent peptide QSFSLPIPIDRPL 10 monoclonal picks, clones labelled MA 1-10 represent peptide QSMAVPTTVENTV, clones labelled ST 1-10 represent phage targeting peptide MDSTPDFPAFNEPM, clones labelled PA 1-10 represent phage targeting peptide PAMAVPSTTSVESTVL, clones labelled LA 1-10 represent phage targeting peptide PSLALPSSSAAEPAAVH, clones labelled SM 1-10 represent phage targeting peptide PSMAVPSTTPVENQVH, clones labelled QS 1-10 represent phage targeting peptide QSLALPTPIDRAF, clones labelled LV 1-10 represent phage targeting peptide QSLAVPTTVENTV and clones labelled TM 1-10 represent phage targeting peptide MDTMPEFPPFAEGM. Peptides (10 µg/ml, A) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) for each individually picked colony were used to detect immobilised peptides and anti-elastin phage was used to detect immobilised elastin or no-antigen control. Bound phage were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

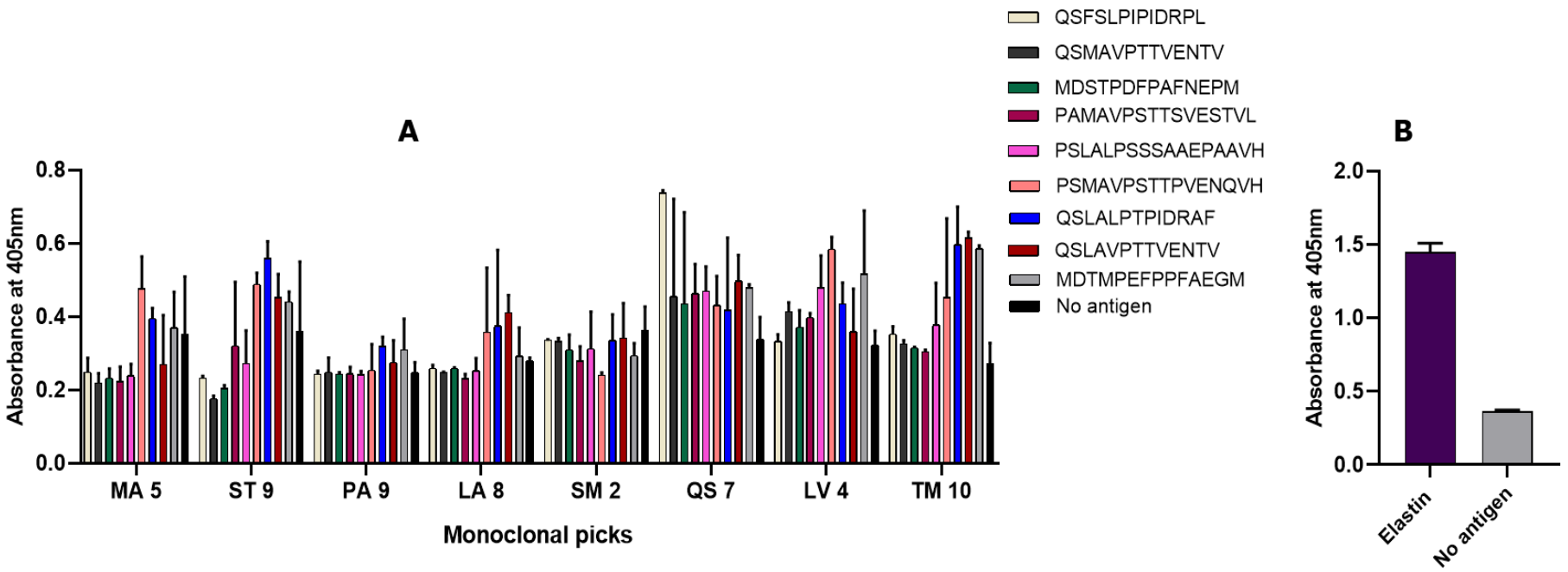


Figure 6:9: Repeat binding of monoclonal phage antibodies to distinct *Eimeria* species-specific peptides from Gam56 protein. V_HH phage were produced for 8 clones that had produced a positive signal from the initial colony pick ELISA. Peptides (10 µg/ml, A) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) for each clone were used to detect immobilised peptides. Bound phage were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed 1-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

6.2.3.1 NGS data analysis; identifying V_HH clones with target specificities

The NGS data was received in a compressed FASTQ file format from the University of Pennsylvania and the data was de-compressed and de-multiplexed (refer to materials and methods **chapter 2** subsection 2.7.8).

The NGS analysis was carried out the same way as in **chapter 5** with the exception that in the analysis of the files generated from the comparison of positive versus negative barcodes, HCDR3 sequences that were present in at least 3 out of the 6 positive replicates were selected, sorted and counted according to the number of times they appeared in the positive barcodes (in chapter 5 HCDR3s that were present in at least 4 positive replicates were selected) (Figure 6.10, Table 6.3). QSFSLPIPIDRPL (peptide unique to *E. mitis* in region 18 to 34 amino acid) had no CDR3 that was present in all six or 5 positive replicates, 3 CDR3s that were present in four positive replicates and 6 CDR3s that were present in three positive replicates. QSMAVPTTVENTV (peptide unique to *E. necatrix* in region 20 to 30 amino acid) had no CDR3 present in all six or 5 out of six positive replicates, 8 CDR3s present in four positive replicates, 21 CDR3s present in three positive replicates. PAMAVPSTTSVESTVL (peptide unique to *E. acervulina* in region 20 to 30 amino acid) had no CDR3 present in all six positive replicates, 1 CDR3 present in five positive replicates, 3 CDR3s present in four positive replicates and 47 CDR3s present in three positive replicates. PSLALPSSSAAEPAAVH (peptide unique to *E. brunetti* in region 20 to 30 amino acid) had 3 CDR3s present in all six positive replicates, 4 CDR3s present in 5 positive replicates, 21 CDR3s present in four positive replicates and 79 CDR3s present in three positive replicates. PSMAVPSTTPVENQVH (peptide unique to *E. maxima* in region 20 to 30 amino acid) had 32 CDR3s present in all six positive replicates, 45 CDR3s present in 5 positive replicates, 45 CDR3s were present in 4 positive replicates and 98 CDR3s were present in 3 positive replicates. QSLALPTPIDRAF (peptide unique to *E. praecox* in region 20 to 30 amino acid) had no CDR3 present in all six of the positive replicates, 5 CDR3s present in five of the positive replicates, 7 CDR3s present in four of the positive replicates and 12 CDR3s present in three of the positive replicates. QSLAVPTTVENTV (peptide unique to *E. tenella* in region 20 to 30 amino acid) had no CDR3 present in all six or five positive replicates, 2 CDR3s present in four of the positive replicates and 41 CDR3s present in three of the positive replicates. MDSTPDFPAFNEPM (peptide unique to *E. necatrix* in region 411 to 424 amino acid) had no CDR3 present in all six replicates, 5 positive replicates or four positive replicates. Only 3 CDR3s were present in three positive replicates hence some peptides that had appeared in only two positive replicates were selected. MDTMPEFPPFAEGM (peptide unique to *E. tenella* in region 411 to

424 amino acid) had no CDR3 present in all six positive replicates, 1 CDR3 present in five positive replicates, 1 CDR3 present in four positive replicates and 130 CDR3s present in three positive replicates. (summarised in Figure 6.11).

6.2.3.2 V_HH synthesis and rescue

As was conducted in **chapter 5**, amino acid sequences for HCDR3 selected from the NGS data were converted to their optimal nucleotide codons (for *E. coli* expression) and phosphorylated primers were designed to synthesise the optimal V_HH sequence in inverse PCR reactions by each covering half of the V_HH CDR3 sequence and 18 nucleotides into the adjacent framework region of the scaffold V_HH. The primer design strategy and inverse PCR was performed exactly as was carried out in chapter 5 and primers are shown in chapter 2 Table 2.9. Forty-three of forty-five V_HH candidates were correctly synthesised and confirmed via Sanger sequencing (Figure 6.12).

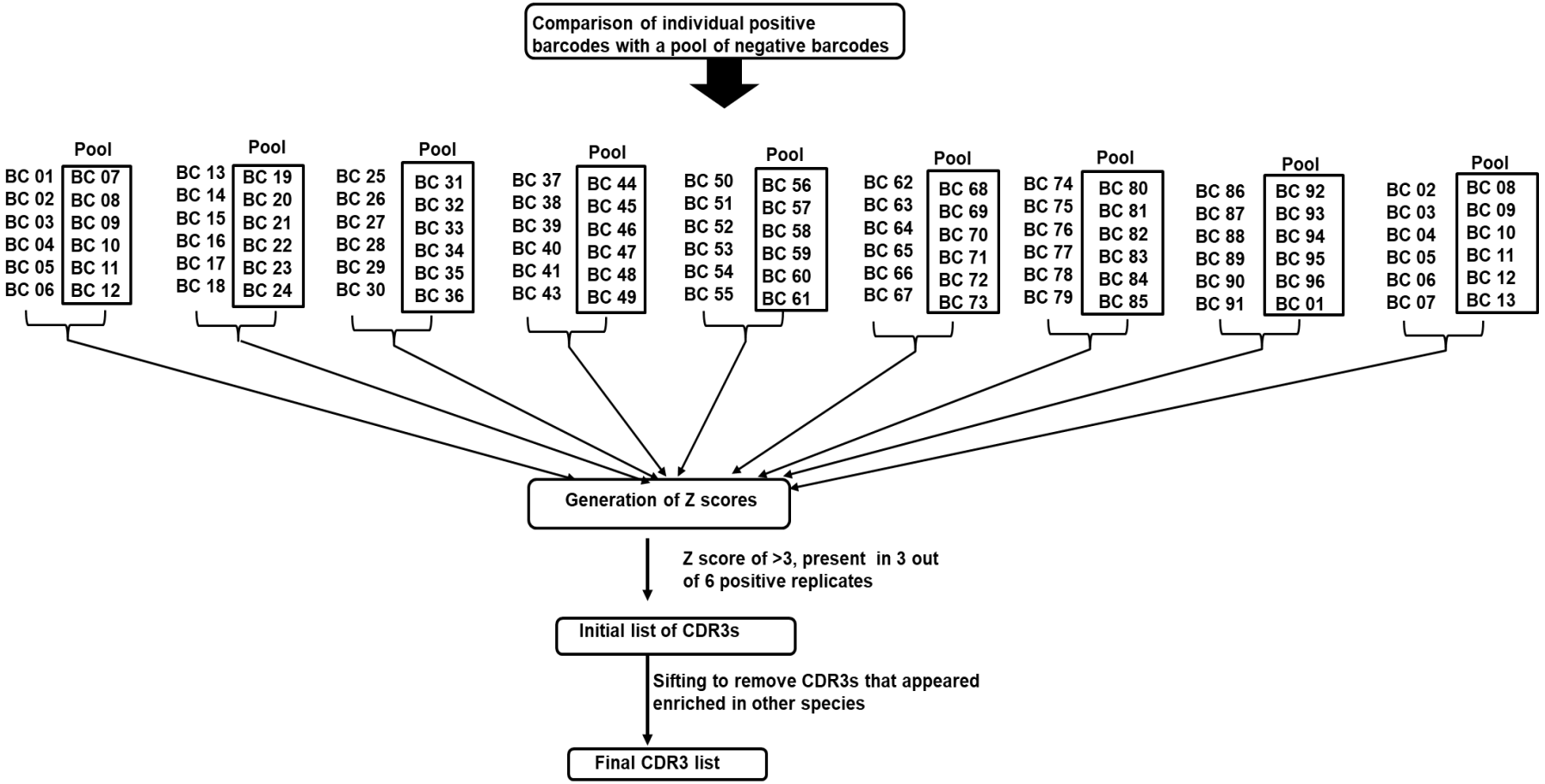


Figure 6:10: Workflow of the strategy employed for the analysis of NGS data. Individual positive barcode replicates were compared against the pool of negative barcodes for all control peptides and Z scores were generated. Analysis was then carried out to select for CDR3s that had a Z score of above 3 and were present in at least 3 out of 6 positive replicates, and an initial list of enriched CDR3s was produced. For each species, the list of CDR3s was sifted to remove CDR3s that appeared enriched when panning against peptides from other species to maximise the chances of isolating species-specific CDR3s. 5 V_HHs were selected against each target peptide.

Table 6:3: A heat map generated for selected V_HHs from panning against the seven related (A) and other two related (B) distinct *Eimeria* species-specific peptides from Gam56 protein

A

CDR3[1]	Specificity[2]	Frequency in positive barcode replicates[3]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate
ILQqKIPRSNKLHIKP	QSFSLPIPIDRPL	3 of 6	4.74	4.14	8.5	0.55	2.34	-1.02
GTNFDMSVMSqLHNLLRFEqS	QSFSLPIPIDRPL	3 of 6	101.08	41.4	269.44	-14.83	-115.38	-438.6
MPISNPGLQLMTSNDG	QSFSLPIPIDRPL	3 of 6	5.12	3.35	8.66	1.76	0.48	0.47
SLNYPRTqHRRKQKNE	QSFSLPIPIDRPL	3 of 6	3.03	3.4	8.37	2.94	-0.43	-4.2
MPNYITPQNVKFLECR	QSFSLPIPIDRPL	3 of 6	3.38	3.35	4.35	2.12	0.98	0.4
VKGQTSGRKNYVNGMREARLq	QSMAVPTTVENTV	4 of 6	208.23	138.49	115.8	22.15	-25.42	-10.99
QWGVCLRLFIRVLLMGLIRLqW	QSMAVPTTVENTV	3 of 6	5.52	4.1	3.2	0.23	2.1	-1.57
qWFFqTSLRTHS	QSMAVPTTVENTV	3 of 6	5.09	3.02	4.28	-1.61	0.14	1.09
FPTSSSVAHLRPRSRS	QSMAVPTTVENTV	3 of 6	6.87	3.56	7.24	-0.72	-0.01	-8.34
MPISNPGLQLMTSNDG	QSMAVPTTVENTV	3 of 6	3.8	3.29	4.73	-0.76	2.12	0.71
CGATqTRGRFITFPASPYASq	PAMAVPSTTSVESTVL	5 of 6	62.99	6.75	6.49	14.97	250.33	-1.41
SKCLHTLSRSqLHPHFQFHDI	PAMAVPSTTSVESTVL	5 of 6	418.99	23.61	4.5	14.65	27.87	-81.68
DKFLAqHVDqTFqqHTHYQDA	PAMAVPSTTSVESTVL	6 of 6	50.68	32.5	8.97	3.75	7.12	6.75
YYSIPLqDNTWV	PAMAVPSTTSVESTVL	3 of 6	23.72	21.7	3.18	-15.54	-68.76	-69.9
AqPIRGKVTIVL	PAMAVPSTTSVESTVL	4 of 6	192.39	253.36	19.87	3.07	-6.37	-29.17
QYTqKKAGNqTKKPHPKRKAA	PSLALPSSSAAEPAAVH	6 of 6	8.42	7.24	7.8	12.26	7.81	6
DYGPCGCYLEQLVqGGCLGGIA	PSLALPSSSAAEPAAVH	6 of 6	3.81	6.38	9.79	5.97	3.82	7.45
ITAWGAPSQRDL	PSLALPSSSAAEPAAVH	5 of 6	64.91	268.39	235.11	96.48	224.16	-52.19
PAIqPTRSYARHGPPR	PSLALPSSSAAEPAAVH	4 of 6	9.98	5.99	14.21	23.51	-18.71	-18.71
IERNERPDLPSERMNSATqSL	PSLALPSSSAAEPAAVH	4 of 6	9.59	4.24	3.32	9.7	0	0
QHRPRPHRPTKRRKSY	PSMAVPSTTPVENQVH	6 of 6	12.95	17.86	19.6	17.58	14.65	11.98
VNSKRKHWHRSR	PSMAVPSTTPVENQVH	3 of 6	8.43	10	11.53	0	0	0
KMPqTPNKKPHRKWKR	PSMAVPSTTPVENQVH	6 of 6	14.84	34.12	38.85	32.2	29.68	29.19
HKSRDGKTSKNHqLLKQKHLK	PSMAVPSTTPVENQVH	6 of 6	15.54	17.27	21.15	37.51	30.9	23.85
SqPFGPPMYYKL	PSMAVPSTTPVENQVH	6 of 6	28.76	36.85	41.26	51.3	46.3	48.76
WAqYSKPPSTYVYYLGKRYLI	QSLALPTPIDRAF	5 of 6	114.95	81.28	98.07	72.21	-50.17	83.75
MLHSSEFIqHSYGDND	QSLALPTPIDRAF	4 of 6	31.71	9.69	0.53	15.31	16.47	-0.57
ASSNSVHLSEqPLHHP	QSLALPTPIDRAF	5 of 6	30.95	27.38	41.44	51.49	52.07	-2.83
qTKANMKGLqTPGYGqLGLKF	QSLALPTPIDRAF	5 of 6	68.75	-43.19	88.95	65.73	68.31	99.51
NRGHqSPIQYTRGMTGRGRHS	QSLALPTPIDRAF	5 of 6	7.97	5.54	3.17	-3.86	5.55	5.73
PEPTWRNAQVqFWSRLPTMRN	QSLAVPTTVENTV	4 of 6	10.62	8.89	18.63	3.03	-20.76	2.66
MYERVYSLYYqLFSNLLMSDY	QSLAVPTTVENTV	4 of 6	5.18	6.86	5.81	3.74	1.32	2.13
YYLSVqHGAVCTSDSNNNDAK	QSLAVPTTVENTV	3 of 6	3.99	13.16	7.59	-13.4	-24.22	0.83
YTGLRWLWqPITCqMPDSNLC	QSLAVPTTVENTV	3 of 6	5.56	9.71	4.03	-19.52	-3.43	1.67
RNHLPKqRKRIPKqNT	QSLAVPTTVENTV	3 of 6	6.5	5.52	3.36	-17.52	-4.31	2.11

B

CDR3[1]	Specificity[2]	Frequency in positive barcode replicates[3]	Replicat	Replicat	Replicat	Replicat	Replicat	Replicat
PHLKFPVLMFCqHHGS	MDSTPDFPAFNEPM	3 of 6	51.58	9.34	74.9	-2.41	-2.16	-140.1
HLVKRSqqNQTSCDVR	MDSTPDFPAFNEPM	3 of 6	10.93	3.94	9.28	-0.07	-3.61	0.46
YSCQNASVWTPM	MDSTPDFPAFNEPM	3 of 6	5.66	4.12	131.52	0	0	0
RPAqIDASDSSLVqRq	MDSTPDFPAFNEPM	3 of 6	4.39	166.48	5.41	-1.41	0.65	0.64
SEFHSKNQHqCLADTS	MDSTPDFPAFNEPM	3 of 6	111.52	3.29	3.11	-1	-1	1.29
KLQqTYVVRSqASRSGC	MDTMPEFPPFAEGM	5 of 6	5.07	18.23	27.72	6.37	26.01	0.15
NRGHqSPIQYTPYFNRq	MDTMPEFPPFAEGM	4 of 6	7	6.48	22.93	7.81	0	0
WRVTHRHHqPHRRRPR	MDTMPEFPPFAEGM	3 of 6	10.11	24.92	3.44	-1	-1	-1
SqPFGPPMYYKL	MDTMPEFPPFAEGM	3 of 6	20.15	60.24	5.83	0	0	0
SIPTHPPMTLTFHNQR	MDTMPEFPPFAEGM	3 of 6	4.24	13.38	4.47	0	0	0

[1] CDR3s of selected V_HH

[2] The peptides the V_HHs were raised against

[3] The number of positive screening replicates the V_HH appeared in that had a Z score of >3

[4] Z scores in the positive replicate barcodes (Blue-red >50)

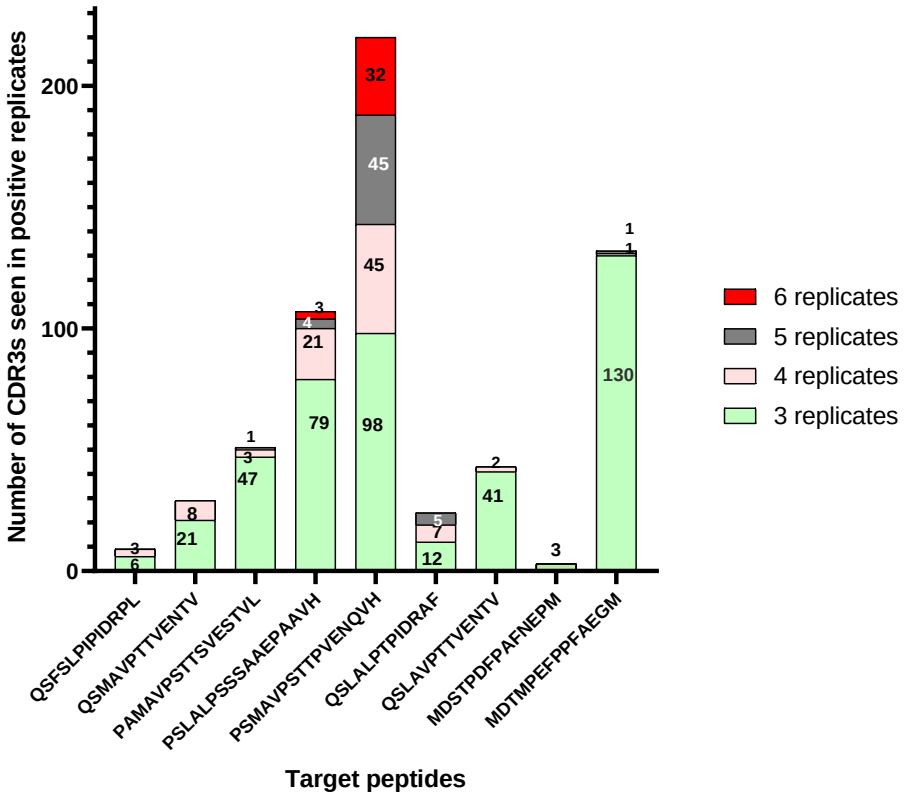


Figure 6:11: The numbers of CDR3s seen in between 6 positive replicate samples. Following screening of each phage with the peptide it was raised against as well as the other six negative related peptides, alongside screening the two related peptides with each other, samples were sequenced by Ion Torrent sequencing and data analysed using a two proportion Z-test. CDR3s were selected based on those that were seen having Z score of above 3 and present in at least 3 of the positive replicate samples.

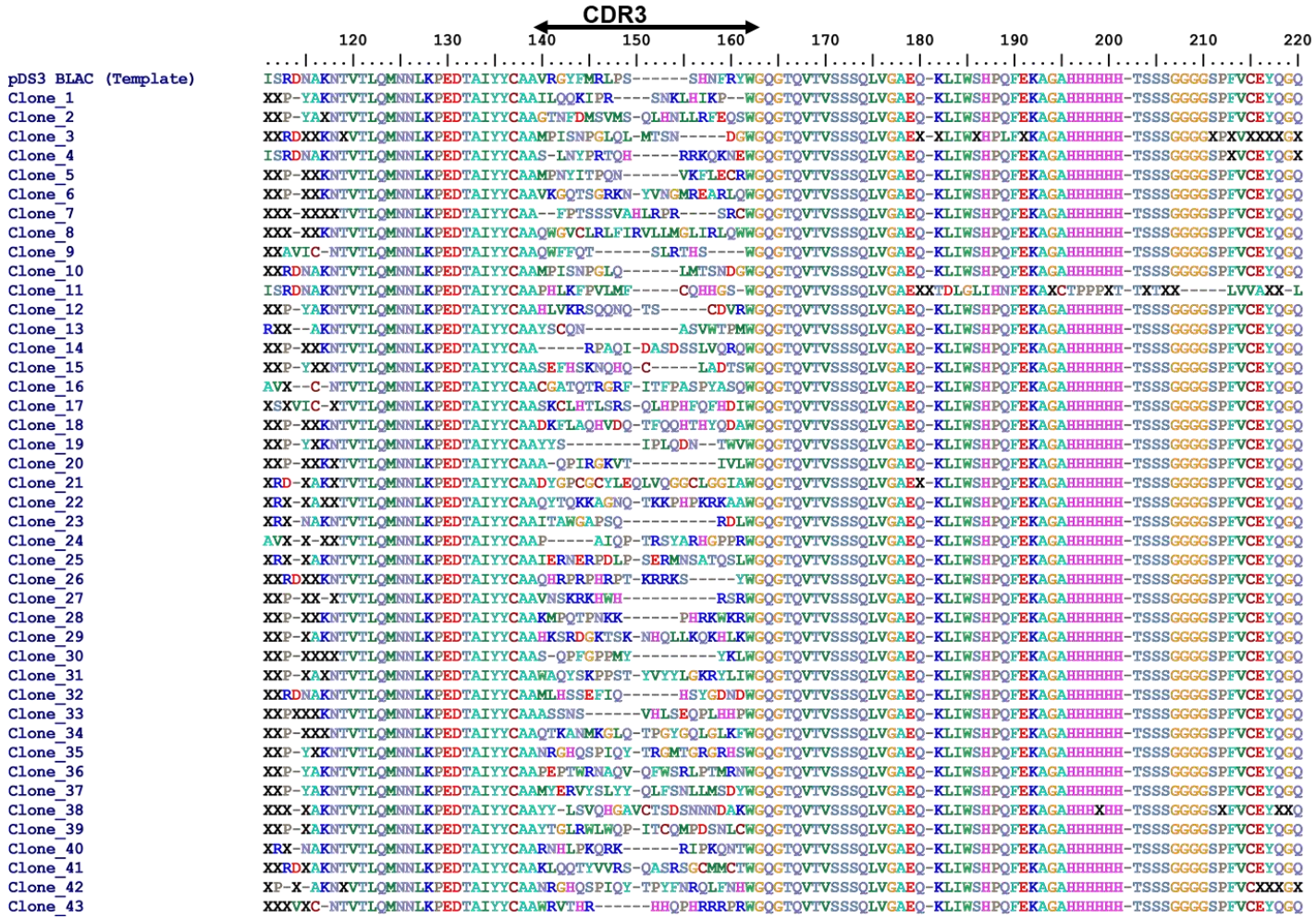


Figure 6:12:Sanger sequences of V_HH clones panned against distinct oocyst wall peptide Gam56. pDS3 containing an anti-beta lactamse V_HH gene was the template used to synthesize the selected clones. Following bacterial transformation of V_HH clones, two colonies were miniprepped and Sanger sequenced, at least 1 was confirmed as containing the target CDR3 sequence. Sequences were aligned using ExPASy alignment tool.

6.2.3 Screening of isolated clones

From *in silico* analysis of NGS data, binders with potential specificity to one of the nine peptides were identified, synthesised and Sanger sequenced to confirm their identify. The binding properties of all 43 clones successfully synthesised were assessed via ELISA to identify actual binders and specificity to target species. Of all the 43 clones screened, none produced ELISA signals above background levels. Representative data for some of the target peptides is shown.

Phage were prepared (10^{11} PFU/ml) for individual rescues ILQqKIPRSNKLHIKP GTNFDMSVMSqLHNLLRFEqS, MPISNPGLQLMTSNDG, SLNYPRTqHRRKQKNE and MPNYITPQNVKFLECR (all raised against peptide QSFSLPIPIDRPL (specific to the oocysts of *E. mitis*) as well as monoclonal V_HH anti-elastin phage control and the binding properties were assessed in a phage ELISA on streptavidin coated plates using a rabbit anti-fd and goat anti-rabbit AP antibodies; anti-elastin phage was assayed on a Nunc Maxisorp® plate. These rescues were screened for binding activity to the target peptide and the six related peptides. Absorbance signals produced after 2 hours of incubating with the pNPP substrate remained at background levels for all clones ~ 0.2-0.4, while the positive control produced a signal of 2.1 (Figure 6.13). The positive signal was about 4X background signals. Phage were prepared for individual rescues VKGQTSGRKNYVNGMREARLq, FPTSSSVAHLRPRSRS, QWGVCLRLFIRVLLMGLIRLqW and qWFFqTSLRTHS (all raised against peptide QSMAVPTTVENTV which is specific to *E. necatrix*) along with the anti-elastin positive control and the binding properties assayed exactly as performed above. Absorbance signals were comparable to the no antigen control after 1 hour of adding substrate at ~0.2-0.3 and the positive control produced an absorbance signal of 1.4 (Figure 6.14).

Following the background signals obtained after assessing the binding properties of the first 2 batches of V_HHs, it was then thought to determine if the peptides were being immobilised in the streptavidin coated plates. A control peptide (SAF 70-peptide) was obtained that is the epitope for a commercial monoclonal antibody (SAF 70) and was immobilised on the plate as well as the other test peptides. Phage was prepared for the V_HH rescues CGATqTRGRFITFPASPYASq, SKCLHTLSRSqLHPHFQFHDI, DKFLAqHVDqTFqqHTHYQDA and YYSIPLqDNTWV (all raised against peptide PAMAVPSTTSVESTVL). The assay was performed exactly as above with the exception that the SAF 70 was the positive control on the same plate. Absorbance signals produced for the peptides were comparable to the no antigen control levels at 0.3-0.5 after 2 hours of incubation with the pNPP substrate and the SAF 70 positive

control produced absorbance signals of 2.1 which was 4X background levels (Figure 6.15).

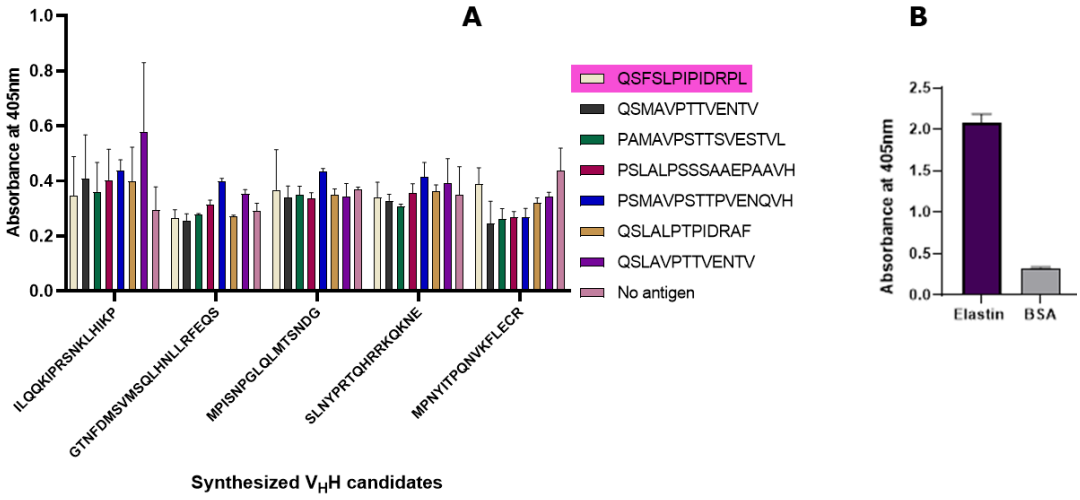


Figure 6:13: Monoclonal V_HH phage ELISA for distinct *Eimeria* species-specific peptides from Gam56 protein, V_HH candidates were raised against the shaded peptide. V_HH phage were produced for individual rescue, and the anti-elastin positive control. Peptides (10 µg/ml, A, the immobilised peptides are indicated in the key) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised antigens and those remaining after the wash steps were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed a 2-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

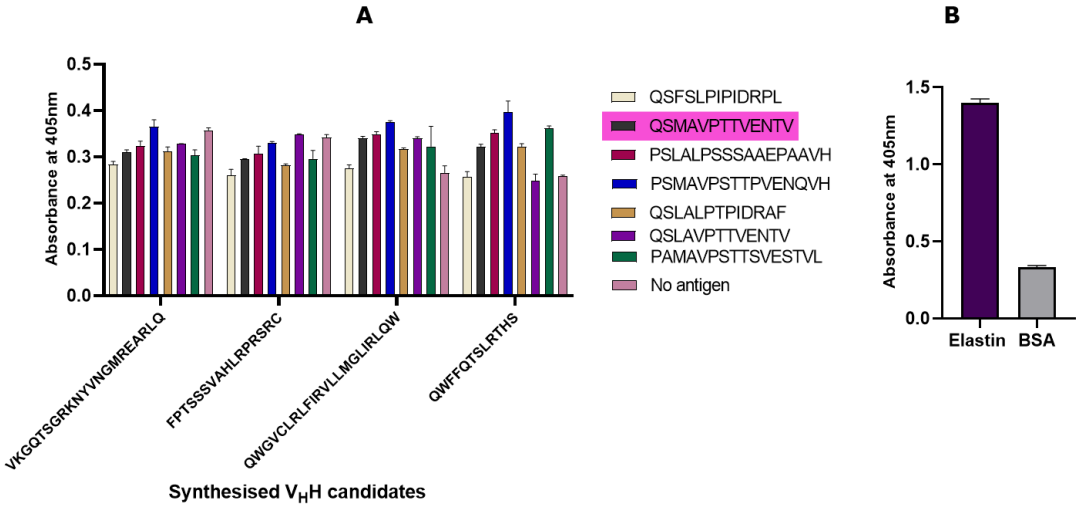


Figure 6:14: Monoclonal phage ELISA for distinct *Eimeria* species-specific peptides from Gam56 protein, V_HH candidates were raised against the shaded peptide. V_HH phage were produced for individual rescue, and the anti-elastin positive control. Peptides (10 µg/ml, A, the immobilised peptides are indicated in the key) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised antigens and those

remaining after the wash steps were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed a 1-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

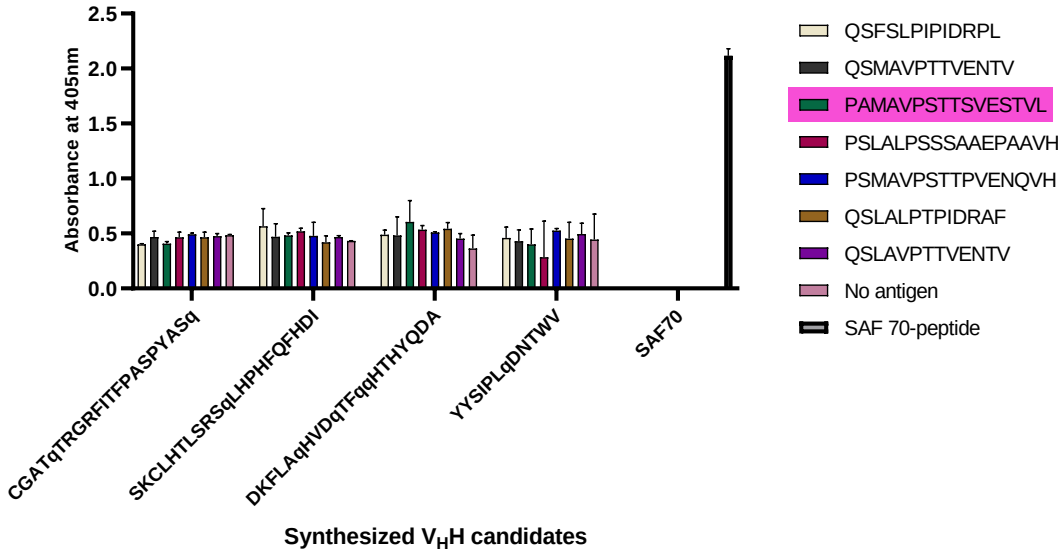


Figure 6:15: Monoclonal phage ELISA for distinct *Eimeria* species-specific peptides from Gam56 protein, V_HH candidates were raised against the shaded peptide. V_HH phage were produced for individual rescues. Target peptides or SAF 70-peptide was immobilised overnight at 10 µg/ml on Pierce™ Streptavidin coated plates; the immobilised peptides are indicated in the key). Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised Gam56 peptides while anti-SAF 70 was used to detect SAF 70-peptide, antibody remaining after the wash steps were then detected with rabbit anti-fd and goat anti-rabbit-AP for V_HH-phage or anti-SAF 70 and goat anti-rabbit-AP for SAF 70. The pNPP substrate was allowed a 2-hour incubation before absorbance was read at 405 nm.

After determining that the reagents and plate allowed the immobilisation of peptides (using the SAF70-peptide control) and detection of bound V_HH-phage (using the anti-elastin V_HH-phage clone) it was proceeded to assay the rescued phage raised against the other target peptides. Phage were prepared (10^{11} PFU/ml) for individual rescues YYLSVqHGAVCTSDSNNNDAK, YTGLRWLWqPITCqMPDSNLC, MYERVYSLYYqLFSNLLMSDY RNHLPKqRKRIPKqNT (all raised against QSLAVPTTVENTV) and the binding activity assessed for the seven related peptides exactly as described above. Absorbance signals produced after 2 hours of incubating with the pNPP substrate were about ~ 0.3-0.6 while the positive control produced a signal of 1.65 (Figure 6.16). This assay was repeated, and absorbance signals produced were at background levels at 0.2-0.3 while the positive control produced an

absorbance signal of 1.3 after 2 hours of incubating with the pNPP substrate (data not shown).

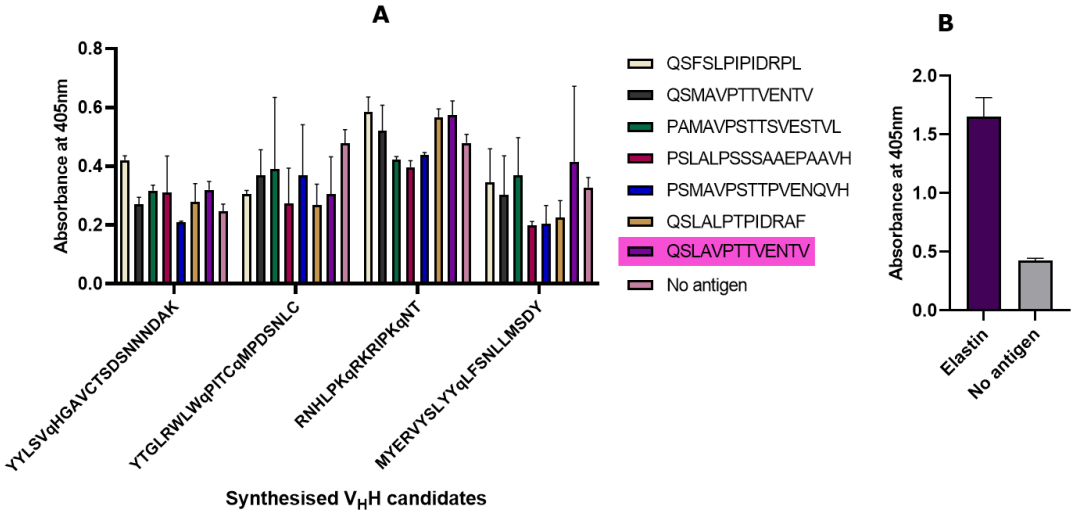


Figure 6:16: Monoclonal phage ELISA for distinct *Eimeria* species-specific peptides from Gam56 protein, V_HH candidates were raised against the shaded peptide. V_HH phage were produced for each individual rescue as well as the anti-elastin positive control. Peptides (10 µg/ml, A, the immobilised peptides are indicated in the key) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised antigens and those remaining after the wash steps were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed a 2-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

For V_HH rescues from the two related peptides; MDSTPDFPAFNEPM and MDTMPEFPPFAEGM. Phage were prepared (10^{11} PFU/ml). For individual rescued clones PHLKFPVLMFCqHHGS, HLVKRSqqNQTSCDVR, YSCQNASVWTPM, RPAqIDASDSSLVqRq and SEFHSKNQHqCLADTS (all raised against MDSTPDFPAFNEPM) and the V_HH were assessed in a phage ELISA using the anti-elastin phage as control. Absorbance signals produced after 2 hours of incubating with the pNPP substrate remained at background levels for the test V_HHs at ~ 0.4-0.5 while the positive control produced a signal of 1.9 (Figure 6.17). This assay was repeated and the valued remained at background level of 0.2-0.3 after 1 hour of incubation whilst the positive control had absorbance signals of 1.5 (data not shown). Phage were prepared (10^{11} PFU/ml) for individual rescues KLQqTYVVRSqASRSGCMMCT NRGHqSPIQYTPYFNRqLFNH, WRVTHRHHqPHRRRPR (all raised against MDTMPEFPPFAEGM) and the binding properties assessed exactly as described above. Absorbance signals produced after

2 hours of incubating with the pNPP substrate remained at background levels at ~ 0.4-0.5 while the positive control produced a signal of 1.8 (Figure 6.18).

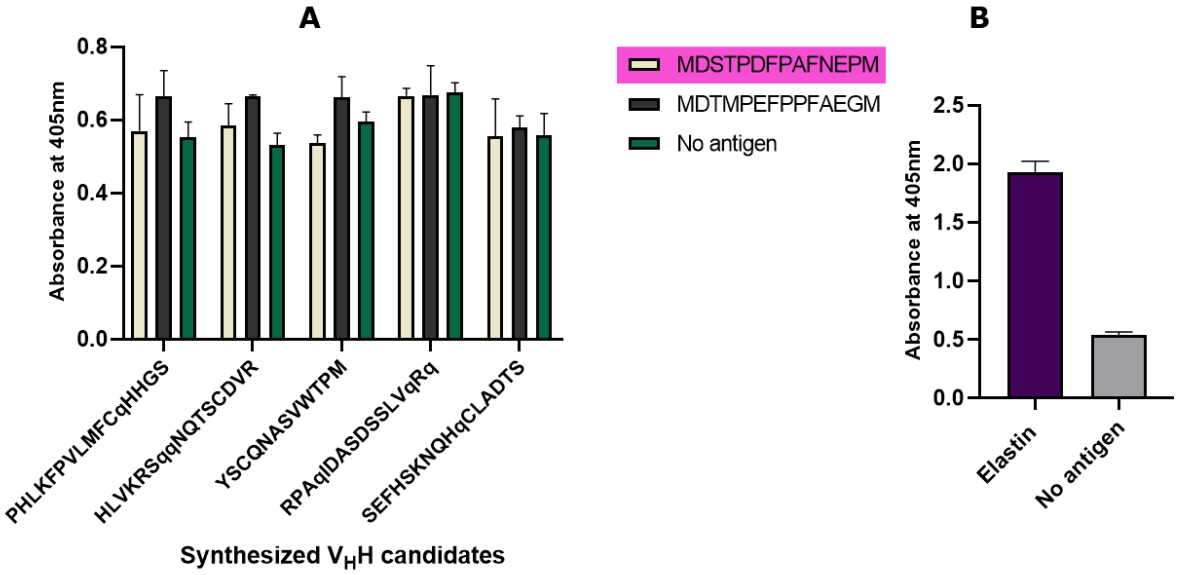


Figure 6:17: Monoclonal phage ELISA for distinct *Eimeria* species-specific peptides from Gam56 protein, V_HH candidates were raised against the shaded peptide . V_HH phage were produced for each individual rescue as well as the anti-elastin positive control. Peptides (10 µg/ml, A, the immobilised peptides are indicated in the key) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised antigens and those remaining after the wash steps were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed a 2-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

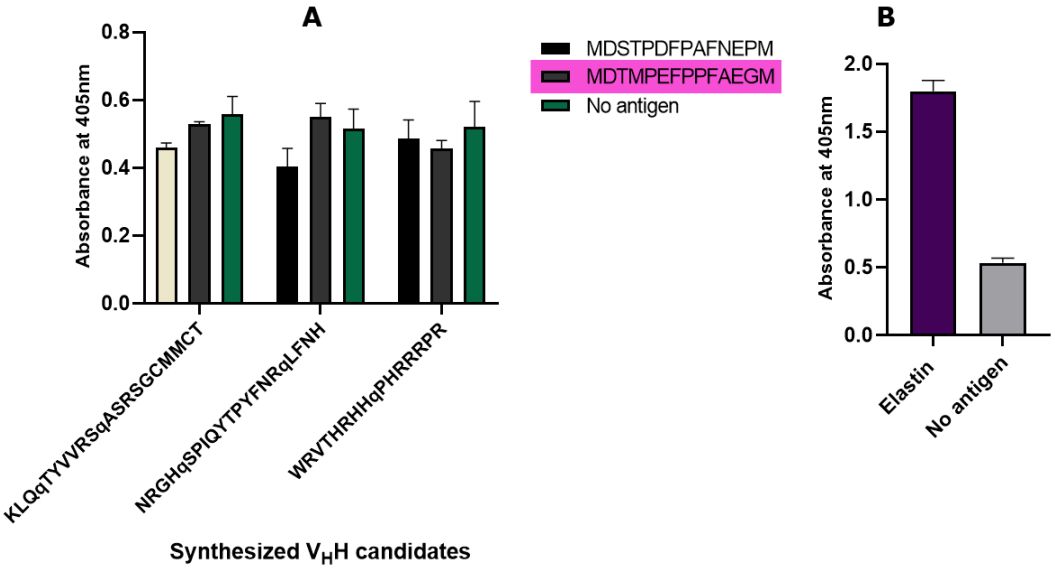


Figure 6:18: Monoclonal phage ELISA for distinct *Eimeria* species-specific peptides from Gam56 protein, V_HH candidates were raised against the shaded peptide. V_HH phage were produced for each individual rescue as well as the anti-elastin positive control. Peptides (10 µg/ml, A, the immobilised peptides are indicated

in the key) or elastin (20 µg/ml, B) were immobilised in 100 µl aliquots on Pierce™ Streptavidin coated plates and Nunc MaxiSorp® plates, respectively. Monoclonal V_HH phage (100 µl of ~10^{11}-10^{12} PFU/ml) were used to detect the immobilised antigens and those remaining after the wash steps were detected with rabbit anti-fd and goat anti-rabbit-AP. The pNPP substrate was allowed a 2-hour incubation before absorbance was read at 405 nm. (A) represents monoclonal phage detecting peptides immobilised on Pierce™ Streptavidin coated plates while (B) represents the positive control phage detecting elastin on Nunc MaxiSorp® plates.

All 43 individual V_HHs that were identified by *in silico* analysis and synthesised by inverse PCR were grown and their binding activity assessed showing that absorbances produced were around background level. None of the rescued V_HH assesed bound their target peptide in this assay format.

6.3 Discussion

This chapter aimed to identify and isolate binders against distinct peptides of Gam56 antigen using a combination of conventional phage display and next generation phage display. The target antigen, a 56 kDa constrained outer wall protein of oocysts is one of the most abundant surface proteins of oocysts and has been widely characterised and described (Belli *et al.,* 2009). Extensive studies have been carried out on Gam56, its functional genomics was characterised as well as its role in oocyst wall formation in *E. maxima* in a study conducted by (Belli *et al*., 2002b). The authors confirmed that it was a structural component of the oocyst wall when a specific antibody to the protein recognised the wall forming bodies in macrogametocytes, and the walls of oocysts and sporocysts of *E. maxima*. Gam56 was selected for this study because in addition to it having been one of the two immunodominant proteins involved in oocyst wall formation, protein database sequences for Gam56 are more abundant and complete as compared to Gam82 which was missing at least one species, *E. praecox*. A low molecular weight antigen; 12 kDa protein had been described as a major protein component of the oocyst wall in *E. tenella* by *(Karim et al., 1996)* but no further studies have been carried out on this protein and the sequences are not readily available in protein databases.

This target antigen in this chapter had the potential to be a much better target for antibody isolation over oocyst homogenate or whole oocysts that were previous used as targets for antibody production. These peptides are far simpler and well defined and the final assay format could still possibly target oocyst homogenates on ELISAs, denaturing western or dot blots. All relatively high throughput assay formats. Binding of phage antibodies to well defined peptides also allowed the focus on the important single peptide specificity and the avoidance of binding to the possibly vast majority of epitopes present in complex antigens such as oocyst homogenates, common epitopes that are unlikely to distinguish the different species.

In this study, Gam56 sequences available in the database for all *Eimeria* species were extracted, aligned and assessed and areas containing differences were noted between the sequences. Between residues 18 to 34 of the amino acid sequences, areas of at least 4 amino acid differences were found in five of seven species of *Eimeria* with the exception of *E. necatrix* and *E. tenella* which each had just one amino acid change between the residues.

All sequences were selected and peptides were synthesized for each species. Peptides were biotinylated allowing immobilisation to streptavidin coated plates. Like the preceding chapter, this chapter approached the study with three panning rounds

against distinct peptides as well as subtraction steps. A pre-panning subtractive step was performed for the 2nd round of panning and a pre-panning and post-panning subtraction of phage were conducted for the 3rd panning round. In this present chapter, the target consisted of 9 peptides altogether of which 7 were related and unrelated to the other two while the other two were related to each other. For the seven related peptides, phage raised against each of the seven targets was subtracted from the other six while for the other two related peptides, phage were subtracted against each other.

There have been reports about the immunogenicity of, and cross reactivity of immune responses to the 56 kDa gametocyte antigen for at least three *Eimeria* species. In the study by (Belli *et al*., 2009), the authors reported that antibodies to *E. maxima* gametocyte wall forming proteins 56 kDa and 82 kDa recognised the same proteins in *E. tenella* and *E. acervulina*. The poteins themselves have been seen to share areas of tyrosine richness and strong homology in their amino-terminal where protective antibodies bind. The study inferred that antibodies to gametocyte antigens of *E. maxima* cross-reacts with other species. The antibody isolation methodology used in this study has advantages over immunisation techniques where it is difficult to avoid antibody responses to cross-reactive, immunodominant epitopes, this technique does not rely on immunogenicity being performed *in vitro* hence it can be applied to selecting antibodies to differential areas of antigens.

Monoclonal antibodies have been produced for several individual proteins/peptide targets of different pathogens including *Eimeria*. In a study by (Wallach *et al*., 1990) monoclonal antibodies were produced against the gametocyte antigen 56 kDa of *E. maxima*, the authors immunized mice with preparations enriched with 56 and 82 kDa antigens and several monoclonal antibodies with specificity to the 56-kDa antigen were isolated. When one of these antibodies or immune chicken sera raised against affinity-purified 56- and 82-kDa antigens were used to passively immunise chicks, the antibodies were found to confer partial protection against *E. maxima* challenge infections. One of these monoclonal antibodies of the immunoglobulin M subclass, provided 40 to 50% inhibition against *E. maxima* challenge infections. Similarly, a monoclonal antibody was produced against the 12 kDa oocyst wall protein of *E. tenella* by injecting mice with the 12 kDa protein. When its reactivity with oocyst wall fragments and sporozoites of *Eimeria* were tested, it was found to be pan-specific to all seven *Eimeria* species and restricted to the gametocytes and oocyst stages of the parasite. Immunogold labelling revealed that the antibody bound to an epitope on the inner face of the oocyst wall and when chickens were passively immunized with the MAb, oocyst output was significantly reduced by 42-50% after homologous *E. tenella*

infection or 35% after heterologous E. *maxima* infection compared with the control groups.

In the study by (Tong *et al*., 2015), peptide based monoclonal antibodies were produced against *Toxoplasma gondii* vacuolar proton pyrophosphate (TgV-PPase) by synthesising a synthetic N-terminal peptide, TgVP1-1 corresponding to amino acids 292-320 of TgVP1 (NH2-YTKAADVGADLSGKNEYGMSEDDPRNPAC-COOH). The peptide without post-translational modification sites was then used to immunise mice to obtain monoclonal antibodies. Three hybridomas were isolated, confirmed by ELISA, immunofluorescence and western blotting, and were found to be specific to YTKAADVGADLSGKNEYGMSEDDPRNPAC and they all reacted with an 85 kDa protein band of *T. gondii*.

Several other studies have reported producing antibodies to individual proteins and peptides (Toloudi *et al.,* 2016) hence this is a valid technique for generation of polyclonal and monoclonal antibodies.

The panning strategy included screening after round 3 of selection to produce positive samples (each peptide phage versus the peptide it was raised against in 6 replicates) and negative samples (the same peptide phage versus the other six peptides excluding the one it was raised against). Alternatively, for the two related peptides, screening was against the target peptide (6 positive replicates) or the non-target peptide (6 negative replicates). This screening step enabled the direct comparison of negative and positive samples during NGS analysis of data aiding isolation of positive binders that are potentially species-specific. Initial polyclonal phage ELISA performed using streptavidin coated plates and the original V_HH library as a negative control did not demonstrate any binding activity to peptides. Positive control used was elastin which was immobilised on a separate plate, Nunc Maxisorp®, and produced signals at least 2-3X more than the background signals. As was performed in the previous chapter, colonies were randomly picked from each target's round 3 and phage ELISA was performed. Some clones demonstrated binding activity to peptides having 2X background signals but on repeat analysis, only one was shown to have binding activity to a peptide, and this was against a peptide the monoclonal phage-V_HH wasn't raised against. On further screening of the clone, phage produced from the clone did not produce higher signals than background.

As was mentioned in the previous chapter, colony picking has been proven to be an effective way of isolating enriched binders within a sub-library but in the present chapter, only 10 clones were picked and screened per target which may not be a true representation of the diversity of enriched clones within the sub-library; typically between 100-1000 clones are screened for binders in traditional colony picking

(Barreto *et al.,* 2019, AC't Hoen *et al.,* 2012, Yang *et al.,* 2017). However, the most common clones are likely to be picked and so these did not bind to the target peptide. However, (Ngubane *et al*., 2013) in a study isolating peptides that bound to Mycobacteria, demonstrated that random clone picking may not necessarily identify highly enriched clones as they missed picking a peptide that constituted 80% of the round 5 polyclonal phage mixture.

In the present chapter, just like in the preceding chapter, bioinformatics analysis was geared towards finding peptide specific clones by directly comparing the positive replicates for each peptide to the pool of the negative samples, generating Z scores (Zhang *et al.,* 2011). The V_HH CDR3 sequences of each the seven related peptides were analysed, pooling the CDR3s for the other six peptides and comparing each positive replicate CDR3s with this negative pool. Similarly, the other two related target peptides had the CDR3s of the other peptide as the negative pool, using 6 repeats. The top 5 candidates based on the criteria applied were synthesised and screened. The synthesis of the clones by inverse PCR into a scaffold V_HH sequence resulted in the successful synthesis of 43 out of 45 clones which was a 95.6% rescue rate. As mentioned in the previous chapter, this was a considerable improvement from the strategy used in chapter 3 for scFv rescue of clones of low abundance from polyclonal phage pools. In phage ELISAs, the V_HH clones did not demonstrate any binding activity to the target peptides. Interestingly, ITAWGAPSQRDL panned against peptide PSLALPSSSAAEPAAVH had a high frequency in the positive replicates ~ 10% but no binding activity was observed when it was screened against peptides. It would have been expected that this clone would show some binding activity given the high frequency of the clone in positive replicates as well as when compared with the other six negative control. There may have been a possibility of having more binders if the stringency of the bioinformatic analysis was reduced across different parameters (this is discussed in detail in **chapter 7**). Also, subtractive steps during panning may have removed the high affinity binders thereby a more sensitive screening method may have been required to produce signals.

When taken together, in the present chapter, the data indicates that panning experiment including extensive subtraction steps and bioinformatics analysis using the two proportion Z score analysis for ranking V_HHs was not very efficient in isolating binders to distinct peptides varying in several amino acid residues in short stretches of the Gam56 antigen. The panning strategy used in the present chapter for this target may have led to the subtraction of high affinity binders leaving behind non-binders, low affinity binders or binders to low abundant epitopes.

The present chapter, just like previous chapters prioritised the isolation of species-specific antibodies hence the reason for the application of stringent subtraction steps. In retrospect, subtraction steps could have been less stringent; subtracting only in one panning round which may have been sufficient for the isolation of species-specific V_HHs while still retaining high affinity binders. Pre-selection subtraction of phage has been successfully employed in many studies to reduce non-specific binding of phage to target. Subtractive panning has been employed for viruses (Lebani *et al.,* 2017), fungi (Portes *et al.,* 2017) whole cells (Alfaleh *et al.,* 2017) and various other targets. In most panning strategies that have incorporated subtraction steps, there is an initial depletion step before a positive selection step. Because species-specific binders to Gam56 might be rare, post-panning subtraction was performed in addition to pre-panning in this chapter as a more effective subtraction technique to maximise enrichment of specific binders. Figure 6.2 shows the reduction in the recovered phage across rounds of panning which may suggest the subtraction of the majority of phage which might have left only low affinity binders.

There are a few studies that have tried to alter depletion steps to maximise the selection of high affinity, target specific ligands. In the study by (Togtema *et al*., 2019), immune V_HH library was panned against a recombinant viral protein of human papilloma. Pre-selection subtraction was performed against maltose binding protein and polyhistidine (His6) tag before phage selection against the protein. The protein concentration of the subtraction step was more than the target protein to enhance selective pressure for high affinity, target specific V_HHs. The blocking agent was also switched in the second panning round to remove binders to the blocker. The present study may have benefitted from subtraction of a higher concentration of protein in the negative targets before the selection round and eliminating post-panning subtraction, this might have been sufficient to remove non-specific targets. Very few studies have reported two negative selection steps in a single panning round (Lim *et al.,* 2019).

The bioinformatics approach may also have been improved by considering published strategies even though to-date not many phage display studies have applied the V_HH antibody format with next generation sequencing to isolate binders (Miyazaki *et al.,* 2015, Turner *et al.,* 2016, Rafique *et al.,* 2019). In all of these studies, immune libraries were used rather than the single pot libraries used within this study which introduces bias towards the isolation of antibodies tailored against the target antigen. (Miyazaki *et al*., 2015) used an immunised Llama V_HH library to isolate binders to a transmembrane protein IZUMO and the study analysed the base library as well as the data after panning round and compared the diversities; only one panning round was conducted before sequencing (illumina). The frequencies of clones before and

after panning were compared and enrichment of clones identified. The top 100 most frequent candidates were selected and phylogenetic tree analysis applied and they found that 75% of the candidates had a high homology with 13 previously isolated individual colony picked candidates. This study identified three sequences by high throughput sequencing that were different from the previously isolated binders and when they were screened, two out of three bound to the target antigen. The results of the study suggest that biopanning followed by next generation sequencing and bioinformatics analysis is a valid method for identifying diverse antigen-specific clones

(Turner *et al*., 2016) only sequenced (Illumina) and analysed the base library, an immune V_HH library against Staphylococcal enterotoxin B (SEB) without performing any panning rounds. The sequencing data was probed with a known SEB binder sequence previously isolated using colony picks. They conducted a BLAST search to identify similar clones and new antibodies were identified. When screened, these were shown to have affinities and melting temperatures similar to those obtained by colony picks. The study demonstrated that utilising NGS coupled with phage displayed biopanning enables the isolation of high-quality antibodies from an immune library even when they are low in frequencies. The bioinformatics strategies described above both compared the frequencies of antibodies in naïve libraries versus panning rounds and diversity of naïve libraries versus panning rounds. Even though the base library in the present chapter was not sequenced prior to panning and there was no reference point to compare the frequencies of the screening round with the base library, the sequences of most of the isolated V_HH candidates were about 1-8 %. Also, some of the V_HHs that had very high frequencies were present in only one or two of the positive replicates; again having the selected V_HH candidates represented in at least three positive replicates was given preference in the present chapter.

The stringent subtraction steps employed in the present chapter most likely led to the removal of high affinity binders leaving behind low affinity binders and as was mentioned in the previous chapter, a more sensitive assay may have revealed binders to the target or the bioinformatics analysis may have been less stringent or looked at more parameters to identify true binders to targets.

The next chapter looked at the bioinformatics analysis performed, assessing and re-evaluating the strategies used.

7. Assessment and optimisation of bioinformatics analysis methods

7.1 Introduction

Next-generation sequencing which is a method of simultaneously sequencing millions of fragments of DNA (or complementary DNA), has revolutionized genomic and genetic research (Quail *et al.,* 2012). NGS technologies have evolved with a rapid pace as new sequencing platforms have been released over the years and offering different ranges of read depth, read length and accuracy. New applications for NGS and bioinformatics sequence analysis tools continue to be developed to address current limitations and improve performance (Quail *et al.*, 2012, Yohe and Thyagarajan, 2017). NGS has been rapidly adopted in the discovery of ligands by phage display because of its ability to simultaneously analyse a very high number of binding events within a single test compared to traditional methods (Yohe and Thyagarajan, 2017). The combination of phage display and NGS has the potential of revolutionising the discovery of new ligands. The present chapter aimed to asses and re-evaluate the bioinformatics analysis approaches for the NGS data within the thesis in relation to published strategies that have been used in the NGPD field as a whole while proffering strategies that could enhance ligand identification in the future. The NGS data from chapter 3 (panning whole oocysts with Griffin library) was first interrogated and the effect of HCDR3 flanking region changes on the selection of the scFvs from both analyses were identified. The first set of analysis performed on the data compared growth and selection phases and selected scFvs that had been enriched in selection phases and not growth only phases (refer to chapter 3, subsection 3.2.3.1). With this analysis, five binders were identified that bound to *Eimeria* species oocysts (refer to **chapter 3**, table 3.9). The second analysis was tailored towards finding species-specific scFvs by comparing each species versus the rest and making selections based on Z scores ranking (refer to **chapter 3**, subsection 3.2.4). With this analysis, difficulties were encountered in the rescue of candidate scFvs but out of the two rescued, one was found to be binding to *Eimeria* species. In the present chapter, the analysis also then considered if the percentage frequency values of clones are an indication of binders that are positive in ELISAs and what percentage frequency were the true binders. This was also compared to the frequencies of the V_HHs (from the sequencing data in **chapters 5 and 6**) that were selected and tested, to see if some of them were expected to be positive via ELISA In that they displayed comparable frequencies to true binders from **chapter 3**. This also considered if true binders at high frequency were removed due to high stringency bioinformatic subtraction steps performed in **chapters 5 and 6**. The analysis also

considered if there were any traits of the known positive scFvs that could be used to select V_HHs.

7.2. Results

7.2.1 Flanking region changes

7.2.1.1 ScFv flanking region changes

The flanking regions of HCDR3 from the scFv library used are not fixed hence the degeneracy of the flanking sequence permitted when identifying the HCDR3 sequences could influence the datasets analysed, and the scFvs selected and tested. In this chapter, re-analysis of the scFv dataset from **chapter 3** was performed to consider this effect of degeneracy allowances within the HCDR3 flanking regions on scFv candidate selection. In chapter 3, the original datasets allowed 1 amino acid sequence change in each of the flanking regions (VYYCAR and WGQGT). In the present chapter, 0 and 2 changes per flanking sequence was allowed. This was performed for both sets of analysis (comparison of growth and selection and species-specific selection). This analysis was aimed at seeing whether the original scFvs

identified allowing 1 sequence change would still be within the data and if they would still be the candidates that would be selected for rescue or whether they had been replaced by other scFvs containing more (2) or less (0) changes within their flanking regions.

The scFvs identified by the original '1-change allowed' analysis were selected based on those that had Z score of >5, were present at 5 or more reads and were enriched in at least two panning rounds when compared to the preceding panning round as described in the materials and methods chapter. scFvs were then selected from these candidates based on those that were enriched in selection rounds and not growth only phases, and 43 scFvs met these criteria (**chapter 2** subsection *3.2.2.1*). In the second set of analysis, scFvs enriched against each individual species were compared against scFvs enriched against the other species generating six different sets of Z scores and then the top scFvs that were specifically enriched against a particular *Eimeria* species and the 10 that had the highest Z scores were selected for rescue. *Eimeria tenella* analysis versus the rest generated only 9 scFvs that fit the criteria and as a result, 69 scFvs were selected in total for the rescues.

From the first set of analysis comparing growth and selection, within the '0- change allowed, dataset, 6 (14%) out of the original 43 candidates were missing from the data (Table 7.1). This suggests that they had at least 1 change within their flanking regions and had been omitted by the search criteria. For the '2 change allowed' data, an

additional 7 candidates (KMSEHTSDG, ICNINSS, LIPATSYAR, MSLLFDY, NTLYH, GKPACS, FDVENWAW) that met the search criteria were isolated in addition to the 43 that had been originally selected (Table 7.1).

Table 7:1: ScFv candidates selected for panning against whole oocysts from the growth and selection analysis with the original '1 change allowed' within each flanking sequence versus '0 change allowed and '2 change allowed'

scFvs candidates selected		
Original 1 change allowed	**0 change allowed**	**2 changes allowed**
DHTPKPq	DHTPKPq	DHTPKPq
DHTPKRq	***	DHTPKRq
DHTPKWq	DHTPKWq	DHTPKWq
DHTPKWQ	DHTPKWQ	DHTPKWQ
DRTPKWq	DRTPKWq	DRTPKWq
DGKPPSE	DGKPPSE	DGKPPSE
DGKPPSG	DGKPPSG	DGKPPSG
DHAPKWq	DHAPKWq	DHAPKWq
DHMPKWq	***	DHMPKWq
DHTLKWq	DHTLKWq	DHTLKWq
DHTPKpq	DHTPKpq	DHTPKpq
DHTPKqq	DHTPKqq	DHTPKqq
DHTPKRq	DHTPKRq	DHTPKRq
DHTPKWo	DHTPKWo	DHTPKWo
STqHP	STqHP	STqHP
STQLP	STQLP	STQLP
VDISAWSFME	***	VDISAWSFME
AWTVNQTTNY	AWTVNQTTNY	AWTVNQTTNY
AYPA	***	AYPA
DRSYTACDTSK	DRSYTACDTSK	DRSYTACDTSK
EDRqCKHG	***	EDRqCKHG
GGKPPSE	GGKPPSE	GGKPPSE
LMVYKH	LMVYKH	LMVYKH
LWFNY	LWFNY	LWFNY
MIMDG	***	MIMDG
MWAR	MWAR	MWAR
QDFPA	QDFPA	QDFPA
QTGMN	QTGMN	QTGMN
RDLTqDMWR	RDLTqDMWR	RDLTqDMWR
RRLA	RRLA	RRLA
SPRLPFDY	SPRLPFDY	SPRLPFDY
STAVTV	STAVTV	STAVTV
VAADPFDY	VAADPFDY	VAADPFDY
YIRYTSPV	YIRYTSPV	YIRYTSPV
DKSSqGT	DKSSqGT	DKSSqGT
KDVGVVPK	KDVGVVPK	KDVGVVPK
ESSqMNS	ESSqMNS	ESSqMNS
SSDAP	SSDAP	SSDAP
STQHP	STQHP	STQHP
IHRMVYL	IHRMVYL	IHRMVYL
SAqHP	SAqHP	SAqHP
TTALP	TTALP	TTALP
SRSKNYLWRSM	SRSKNYLWRSM	SRSKNYLWRSM
		KMSEHTSDG
		ICNINSS
		LIPATSYAR
		MSLLFDY
		NTLYH
		GKPACS
		FDVENWAW

[1]The amino acid sequence of the original HCDR3 being rescued based on the 1-change allowed dataset.
[2] The data was re-analysed permitting 0 changes within each flanking sequence, presence or absence of sequence in the dataset
[3] The data was re-analysed allowing 2 changes within each flanking sequence, presence or absence of sequence in the dataset. Blue highlight indicates additional scFvs identified based permitting 2 changes in each flanking sequence
*** Missing scFvs

For the second set of analysis, out of the 69 candidate scFvs selected from the original '1 change allowed', 13 scFvs (19%) were missing from the final datasets when no changes were allowed in the flanking regions (Table 7.2). This suggests that they had at least 1 change within their flanking regions and had been excluded by the search criteria. Comparison of the final list from the 0 and 2 changes datasets were performed against the original list from the '1 change allowed' analysis to see whether the original candidates still made the top 10 candidates for each species (Table 7.2). For *E. mitis*, only five (50%) of the original 10 top candidates emerged among the top ten. For *E. necatrix, E. acervulina and E. praecox*, only four (40%) of the original candidates emerged as the top ten. For *E. brunetti*, 7 (70%) of the original candidates still emerged as the top 10 while for *E. maxima*, only 2 (20%) out of the original scFvs selected emerged as the top 10. For *E. tenella*, 5 (50%) out of the original scFv candidates emerged as the top 10 (Table 7.2). Taken all together, whilst all scFvs were present in the datasets when 2 changes were allowed in the flanking regions, only 45% of the top candidates from the '1 change allowed' were selected under the same criteria when using the '2 changes allowed' analysis. For 'no changes allowed' analysis, all 81% of the scFvs present in the datasets were selected in the top 10s.

Table 7:2: Original list of rescue candidates for panning against whole *Eimeria* species oocysts for the species-specific selection based on 1 change allowed within each flanking sequence compared to 0 or 2 changes

Original Rescue [1]	**Specificity [2]**	**0 change [3]**	**2 changes [4]**	**Still top 10 candidate[5]**	
				0 change	**2 changes**
ANACID	*E. mitis*	Yes	Yes	Yes	Yes
RWRAMR	*E. mitis*	Yes	Yes	Yes	Yes
SEYCEKTPGG	*E. mitis*	No	Yes	N/A	Yes
LWRNYYSS	*E. mitis*	No	Yes	N/A	Yes
CGNVN	*E. mitis*	Yes	Yes	Yes	Yes
TCTTLPIGLQA	*E. mitis*	Yes	Yes	Yes	No
DDTINVEV	*E. mitis*	Yes	Yes	Yes	No
KHICRFDY	*E. mitis*	Yes	Yes	Yes	No
VWSTESI	*E. mitis*	Yes	Yes	Yes	No
PHYVLM	*E. mitis*	Yes	Yes	Yes	No

RQTKYWYDCSD	*E. necatrix*	No	Yes	N/A	Yes
GYRFCHNSRD	*E. necatrix*	Yes	Yes	Yes	Yes
ELPPTPITS	*E. necatrix*	Yes	Yes	Yes	Yes
AFEWKTRVLF	*E. necatrix*	Yes	Yes	Yes	Yes
RRPDLLM	*E. necatrix*	Yes	Yes	Yes	No
LESTSV	*E. necatrix*	Yes	Yes	Yes	No
CWKGQG	*E. necatrix*	Yes	Yes	Yes	No
VNPTKFDY	*E. necatrix*	Yes	Yes	Yes	No
WVPAGWY	*E. necatrix*	Yes	Yes	Yes	No
FEWRPPTSCQG	*E. necatrix*	Yes	Yes	Yes	No
ARWRAEK	*E. acervulina*	Yes	Yes	Yes	Yes
FCVSHFDY	*E. acervulina*	Yes	Yes	Yes	Yes
PSYWRFDY	*E. acervulina*	Yes	Yes	Yes	Yes
CAPEE	*E. acervulina*	Yes	Yes	Yes	Yes
ICSPRATRRQ	*E. acervulina*	Yes	Yes	Yes	No
DQHWQPLAMS	*E. acervulina*	Yes	Yes	Yes	No
EKTWDFDY	*E. acervulina*	Yes	Yes	Yes	No
QQNENSD	*E. acervulina*	Yes	Yes	Yes	No
RDQSHKQEMS	*E. acervulina*	No	Yes	N/A	No
ASNCERE	*E. acervulina*	Yes	Yes	Yes	No
NHMRTFDY	*E. brunetti*	Yes	Yes	Yes	Yes
KWNSGRDAD	*E. brunetti*	Yes	Yes	Yes	Yes
APRWDI	*E. brunetti*	Yes	Yes	Yes	Yes
FAAMCDDV	*E. brunetti*	Yes	Yes	Yes	Yes
TGVENWTL	*E. brunetti*	Yes	Yes	Yes	Yes
MAEPPLTTHT	*E. brunetti*	Yes	Yes	Yes	Yes
HSQVPAVAPqF	*E. brunetti*	No	Yes	N/A	Yes
IWGPQFDY	*E. brunetti*	Yes	Yes	Yes	No
RADRASF	*E. brunetti*	No	Yes	N/A	No
RAILSRNGLHC	*E. brunetti*	Yes	Yes	Yes	No
SPRMF	*E. maxima*	Yes	Yes	Yes	Yes
SFTLVMDHPS	*E. maxima*	No	Yes	N/A	Yes
NRYSKT	*E. maxima*	Yes	Yes	Yes	No
RLFQVE	*E. maxima*	Yes	Yes	Yes	No
ASRDPKMNQVL	*E. maxima*	Yes	Yes	Yes	No
MTTVFR	*E. maxima*	Yes	Yes	Yes	No
WRMPTP	*E. maxima*	No	Yes	N/A	No
ENTMRGFGL	*E. maxima*	Yes	Yes	Yes	No
FMVGDG	*E. maxima*	No	Yes	N/A	No
VEGPRFDY	*E. maxima*	Yes	Yes	Yes	No
KELIQPTV	*E. praecox*	Yes	Yes	Yes	Yes
YTRKRFRRPER	*E. praecox*	Yes	Yes	Yes	Yes
PWPTPNQ	*E. praecox*	No	Yes	N/A	Yes
SGSPPFDY	*E. praecox*	Yes	Yes	Yes	Yes
LRNGKSHMMRTV	*E. praecox*	No	Yes	N/A	No
PAGSRTL	*E. praecox*	Yes	Yes	Yes	No
HRPKQGKA	*E. praecox*	Yes	Yes	Yes	No
RFIMSHS	*E. praecox*	Yes	Yes	Yes	No
LNTNDV	*E. praecox*	Yes	Yes	Yes	No

TRLRPTNLKV	*E. praecox*	Yes	Yes	Yes	No
IGPSA	*E. tenella*	Yes	Yes	Yes	Yes
LVNRSSNEQG	*E. tenella*	Yes	Yes	Yes	Yes
LAPSLSERPV	*E. tenella*	Yes	Yes	Yes	Yes
KRAHGRNEPA	*E. tenella*	No	Yes	N/A	Yes
GGFSMTGLF	*E. tenella*	Yes	Yes	Yes	Yes
ASYRGH	*E. tenella*	Yes	Yes	Yes	No
TIAIGFDY	*E. tenella*	No	Yes	N/A	No
SHQTqWQSSIAE	*E. tenella*	Yes	Yes	Yes	No
RILDLDT	*E. tenella*	Yes	Yes	Yes	No

[1]The amino acid sequence of the original HCDR3 being rescued based on the 1-change allowed dataset
[2] Specificity to target *Eimeria* species
[3] The data was re-analysed permitting 0 changes within each flanking sequence, presence or absence of sequence in the dataset
[4] The data was re-analysed allowing 2 changes within each flanking sequence, presence or absence of sequence in the dataset
[5] determining if the HCDR3s are still top candidates after 0 and 2 changes permitted

7.2.1.2 Using *V_HH flanking region changes to predict error* rates caused by amplification and sequencing

The flanking regions of V_HH are fixed hence no degeneracy of the flanking sequence was permitted when sifting the HCDR3 sequences which could have influenced the datasets that were analysed, and the antibodies that were selected and tested. In this chapter, the V_HH dataset from **chapter 5** (V_HH/oocyst lysate panning) and **chapter 6** (V_HH/peptide panning) were re-analysed to look at the effect of degeneracy allowances within the HCDR3 flanking regions on the number of reads. In **chapters 5** and **6**, the original datasets allowed no amino acid sequence change in each of the flanking regions (IYYCAA and WGQGT) because the V_HH library had a conserved V_HH scaffold and varied only in the HCDR3s. In the present chapter, 1 and 2 changes per flanking sequence was allowed. This analysis was aimed at predicting error rates caused by amplification and sequencing by comparing reads in the pool of positive replicate samples when no degeneracy was allowed to when 1 or 2 changes were allowed in the flanking sequences.

The original 'no change allowed' analysis compared each positive barcode versus the pool of negative barcodes before selecting candidate V_HHs that were seen in at least four positive barcode replicates (for **chapter 5**) and three positive replicates (for **chapter 6**). In the present chapter, the reads in the pool of the positive barcodes for each target was compared for the '0 change allowed' versus the '1 change allowed' and '2 change allowed' for both datasets from V_HH-oocyst lysate panning and V_HH-peptide panning (Table 7.3 and 7.5). The percentage increase in the number of reads when 1 change in the flanking sequences was allowed and when 2 changes in the

flanking sequences were allowed were also calculated (Table 7.4 and Table 7.6). When all data were taken together, the percentage increase in the number of reads V_HH-oocyst lysate dataset from the original analysis and when 1 change was allowed was 9.63%, when 2 changes were allowed, it was 72.74%. For the V_HH-peptide dataset percentage increase in the number of reads from the original analysis and when 1 change was allowed was 8.73% and when 2 changes were allowed the increase in the number of reads was 63.33%.

Table 7:3: Total reads in the pool of positive replicate samples for each target antigen after 0-2 flanking region changes (V_HH-oocyst lysate panning)

Target	0 change	1 change	2 changes
E. mitis	1783738	1939302	3315638
E. necatrix	1509283	1703789	2937447
E. acervulina	1848242	1986376	3242054
E. brunnetti	1846488	2009393	3594353
E. maxima	1649723	1804353	3238848
E. praecox	1918762	2103377	3553536
E. tenella	2000472	2210489	3874890

Table 7:4: Percentage increase in total reads for each round of panning after comparison to 0 flanking region changes

Target	1 change	2 changes
E. mitis	8.72	70.97
E. necatrix	12.88	72.41
E. acervulina	7.47	63.21
E. brunnetti	8.82	78.88
E. maxima	9.37	79.50
E. praecox	9.62	68.94
E. tenella	10.50	75.30

Table 7:5: Total reads in the pool of positive replicate samples for each target peptide after 0-2 flanking region changes (V_HH-peptide panning)

Target	0 change	1 change	2 changes
QSFSLPIPIDRPL	1814107	2062262	3354457
QSMAVPTTVENTV	2999228	3322567	5348484
PAMAVPSTTSVESTVL	2657702	2914342	4836475
PSLALPSSSAAEPAAVH	4063810	4422689	7234345
PSMAVPSTTPVENQVH	3250358	3527839	5752552
QSLALPTPIDRAF	3607170	3934764	6725366
QSLAVPTTVENTV	2046599	2194847	3398484
MDSTPDFPAFNEPM	3542695	3739930	6546478
MDTMPEFPPFAEGM	5594378	5883836	8998373

Table 7:6: Total reads in the pool of positive replicate samples for each target peptide after 0-2 flanking region changes (V_HH-peptide panning)

Target	1 change	2 changes
QSFSLPIPIDRPL	13.68	62.66
QSMAVPTTVENTV	10.78	60.97
PAMAVPSTTSVESTVL	9.66	65.95
PSLALPSSSAAEPAAVH	8.83	63.57
PSMAVPSTTPVENQVH	8.53	63.06
QSLALPTPIDRAF	9.08	70.92
QSLAVPTTVENTV	7.24	54.84
MDSTPDFPAFNEPM	5.56	75.04
MDTMPEFPPFAEGM	5.17	52.93

7.2.2: Reanalysing V_HH data using percentage frequency and comparison of percentage frequency of known scFvs binders versus V_HH selected rescues

The bioinformatics analysis strategy employed in **chapter 3** were based on two approaches, all of which relied on Z score analysis to define enrichment of a particular antibody. The first analysis generated Z scores for each scFv based on the frequency and ratio of binding when comparing progressive rounds of panning (from round 1 to round 2, round 2 to round 3, round 3 to round 4, and round 4 to round 5). Followed by a comparison between the frequency of scFvs in growth only phases and selection+growth phases (refer to **chapter 2**, subsection 2.3.6). Following this analysis and testing of selected scFvs, six binders were found to bind to *Eimeria* species out of 13 rescued scFvs from 2 sets of analysis.

The second strategy aimed at enhancing the recovery of species-specific clones identifying enrichment by comparing scFvs identified against each species versus the rest. Of the two scFvs rescued, 1 was found to bind to all species of *Eimeria*. The bioinformatics analysis conducted in **chapters 5** and **6** utilised the same latter strategy and so had very strict selection criteria to try and select for species specific CDR3s to individual *Eimeria* species.

Many published NGS phage display studies have based their candidate selection on overall frequency generating a ranking system to identify enrichment but without comparing binding to the target versus controls. Alternatively, studies compared the enrichment of individual candidates across panning rounds as was performed in **chapter 3** of this study (Miyazaki *et al.,* 2015). Analysing and comparing the enrichment of candidates across panning rounds could not be conducted for **chapters 5** and **6** because sub-libraries from the panning rounds were not sequenced. The highly stringent panning steps including pre-panning subtraction and post-

selection subtraction of phage, this coupled with the strict bioinformatic strategy may have led to the failure to identify true target-specific binders. To determine the possibility of a candidate frequency-based bioinformatics analysis changing the candidate binders that were identified, the V_HH NGS datasets of **chapter 5** (panning against oocyst lysates) and **chapter 6** (panning against distinct peptide regions of Gam56 protein), were reanalysed by ranking HCDR3s according to percentage frequency in positive replicates. This would select for binders without imposing comparative enrichment criteria in the bioinformatic steps.

To support this approach, the frequency values of scFvs from **chapter 3** that were true binders were determined. These frequency values were scrutinised to find out whether percentage frequency of scFvs have any relationship with positive ELISA results while comparing to published results to find what percentage of frequency reflect true binders. The V_HH datasets from **chapters 5** and **6** were then compared to the frequencies of true scFv binders to determine if some V_HHs are expected to be binders.

The positive panning replicate's HCDR3 from the round 3 screen were pooled into a single list. This was performed for both the V_HH-oocyst panning and V_HH-peptide panning datasets. The HCDR3 sequences were displayed as a percentage of the total reads. The top 5 frequency candidates are shown in tables 7.7-7.13 for V_HH-oocyst lysate panning and 7.12-7.20 for V_HH-peptide analysis. Any of the V_HH candidates that had been isolated in the original Z score analysis (that selected the top 5 candidates for each target) were highlighted in green.

Table 7:7: The top 5 frequency candidates for *E. mitis* V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	ITAWGAPSQRDL	22.74304979
2	AKSSPQPMTVSLACqP	3.401031858
3	PLVKRSqqNQTSCDVR	2.943183348
4	PAAqYMNTqLqTAWLD	2.878563429
5	LCLNRSqLTVNQ	2.66638172

[1]Frequency ranking of each HCDR3 candidate.

[2] HCDR3 sequence of *E. mitis* V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:8: The top 5 frequency candidates for *E. necatrix* V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	ITAWGAPSQRDL	21.51507369
2	RqPVERQRMTVATAPRKTqRV	3.527579562
3	WAqYSKPPSTYVYYLGKRYLI	2.966832715
4	qRFFqTSLRTHS	2.613457552
5	AKSSPQPMTVSLACqP	2.110471156

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of *E. necatrix* V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:9: The top 5 frequency candidates for *E. acervulina* V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	ITAWGAPSQRDL	22.62404215
2	qPMLTPQYDSqRANYK	6.011086364
3	qRFFqTSLRTHS	4.222185928
4	IGPYDTQMRSDL	3.401540402
5	PLVKRSqqNQTSCDVR	2.730603522

[1] Frequency ranking of each HCDR3 candidate.

[2] HCDR3 sequence of *E. acervulina* V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:10: The top 5 frequency candidates for *E. brunetti* V_HH dataset

Rank [1]	**Sequence [2]**	**% of total reads (positive) [3]**
1	ITAWGAPSQRDL	17.86912668
2	WAqYSKPPSTYVYYLGKRYLI	4.303990789
3	RqPVERQRMTVATAPRKTqRV	2.795800525
4	qRFFqTSLRTHS	1.983677104
5	PLVKRSqqNQTSCDVR	1.817677564

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of *E. brunetti* V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

7:11: The top 5 frequency candidates for *E. maxima* V_HH dataset

Rank [1]	**Sequence [2]**	**% of total reads (positive) [3]**
1	ITAWGAPSQRDL	24.72562799
2	LCLNRSqLTVNQ	5.267400137
3	LqLLQPHTHIVI	4.244069998
4	RKTAqSTASPTL	3.829539598
5	FVAPIqTFDFPRLLSR	3.180348273

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of *E. maxima* V_HH candidates. Candidates sselected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:12: The top 5 frequency candidates for *E. praecox* V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	ITAWGAPSQRDL	24.72562799
2	LCLNRSqLTVNQ	5.267400137
3	LqLLQPHTHIVI	4.244069998
4	RKTAqSTASPTL	3.829539598
5	FVAPIqTFDFPRLLSR	3.180348273

[1] Frequency ranking of each HCDR3 candidate.

[2] HCDR3 sequence of *E. praecox* V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.

[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:13: The top 5 frequency candidates for *E. tenella* V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	ITAWGAPSQRDL	21.72730227
2	qRFFqTSLRTHS	8.400983933
3	PLVKRSqqNQTSCDVR	3.638119339
4	RqPVERQRMTVATAPRKTqRV	2.589588369
5	LCLNRSqLTVNQ	2.338670425

[1] Frequency ranking of each HCDR3 candidate.

[2] HCDR3 sequence of *E. tenella* V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.

[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:14: The top 5 frequency candidates for peptide QSFSLPIPIDRPL V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	DLNYPRTqHRRKQKNE	62.909426
2	GTNFDMSVMSqLHNLLRFEqS	39.9378282
3	TDHRISqGLHVKKHAT	27.3179573
4	KANHELKLYTRLqPFT	23.0551901
5	GTNFDMSVMSqLHNLLRFEqS	21.3500702

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of QSFSLPIPIDRPL V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:15: The top 5 frequency candidates for peptide QSMAVPTTVENTV V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	qRFFqTSLRTHS	22.76944823
2	QWELPTPFHPCSIDGAHKTqW	10.12577589
3	qSMRHRQQQKSPGYMHNNWHW	8.123404575
4	VKGQTSGRKNYVNGMREARLq	5.406576177
5	QWELPTPFHPCSIDGAHKTqW	3.516875405

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of QSMAVPTTVENTV V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:16: The top 5 frequency candidates for peptide PAMAVPSTTSVESTVL V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	ITAWGAPSQRDL	13.4715976
2	SKCLHTLSRSqLHPHFQFHDI	8.772468238
3	GVqVEFYTNPLNDKLNLRCDq	4.43819767
4	AqPIRGKVTIVL	2.091127716
5	CGATqTRGRFITFPASPYASq	1.786357619

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of PAMAVPSTTSVESTVL V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.

[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:17: The top 5 frequency candidates for peptide PSLALPSSSAAEPAAVH V_HH dataset

Rank [1]	**Sequence** [2]	**% of total reads (positive)** [3]
1	ITAWGAPSQRDL	42.43571481
2	qPPRMYTLIRqKSAAP	10.083282111
3	qHqKSKDGNNPMPKPS	0.502263936
4	CqLTTqSTELRARRKS	0.451572265
5	CQQKLGPPHWTAEqGRCTQAS	0.312839667

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of PSLALPSSSAAEPAAVH V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:18: The top 5 frequency candidates for peptide PSMAVPSTTPVENQVH V_HH dataset

Rank [1]	**Sequence** [2]	**% of total reads (positive)** [3]
1	AKSSPQPMTVSLACqP	34.88778404
2	ITAWGAPSQRDL	20.35677877
3	PAAqYMNTqLqTAWLD	6.715581159
4	FLLHNNqNLLLQRMLL	4.357467126
5	qTKSFGMGHqNRCVWKRTRTR	3.987730903

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of PSMAVPSTTPVENQVH V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:19: The top 5 frequency candidates for peptide QSLALPTPIDRAF V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	RqPVERQRMTVATAPRKTqRV	22.12875166
2	WAqYSKPPSTYVYYLGKRYLI	18.33659541
3	qTKANMKGLqTPGYGqLGLKF	13.10062677
4	CFGQCEHSqYTRGMTGRGRHS	10.49784645
5	ASSNSVHLSEqPLHHP	9.197479464

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of QSLALPTPIDRAF V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:20: The top 5 frequency candidates for peptide QSLAVPTTVENTV V_HH dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	RqPVERQRMTVATAPRKTqRV	6.0438083
2	WAqYSKPPSTYVYYLGKRYLI	5.128724353
3	CFGQCEHSqYTRGMTGRGRHS	4.810446552
4	QNYLTVTqTRPTKTFH	4.221326355
5	qTKANMKGLqTPGYGqLGLKF	3.503429995

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of QSLAVPTTVENTV V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:21: The top 5 frequency candidates for peptide MDSTPDFPAFNEPM V*H*H dataset

Rank [1]	Sequence [2]	% of total reads (positive) [3]
1	PLVKRSqqNQTSCDVR	11.71091084
2	PHLKFPVLMFCqHHGS	10.10688035
3	PSASYAGMqRRWKQNL	7.322821436
4	YqLTFAHTPAAFVYVFRSVAS	6.654784085
5	IVqTLYMRPVVINAARCRGCT	6.333080391

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of MDSTPDFPAFNEPM V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.

[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

Table 7:22: The top 5 frequency candidates for peptide MDTMPEFPPFAEG V_HH dataset

Rank [1]	**Sequence** [2]	**% of total reads (positive)** [3]
1	ITAWGAPSQRDL	36.3530368
2	LCLNRSqLTVNQ	13.83221656
3	LqLLQPHTHIVI	1.972216944
4	RKTAqSTASPTL	1.872657464
5	qYDYAFHRMTKRYLTA	1.745860873

[1] Frequency ranking of each HCDR3 candidate.
[2] HCDR3 sequence of MDTMPEFPPFAEG V_HH candidates. Candidates selected from the initial NGS analysis were highlighted in green.
[3] The positive pool read count of the HCDR3 candidate expressed as a % of the total reads.

With reference to tables 7.7-7.13 for the V_HH-oocyst lysate dataset, just 11% of the five top V_HH candidates for all targets from the original Z score analysis were identified. With reference to tables 7.14-7.22 for the V_HH-peptide panning dataset, 27% of the 5 top V_HH candidates for all targets from the original Z score analysis were identified. For both datasets; V_HH-oocyst lysate and V_HH-peptide panning, there was a consistent distribution of frequencies within their positive barcodes when three positive replicate barcodes were considered with coefficient of variation (CV) values across the replicates usually <30% (data not shown). The ITAWGAPSQRDL antibody was seen as the top binder for all panning against oocysts and also 2 distinct peptides. The frequencies of the known scFv binders across the five panning rounds were analysed and it was observed that for all identified scFvs except DHTPKWq, there was a steady increase in the frequencies from the rounds 1-3 of panning and then a decline from the round 4 panning. For DHTPKWq, there was a sharp increase in frequency between round 3 and 4 of panning and by the 5th round of panning, it dominated at over 90% and that coincides with other isolated clones declining in frequencies. The conventionally picked scFvs DHTPKWq, STQLP and STqHP as expected had the highest frequencies (Table 7.23). In all 35 barcodes which include the five panning rounds across the seven species the clones identified from the random colony picks ranked amongst the top 10 in frequency with the exception of four barcodes (data not shown). Interestingly, the isolated scFv that was displayed most and had the strongest binding was missing in the round 1 panning of all species with the exception of *E. tenella*. Frequencies of the known binder STqHP ranged

between ranged between 0.000284 -8.9%, it is the second most abundant scFv followed by STQLP with a percentage frequency range of between 0.0 to 0.8%. The percentage frequency of the known binder SAqHP was between 0.0000184 to 0.028849% across panning rounds in different species while the frequency of SRSKNYLWRSM was between 0.000129 to 0.5%. The frequency of YTRKRFRRPER was between 0.000108 to 1.4% across panning rounds (Table 7.23). Overall, the highest frequencies of five of the six true binders in any panning round ranged from 0.03% to 8.9%. The frequencies of the selected candidate V_HHs that were screened from both analyses are usually much higher than the frequencies of the known scFv binders oocysts panning.

Table 7:23: Percentage frequency of known scFv binders across panning rounds for all seven *Eimeria* species

Target Eimeria species/ Panning rounds [1]	DHTPKWq	STQLP	STqHP	SAqHP	SRSKNLYWRSM	YTRKRFRRPER
E. mitis Round 1	0.490737	0.001738	0.000284	0.0000184	***	***
E. mitis Round 2	0.823809	0.009939	0.001389	***	0.201714	***
E. mitis Round 3	0.0152	0.011161	0.004582	0.0000907	0.061769	0.000386
E. mitis Round 4	86.88682	0.536499	6.262453	0.015779	0.012709	0.002047
E. mitis Round 5	94.97145	0.090527	2.101463	0.003461	0.001923	0.00011
E. necatrix Round 1	0.909633	0.023845	0.053234	0.00016	***	***
E. necatrix Round 2	2.555651	0.359373	1.393162	0.003248	0.003061	***
E. necatrix Round 3	48.22387	0.787788	6.146705	0.006911	0.031367	0.005966
E. necatrix Round 4	83.9549	0.568431	8.91122	0.015947	0.003621	0.001842
E. necatrix Round 5	91.7641	0.234299	5.303911	0.010837	0.000542	0.000108
E. acervulina Round 1	0.704863	0.002458	0.032933	0.000492	***	***
E. acervulina Round 2	0.782861	0.005	0.52829	***	0.000134	***
E. acervulina Round 3	25.36691	0.21225	1.447962	0.003624	0.000129	0.018895
E. acervulina Round 4	85.47844	0.585268	7.034054	0.01507	0.002374	0.001548
E. acervulina Round 5	92.40877	0.22588	4.427293	0.01111	0.001165	***
E. brunetti Round 1	1.333894	0.109374	0.238495	0.000143	***	0.003848
E. brunetti Round 2	9.661929	0.49409	1.258969	0.002682	0.02648	0.000112
E. brunetti Round 3	65.48298	0.3497	2.559171	0.004769	0.543625	0.012716
E. brunetti Round 4	90.39023	0.295676	4.348985	0.00587	0.001791	0.000597
E. brunetti Round 5	96.71434	0.065268	1.292457	0.001784	0.00021	***
E. maxima Round 1	0.232144	0.001219	0.004878	***	***	0.000152
E. maxima Round 2	5.109374	0.045683	0.248745	***	0.007016	***
E. maxima Round 3	17.38644	0.22715	1.319281	0.002128	0.268511	0.061974
E. maxima Round 4	86.85292	0.577737	6.92758	0.010867	0.008694	0.003557
E. maxima Round 5	94.62543	0.170525	2.723774	0.007046	0.000604	***
E. praecox Round 1	0.962854	0.002493	0.022583	0.000293	***	***
E. praecox Round 2	4.712347	0.017818	0.187473	***	0.328998	1.4773
E. praecox Round 3	7.610627	0.046874	0.561986	0.001512	0.084298	0.211564
E. praecox Round 4	89.31016	0.669139	5.083965	0.01193	0.019107	0.009699
E. praecox Round 5	95.94472	0.147139	1.794253	0.002988	0.001292	0.000404
E. tenella Round 1	0.263431	0.002876	0.028239	0.00012	0.001558	***
E. tenella Round 2	0.79224	0.061909	0.354748	0.000531	0.001993	0.000733
E. tenella Round 3	64.23593	0.581749	7.218855	0.018306	0.04704	0.023868
E. tenella Round 4	84.28771	0.525592	8.280912	0.020315	0.004382	0.000996
E. tenella Round 5	93.24491	0.178326	3.913582	0.028849	0.006474	0.000226

[1] Species of *Eimeria* oocysts and panning rounds; five panning rounds were conducted

*** scFv missing in the barcode

7.2.3: Reanalysing V_HH data using an alternative *in silico* analysis

This *in silico* analysis was performed in an attempt to further identify and test HCDR3s that were binding to oocysts of the seven *Eimeria* species during NGS analysis but cannot be verified by ELISA possibly due to the latter having a lower assay sensitivity. This analysis was applied to datasets from panning against V_HH-oocyst lysates. The failure to find any binder to *Eimeria* oocysts in both V_HH-oocyst lysate panning dataset using conventional ELISA informed this analysis.

In this screen, HCDR3s were selected in a training cohort of samples using the criteria 1) Z scores >3 in positive replicates 2) those identified in at least 2 out of 3 positive replicates which served as the 'training' cohort. HCDR3s identified that met these criteria were then screened in a test cohort (n=3) of samples under the same criteria. For *E. mitis*, 10 HCDR3s met the selection criteria for both the training and test (Table 7.24). When *E. necatrix* positive replicate barcodes were screened, 14 HCDRs met the criteria (Table 7.25). For *E. necatrix* dataset, there were only five replicate barcodes (refer to **chapter 5** subsection 5.2.1.5). For *E. acervulina* dataset, 15 HCDR3s met the criteria (Table 7.26). For *E. brunetti* dataset, 16 HCDR3s met the criteria (Table 7.27) while 125 HCDR3s met the criteria when *E. maxima* dataset was considered hence the Z score cut off was increased to 5.0 then screened again and 38 HCDR3s were found to meet the criteria (Table 7.28). No HCDR3 met the selection criteria when dataset of *E. praecox* was considered and only 2 HCDR3s met the criteria when *E. tenella* dataset was considered (Table 7.29).

Table 7:24: HCDR3s identified when screening training and test cohorts for *E. mitis*

		Test cohort3		
HCDR3[1]	**Training cohort[2]**	**BC 04**	**BC 05**	**BC 06**
ITAWGAPSQRDL	3/3	31.33	0	72.68
AKSSPQPMTVSLACqP	2/3	50.32	12.27	0
TSTGTLQSSRPPKHSH	2/3	11.31	5.52	0
AqSHAqCRLSNKELKR	2/3	4.72	4.58	9.15
DqRSPNSLCAqC	2/3	3.72	3.68	9.64
YAYSIMS	2/3	3.24	0	3.37
WFSSRGRQAPqLHYTG	2/3	0	4.48	6.37
RPQCHWTLNGGqLPqN	2/3	0	59.11	6.89
qEPLHRFLSLqT	2/3	4.62	8.65	0
LKKPRqKKPPKHHSLR	2/3	4.11	3.95	0

[1] HCDR3s raised against *E. mitis*, green highlight are HCDR3s that were selected in the original analysis
[2] BC01, BC02 and BC03 served as training cohort and HCDR3s were analysed to determine if they appeared at least 2 out of 3 times
[3] BC04, BC05 and BC04 served as test cohort in which selected HCDR3s from training cohort were searched for using the same criteria the HCDR3s from the training cohort were selected.

Table 7:25: HCDR3s identified when screening training and test cohorts for *E. necatrix*

		Test cohort[3]	
HCDR3[1]	**Training cohort[2]**	**BC 17**	**BC 18**
WaqYSKPPSTYVYYLGKRYLI	3/3	42.59	13.57
YLRSqCEqHNFLLDALNVEDA	2/3	31.32	8.72
RqPVERQRMTVATAPRKTqRV	3/3	5.89	25.29
CFGQCEHSqYTRGMTGRGRHS	2/3	20.46	7.20
SqPHHTSRLINI	3/3	18.59	3.08
qTKANMKGLqTPGYGqLGLKF	3/3	32.73	8.4
QNYLTVTqTRPTKTFH	3/3	37.46	6.01
qCMLVIRVDEWG	3/3	16.91	3.44
YLRSqCEqHNFLLDALNVEDA	3/3	31.32	8.72
TAPDPLqLVAVPWYRHYPYKG	2/3	7.76	3.1
RPQCHWTLNGGqLPqN	2/3	47.85	8.26
PSPAKTPLPTPEERPN	2/3	6.93	3.28
PQVqRIRNHVTTPRYM	2/3	9.23	55.93
NCSPAPSPPFMNLIIC	2/3	3.89	10.16

[1] HCDR3s raised against *E. necatrix*, green highlight are HCDR3s that were selected in the original analysis
[2] BC13, BC14 and BC15 served as training cohort and HCDR3s were analysed to determine if they appeared at least 2 out of 3 times
[3] BC16 and BC17 served as test cohort in which selected HCDR3s from training cohort were searched for using the same criteria the HCDR3s from the training cohort were selected.

Table 7:26: HCDR3s identified when screening training and test cohorts for *E. acervulina*

		Test cohort[3]		
HCDR3[1]	**Training cohort[2]**	**BC 28**	**BC 29**	**BC 30**
YqLTFCSYACCVLFMFFRSVAS	2/3	44.92	20.38	6.51
PSASYAGMqRRWKQNL	3/3	36.90	13.95	0
IHWqREKqLTTqSRTVPAHNM	3/3	4.33	4.96	8.38
MVVWKqHSSLAKTRCY	2/3	0	6.20	11.8
PHLKFSCFpCFCqHHGS	2/3	4.45	0	6.83
LYNqVKKQPRWVCqRQ	2/3	6.71	5.93	3.91
VRGPESIMNGPHSTTR	2/3	8.93	13.56	4.67
SqPFGPPMYYKL	2/3	0	7.82	15.16
SNMSSRWVTqNA	2/3	3.99	4.78	12.11
RTSDQHHNY	2/3	0	4.09	14.71
RTDKGHEGSqSFHDVP	2/3	0	8.89	9.16
NCIMTMSMLRTTTITR	2/3	8.99	7.90	6.55
MVVWKqHSSLAKTRCY	2/3	3.09	17.11	4.71
MqYVMTPQPqHLVSNL	2/3	11.67	5.19	6.01
KLRFRLTqRSDDHqKRYPRHS	2/3	13.91	4.12	3.51

[1] [1] HCDR3s raised against *E. acervulina*, green highlight are HCDR3s that were selected in the original analysis

[2] BC25, BC26 and BC27 served as training cohort and HCDR3s were analysed to determine if they appeared at least 2 out of 3 times

[3] BC28, BC29 and BC30 served as test cohort in which selected HCDR3s from training cohort were searched for using the same criteria the HCDR3s from the training cohort were selected.

Table 7:27: HCDR3s identified when screening training and test cohorts for *E. brunetti*

		Test cohort[3]		
HCDR3[1]	**Training cohort[2]**	**BC 40**	**BC 41**	**BC 43**
YNRLqRTGNATT	2/3	8.14	33.92	0
HsqLCKVPCVTRPRNY	2/3	0	5.18	51.55
IcqLLLHPLCAL	2/3	6.05	41.99	0
NyqPNWNPqKTS	2/3	31.29	0	4.80
IMGPMSTANKMNTMPQ	2/3	3.89	26.57	0
GKLGPGSLYTSq	2/3	21.91	3.87	0
VVILTQCTTKDY	2/3	14.19	0	5.78
VLGIHNGqIQYLILETLQq	2/3	8.72	8.11	0
SYWNSNVGLRKLSQqW	2/3	9.89	4.89	0
TNFCANPLMPTS	2/3	7.98	0	4.12
SYWNSNVGLRKLSQqW	2/3	19.46	5.18	0
NHSSPLSTNSPYTGqP	2/3	9.87	0	7.17
HSqLCKVPCVTRPRNY	2/3	0	8.81	3.90
DEqHENTMTYSA	2/3	6.18	0	4.77
ASESSVqLPERL	2/3	0	10.19	6.89
IVNMIRSLRNRP	2/3	0	6.56	4.18

[1] [1] HCDR3s raised against *E. brunetti*, green highlight are HCDR3s that were selected in the original analysis

[2] BC37, BC38 and BC39 served as training cohort and HCDR3s were analysed to determine if they appeared at least 2 out of 3 times

[3] BC40, BC41 and BC43 served as test cohort in which selected HCDR3s from training cohort were searched for using the same criteria the HCDR3s from the training cohort were selected.

BC42 was not used during the panning

Table 7:28: HCDR3s identified when screening training and test cohorts for *E. maxima*

		Test cohort[3]		
HCDR3[1]	**Training cohort[2]**	**BC52**	**BC53**	**BC54**
LCLNRSqLTVNQ	2/3	33.13	23.47	28.45
DLNYPRTqHRRKQKNE	2/3	22.04	19.80	17.40
SPVDTVRSGGAV	3/3	8.62	9.13	8.29
GPMQTAWqYAGF	2/3	7.8	0	8.28
qQPPPMHPHTYIPPYM	2/3	5.88	19.09	13.31
qQLFTWCLTRAAETHC	2/3	11.27	17.35	10.64
qPGDYLTPILVV	2/3	22.24	17.34	22.75
NMYSKCGK	3/3	9.49	6.85	0
NCSPAPSPPFMNLIIC	3/3	7.44	12.25	9.07
MPSLRTALTNSAFHIq	2/3	8.51	5.76	5.42
LKDRVSTGQKSCLHLP	2/3	14.21	9.28	0
KMPWMDHHNVqIGLHT	2/2	5.7	0	11.55
GRPNSqNHNRqRDGSD	2/3	6.8	12.39	11.48
FVSTHHqDLIPRTHTR	2/3	22.09	11.23	12.98
FDqGVTVqEHKDYLKR	2/2	12	9.1	10.43
ETqMMqCPGIRSFRLR	3/3	8.34	5.89	0
AISTSTSCLCqL	3/3	0	6.11	0
YITDCRVITGLF	2/3	13.41	8.09	5.11
YADqYASKSFKL	2/3	0	21.08	7.71
VPSTGDqPNVLCTIWL	3/3	11.13	10.9	11.96
VNSDHRRqYLRACSPC	3/3	9.18	8.85	7.96
VKQFAAqPQLPATGGR	2/3	10.88	9.5	8.56
VDVEKSDYNRCWLPLqqCPQT	2/3	9.11	7.33	16.26
THPNTQFWKMDTCVAL	3/3	7.78	6.38	6.16
SRARQARGTTPRRQIY	3/3	0	8.63	16.57
SDDLFRRLKTSL	2/3	17.11	18.42	0
SALTqHqDRKINQSDG	3/3	0	13.02	11.22
RNLAKAHYWKLMSqRRIMqHN	3/3	0	14.35	15.02
RLPLHPqRSSYS	3/3	12.49	0	11.7
RHHNCHVFVSAVGVGAITqQY	3/3	12.26	11.94	0
PSPAKTPLPTPEERPN	2/3	2.88	11.5	12.01
NFAFPNGGPPPA	2/3	8.58	10.77	10.36
MVqAGPKPASTCSEAYPPVVL	3/3	7.54	11.27	12.07
HLLTSPSYTSPPNGYE	2/3	12.59	11.34	10.03
KSSHIPPVTTNHKANq	2/3	12.18	12.53	0
ADPYYSVAqREFPKLH	2/3	9.62	0	10.37
WKSqqRTCPTCHYLqH	2/3	8.47	10.85	9.41
SLENSqGNNSWRSSGQ	2/3	7.75	9.32	8.9

[1] HCDR3s raised against *E. maxima*, green highlight are HCDR3s that were selected in the original analysis

[2] BC49, BC50 and BC51 served as training cohort and HCDR3s were analysed to determine if they appeared at least 2 out of 3 times

[3] BC52, BC53 and BC54 served as test cohort in which selected HCDR3s from training cohort were searched for using the same criteria the HCDR3s from the training cohort were selected.

Table 7:29: HCDR3s identified when screening training and test cohorts for *E. tenella*

		Test cohort[3]		
HCDR3[1]	**Training cohort[2]**	**BC 76**	**BC 77**	**BC 78**
ASSNSVHLSEqPLHHP	2/3	7.47	50.25	5.73
VNqMHYTIRDLV	2/3	5.7	5.09	0

[1] HCDR3s raised against *E. tenella*, green highlight are HCDR3s that were selected in the original analysis

[2] BC73, BC74 and BC75 served as training cohort and HCDR3s were analysed to determine if they appeared at least 2 out of 3 times

[3] BC76, BC77 and BC78 served as test cohort in which selected HCDR3s from training cohort were searched for using the same criteria the HCDR3s from the training cohort were selected.

From the V_HH-oocyst lysate dataset analysed, a number of V_HH candidates appeared across the positive barcode replicates having a relatively high Z score. The data indicates the antibodies are consistently enriched across multiple replicate analyses which may reflect true binders. However, very few antibodies were consistent in each of the six replicates.

7.3 Discussion

The emergence of next generation sequencing and coupling NGS analysis with phage display has the potential to transform the discovery of new ligands. The sheer amount of data output from deep sequencing should enable the analysis of complex phage display binding events and could potentially circumvent conventional laborious colony picking and functional screening of individual phage clones. The identification of potential candidates after phage display biopanning has been a major challenge associated with the phage display technique. Traditionally, <1000 individual clones from the final enriched sub libraries are randomly picked and amplified. Then functional testing of clones, usually ELISA based techniques can be used to distinguish the specific binders before the sequence of the displayed ligand with specific binding could be determined by Sanger sequencing.

Hence NGS methods are increasingly being coupled with phage display studies to allow for much greater insights into library diversity both before and after panning (Rouet *et al.,* 2018). Next generation sequencing has been applied in various studies of antibody display libraries (Saggy *et al*., 2012a, Sasso *et al*., 2015, Venet *et al*., 2012, Chen *et al*., 2012). There are different sequencing platforms and since NGS technologies differ in terms of chemistry, template amplification and detection principles, they offer different ranges of read depth, read length and accuracies. Of all the platforms, Life Technologies Ion Torrent (400bp) and Illumina's paired end MiSeq are the most widely used with antibody phage display studies.

In the present chapter, flanking region motif degeneracy for the scFv panning in **chapter 3** were changed to determine whether antibodies selected would change. The Griffin library human scFv HCDR3 flanking region sequence was VYYCAR HCDR3 WGQGT but the natural HCDR3 flanking sequences are not fully conserved, hence some level of degeneracy was allowed within the flanking sequences (1 change in each flanking sequence was used in the original analysis). The NGS data from **chapter 3** was re-analysed in the present chapter allowing 0 or 2 changes within the HCDR3s flanking region sequences. The first analysis that was performed in **chapter 3** permitted 1 change within the flanking region sequences and looked at growth and selection patterns of scFvs across panning rounds and 43 scFvs with potential binding to *Eimeria* oocysts were isolated. In this chapter following the 0-change allowed, 6 out of the original 43 candidates were missing from the data. This suggests that 14% had at least 1 sequence change in the flanking regions. When 2 changes were permitted, another 7 scFvs (above the original 43) that met the

selection criteria of growth and selection patterns were identified which is a 14% increase.

In the second analysis that looked at identifying species specific scFvs by analysing each species versus the rest, when 0 change in the flanking sequences were permitted, 19% of the identified clones were missing and the other candidates identified in the original 1 change allowed still remained among the top ten candidate binders for each *Eimeria* species. When 2 changes were allowed, all scFv sequences were present in the datasets, however, only 45% of the original identified scFvs still remained among the top 10 candidates. In published papers, any degeneracy allowed in flanking regions are often not reported and only a few studies have identified the number of changes they have allowed in the flanking region. The study by (Yang *et al*., 2017) using chicken immune scFv libraries and known flanking sequences, permitted up to two changes within the flanking region. The authors did not describe changing levels of flanking region sequences as was performed in the present study. Other studies have reported permitting 1 change per sequence (Spiliotopoulos *et al.,* 2015, Lövgren *et al.,* 2016) also not attempting to change the flanking region sequences, hence there is no report on the optimal flanking region sequence degeneracy.

Here, the more stringent flanking sequence criteria may have prevented the isolation of more candidates in the initial analysis. It is not very clear what degeneracy should be allowed in the flanking sequences but it may be beneficial to allow 2 changes to maximise the selection of HCDR3s given that more candidates were identified. In the species-specific analysis permitting two changes in the flanking regions, about 50% of the candidate selected scFvs changed. The future rescue and testing of the scFvs identified by both methods would define which is the more accurate method for identifying true binders.

When considering the V_HH libraries, the flanking regions of the HCDR3s were conserved hence any degeneracy allowed in the flanking regions suggests PCR and/or sequencing errors. Analysis clearly demonstrated that ~ 10% additional sequences were captured when 1 change was allowed and ~ 60% with 2 changes allowed. This suggests that selected antibodies are likely to change with more changes allowed in the flanking region. This may also reflect the error rate of the NGS method and will reflect errors within the CDR3 sequences as well. It may be beneficial to employ identification of motifs and collapsing of sequences to avoid having many versions of an abundant sequence being pursued.

The bioinformatics strategy focused on generating Z scores (Zhang *et al.,* 2011, Faver *et al.,* 2019) for each sample's CDR3 sequence in all the analysis conducted in this study. In the scFv study in chapter 3, growth and selection patterns were analysed and HCDR3s that displayed enrichment in both growth and selection phases were selected over those that displayed enrichment in the growth only phases. The HCRD3s were then ranked according to their Z scores and candidates were selected on that basis. Similarly, in the species-specific selection in which individual species were compared versus the other species and HCDR3s that displayed binding in the particular species when compared to others were selected, they were ranked according to their Z scores and the top 10 were selected. These analyses generated binders which proved that the use of Z score was a valid approach to isolating binders to any target. The studies by (Zhang *et al*., 2011) and (Naqid *et al*., 2016b) also successfully identified target specific binders using the Z score strategy. (Naqid *et al*., 2016b) conducted peptide phage display using a naïve peptide library in 2 rounds of panning which was aimed at mapping polyclonal antibody responses to *Salmonella* infections. Pre-infection (negative control) and post-infection IgG samples were panned against and binding to each one was compared in the bioinformatics analysis. In the study 22 out of 27 of the most enriched candidates bound to their target. In the study by (Zhang *et al*., 2011), the target and control antigens were panned against in 2 rounds using a naïve scFv library and Roche 454 pyrosequencing was used to sequence the HCDR3 of the scFvs. The target and control data were compared and HCDR3s were ranked according to Z scores. Two clones were recovered and upon testing were found to specifically bind to the target antigens. The authors went on to test the clones in full IgG formats and were also found to bind to the target. Both studies illustrate that defining enrichment and relative binding properties using Z scores is a valid approach.

In this study, **chapter 5** and **6** were analysed by ranking according to Z scores and how many times the HCDR3s were seen in the positive replicates, candidate V_HHs were identified but after testing them, they were not found to be binding the target. In this chapter, the data from **chapter 5** and **6** were re-analysed based on frequency of HCDR3s. The frequency of known binders from scFv-whole oocyst panning in **chapter 3** were also determined in the panning rounds. **Chapter 5** and **6** V_HH panning did not sequence data from panning rounds hence only data from the round 3 screen was available for analysis. Nevertheless, the frequencies of the HCDR3s were compared to that of known binders from **chapter 3** to determine if they had comparable frequencies. Several peptide and antibody phage display studies have employed the use of frequency of clones in different capacities to determine binders

to various targets. The study by (Ryvkin *et al*., 2012) coupled naïve peptide libraries with Illumina sequencing platform. The sequenced data for both naïve library and the three panning rounds were analysed and those that had high copy numbers in the naïve library were excluded. The peptide sequences were ranked by frequency and then analysed using the MEME motif analysis software to identify common motifs that had become enriched within the data. Enrichment was characterised with a gradual rise in the frequency of enriched clones while diversity dropped across panning rounds. Here, the diversity of the panning rounds where not directly determined, the frequency of the clones across panning rounds increased especially the scFv clone DHTPKWq which accounted for over 90% of the phage pool by the 5th panning round for all species which clearly suggests that the phage pool was largely monoclonal. Other studies that used peptide libraries and frequency analysis were studies by (AC't Hoen *et al*., 2012) and (Ngubane *et al*., 2013). In both studies, naïve peptide libraries were paired with Illumina sequencing platform and the sequenced data were analysed for both naïve library and the panning rounds. In the study by (AC't Hoen *et al*., 2012), peptides were ranked according to frequency and top frequency candidates were selected. The authors demonstrated that high affinity target binders can be identified after only 1 panning round. The study by (Ngubane *et al*., 2013) interestingly, conducted 10 random colony picks from the round 5 panning stock and also NGS sequencing of clones and a binder was identified and synthesised after NGS analysis. This binder represented 80% of the round 5 library and yet was not identified in random colony picks. In this study, the scFv clones randomly picked and confirmed via Sanger sequencing clearly mirrored the frequency of the clones in the round 5 sub library. Twenty-one of 23 of the sequenced clones were the DHTPKWq sequence which sequencing data showed was over 90% of the round 5 sub-library. The study by (Rentero Rebollo *et al*., 2014) paired peptide library with Ion torrent sequencing platform. Dataset was sorted by frequency and compared with another dataset in which the same library was used to remove nonspecific binders and after one round of panning a rare target binder peptide was identified.

The study by (Ravn *et al*., 2010c) used a naïve scFv library with Ion torrent sequencing in 3 panning rounds . Sequenced data were ranked according to frequency and the most abundant clones were rescued. Six binders were identified which bound as phage fusions. The studies by (Sasso *et al*., 2015), (D'angelo *et al*., 2014), (Lövgren *et al*., 2016) also used naïve scFv libraries/synthetically randomised libraries and conducted panning. (Sasso *et al*., 2015) performed 4 rounds of panning, (Lövgren *et al*., 2016) conducted 3 panning rounds and (D'angelo *et al*., 2014) conducted 2 panning rounds. In all these studies, sequences were ranked according

to frequencies/ abundance. (D'angelo *et al*., 2014) ranked the top 10 clones in each target according to their relative frequencies of the individual HCDR3s. Percentage frequencies of the top 10 clones ranged between 56.9% and 0.1%. These were found to bind specifically to the target *Lactobacillus acidophilus*. In the present study, when percentage frequencies of HCDRs were ranked, a HCDR3 (ITAWGAPSQRDL) that had a percentage frequency of 42.4% was observed and it did not bind to the target in an ELISA assay.

The ITAWGAPSQRDL scFv identified as a top binder is clearly is non-specific appearing in independent panning experiments (V_HH/oocyst lysate and V_HH/ peptide). It may likely be binding to the blocking solution as that is the only common component For the V_HH/oocyst lysate panning in **chapter 5**, 100 from each panning round were screened hence any scFv at frequency about 5% may likely have been screened which was all negative. An alternative selection criterion may not improve the selection of positives over what had been done in **chapters 5** and **6** given that many of the selected and screened candidates from the original analysis are seen in the alternative analysis performed in this chapter.

The percentage frequencies of the selected HCDR3s in the present study selected for both V_HH-oocyst lysate and V_HH-peptide datasets were relatively high, often much more than the frequencies of the known binders in the scFv-whole oocyst panning. The low frequencies of the true binders in chapter 3 may be attributed to them being outcompeted by the fast propagating binder DHTPKWq which accounted for most of the 'sequence space'. It is well known that some phage have growth advantages over others.

In the present chapter, alternative analysis have shown that selected V_HH candidates were expected to be binders to target and may have shown this in a more sensitive assay such as a sequencing assay as opposed to conventional ELISAs. As was mentioned before, the stringency of the panning strategy vis-a-vis subtractive phage panning may have led to the subtraction of high affinity binders hence a more sensitive assay would have been required to analyse the sequenced data.

The summary of the findings observed in this chapter include;

1) allowing degeneracy in flanking regions can improve the chances of identifying more binders even for libraries that use a known scaffold as more data will be captured.

2) It may be beneficial to analyse for single changes within the CDR and collapsed sequences to avoid isolating many different clones of the same binder and to improve the chances of isolating distinct binders.

3) Analysis; frequency and Z score alternative analysis tools may identify different but overlapping groups of candidates.

4) *In silico* analysis of binding may provide a high sensitivity assay compared to conventional immunoassays but this required much more validation using antibodies with known binding specificities.

5) Analysis of growth vs selection criteria is useful for selecting true binders and it is recommended as a bioinformatics strategy as the analysis enabled the isolation of binders against oocysts.

6) Finally, the use of subtractive panning may remove all binders when looking for very specific binding properties. Post panning subtraction has not been reported in the literature and may be too stringent.

8. General discussion, conclusion and future perspectives

8.1 General discussion

The aim of this research was to isolate recombinant antibodies against the seven species of *Eimeria* in chickens that would be used to develop novel diagnostics able to differentiate between infecting species of *Eimeria* in chickens. It was hypothesised that NGPD and conventional phage panning would allow comparative screening of antibody binding events to identify antibodies to species-specific epitopes of *Eimeria* oocysts and such antibodies could then be used to develop immunoassays to speciate *Eimeria* infections of poultry. The research objectives were to carry out antibody-phage display panning in order to isolate antibodies against oocysts of all seven *Eimeria* species, to screen the antibodies isolated via ELISA and finally, to compare traditional phage panning with NGPD strategies.

Phage display experiments and NGS strategies used in this study in general were geared towards the identification of species-specific binders to *Eimeria* oocysts. In the first phase of this study, the Griffin scFv library was paired with whole oocysts as targets and panning was performed in solution. Oocysts were targeted as they are the most abundant stage of this parasite, and they could easily be applied in any future development of an in-field assay. Two sets of NGS analysis were performed in the scFv phage display analysis; analysis of growth and selection patterns across panning rounds and comparison of each species versus the other species. The first strategy has been used with success in some published research (Sasso *et al.,* 2015). Z scores were generated in both analyses to identify scFv enrichment and six binders were identified across both analyses; 2 binding to all species of *Eimeria* and the other 4 binding differentially to distinct species of *Eimeria*. The panning approach and bioinformatics analysis were successful in the isolation of scFvs against whole oocysts of *Eimeria*. However, major challenges experienced with this approach were 1) rescuing the NGS-identified scFv candidates from the polyclonal phage sub libraries. In most instances they were present in very low frequencies and rescuing them successfully was inefficient despite using published methods that had largely been previously successful (Spiliotopoulos *et al.,* 2015) and 2) Difficulty encountered when attempting to immobilise oocysts on 96 well plates to develop a high throughput assay. Different immobilisation conditions were tried including using published methods (Kramer *et al.,* 2007). All attempts were unsuccessful and eventually a spin down ELISA assay was optimised.

The oocyst stocks used in the present study which were provided by MSD animal health were used whole without processing for the panning experiments carried out in this study. The oocysts were confirmed to be the correct species only by comparing

them microscopically to published guidelines for each species. Also, it was not determined if contamination had occurred in the handling and labelling of samples or at any stage in handling of the oocyst samples upon receiving them from the supplier. In hindsight it would have been useful to verify the purity of the oocyst samples using molecular methods such as PCR or qPCR (Schnitzler *et al.,* 1998, Kawahara *et al.,* 2008). The high sensitivity and specificity of molecular based techniques would have enabled us to completely rule out the possibility of any contamination which could have undermined the data analysis for the selection of species-specific binders. The oocyst stocks were also largely sporulated when viewed under the microscope even though the exact percentage of sporulated oocysts were not determined. This would have been important to know as it would have determined the target of the final assays as oocysts are shed unsporulated. The protein profile of sporulated oocysts have also been reported to be different from unsporulated oocysts. The study by (Wang and Stotish, 1975) on oocysts of *E. tenella* reported a change in the protein profile of the oocyst after sporulation as confirmed by SDS-PAGE while the total protein content remained relatively unchanged after sporulation. A study by (Stotish *et al*., 1978) also reported a protein in the cytoplasm *E. tenella* of 14% of the total soluble protein of the unsporulated oocyst that disappeared from the cytoplasm of the oocyst between the 15th to 20th hour of the 20-hour sporulation process. On the other hand, because sporulation almost always occurs after 1-2 days in at least 50% of oocysts, if oocysts obtained from faecal samples in farms are targeted for the assay format, sporulated oocysts would likely be used (Edgar, 1954, Graat *et al.,* 1994, Norton and Chard, 1983).

The development of a high throughput assays to whole oocysts was unsuccessful. The isolated antibodies after functional testing in a spin down ELISA were then sub-cloned into the pMAL-p5X vector to enable the expression and purification of antibodies and to produce them as soluble antibodies. The antibodies cloned into the MBP vector were confirmed to be expressing in the vector using SDS-PAGE and also Sanger sequencing confirming that they were successfully cloned. This approach proved to be problematic as very low levels of antibodies were recovered when tested via Bradford's assay. This was not unsurprising as many previous studies have reported low levels of recombinant antibodies when sub-cloned into other expression vectors in *E. coli*. The quality of the protein in the crude lysates obtained after subcloning into the vector were not validated. In hindsight, this step could have been conducted to enable the establishment of the stage of the subcloning process that was inefficient. This could have been carried out by analysis on Coomassie or silver

stained gels (Shevchenko *et al.,* 1996, Lanne and Panfilov, 2005, Dyballa and Metzger, 2009).

These difficulties encountered led to an alternative panning strategy whereby antibodies were isolated against oocyst components (crude lysate) that were readily immobilised on ELISA plates. This approach also used a distinct antibody library that had diversity in a single CDR region, the NGS-identified clones could then be very effectively re-synthesised into any expression vector containing a scaffold antibody gene. No binders were identified using this approach despite extensive panning. It may be that the crude oocyst homogenates do not present antigens that are species-specific and relatively abundant and so do not present appropriate targets for the phage display panning aimed at isolating species-specific binders. Panning was then conducted using distinct regions of *Eimeria* peptide Gam56 which had the potential to be a much better target for antibody isolation over oocyst homogenate or whole oocysts that were previously used. This is because the peptides are far simpler, well defined and immobilised in high amounts. However, this approach was again unsuccessful in isolating binders to *Eimeria* antigen. The panning strategy may have contributed to this because stringent subtraction steps were conducted to maximise the chances of isolating species- specific clones, this may have led to the removal of high affinity binders that bind to more than one version of the peptides. Also, species-specific binders may actually be very rare and they were all removed. Alternatively, binders may have been present and can be identified by analysing binding events by NGS, but cannot be the shown to be positive binders in ELISAs, possibly as the latter is a lower sensitivity assay. This remains to be established.

The crude oocyst lysates used for the panning were also not fully validated, though the method of sonication of the oocysts used in this study followed published methods, it would have been useful to confirm the 'quality' of the proteins in before using them for panning. This will prevent the possibility of using degraded proteins which can consequently invalidate the results obtained. This could have been done by SDS-PAGE analysis of soluble fractions to assess the extent of protein release and any gross protein fragmentation. On the other hand, if the protein fragmentation or denaturation is consistent between the assays then the assay would still be valid only that the conformational epitopes may be dependent on a consistent sample preparation method each time.

The final chapter of this study also reassessed the methods used to analyse the NGS data and interestingly, in this study, the frequency of the true scFv binders isolated in **chapter 3**'s against whole oocyst were analysed and they were found to be present in very low frequencies yet they bound to the target. The assessment of bioinformatics

strategies that were used in the analysis of sequenced NGS data in this study as a whole suggest that the growth and selection patterns of HCDR3s may be a more efficient method of determining clonal enrichment to a target when compared to target versus negative control analysis or frequency analysis. The final chapter of this study also focused on the development of the bioinformatics approaches in terms of how it might benefit phage display and isolating novel antibodies from naïve libraries. It was determined that the approaches used for analysis in this study were efficient to select binders if they were present, as when reanalysis was conducted with published methods, the same binders that were selected in this study also emerged as top binders in the alternative analysis. It is recommended not to include any post-panning subtraction of phage as this may well be too stringent an approach and remove most binders. Also, relying on bioinformatic comparisons will be helpful to select for binding properties in future studies as distinct candidate binders can be identified.

At present, there is little discussion in the literature as to what analysis is suitable for different targets, ligand types and phage libraries. A thorough comparison of different data analysis strategies followed by empirical testing of the top candidates will help to shed light on the most effective and efficient methods.

In summary, this entire study validated the hypothesis that NGPD would enable the comparative screening of antibody binding events to identify novel antibodies to epitopes of *Eimeria* oocysts. Six novel antibodies against *Eimeria* species in chickens were isolated. However, none of the antibodies isolated were exclusively specific to one species. This is not surprising since there are regions of homology on the oocyst wall across different species and the oocyst is a highly conserved stage of the parasite lifecycle (Belli *et al.,* 2009). As was mentioned, the stringency of the subtraction steps used have likely contributed to the inability to isolate antibodies that bind species-specific epitopes of *Eimeria* oocysts which are likely present in very low concentrations.

8.2 Future approaches and conclusion

Some approaches may be considered to improve the likelihood of improving the success of the project. Oocysts used for any future experiments would benefit from confirming if there is any form of contamination of oocyst stocks and confirming the species. This will avoid the possibility of introducing mixtures of oocysts in the panning that would undermine the panning approaches.

Future approaches would also likely benefit from confirming the quality of the proteins in the lysates to ensure that degraded proteins are not used for studies which

consequently invalidate the results. Better results are likely to be obtained with higher protein quality.

Targeting oocyst homogenates seems an appropriate approach as does the use of an antibody library with a single region of diversity (to allow effective clone rescue). But the application of less stringent subtractive steps in the panning is likely to enhance the chances of finding binders. In addition, the approach should identify clones that are enriched in selection (not growth only) stages and either have relatively high frequencies or high species-specificity as analysed by Z score analysis. It is recommended not to include any post-panning subtraction of phage. Also, relying on bioinformatic comparisons will be helpful to select the widest range of candidate binders for characterisation.

Appendices

Appendix I

The unique barcode sequences used in barcoded primers 1-96. Each unique barcode sequence was flanked by the sequences: CCATCTCATCCCTGCGTGTCTCCGACTCAG and GTAATCCTTGTGGTATCG

Barcode	Sequence	Barcode	sequence
Akey-BC1-lnk1	CTAAGGTAAC	Akey-BC50-lnk1	CGGACAATGGC
Akey-BC2-lnk1	TAAGGAGAAC	Akey-BC51-lnk1	TTGAGCCTATTC
Akey-BC3-lnk1	AAGAGGATTC	Akey-BC52-lnk1	CCGCATGGAAC
Akey-BC4-lnk1	TACCAAGATC	Akey-BC53-lnk1	CTGGCAATCCTC
Akey-BC5-lnk1	CAGAAGGAAC	Akey-BC54-lnk1	CCGGAGAATCGC
Akey-BC6-lnk1	CTGCAAGTTC	Akey-BC55-lnk1	TCCACCTCCTC
Akey-BC7-lnk1	TTCGTGATTC	Akey-BC56-lnk1	CAGCATTAATTC
Akey-BC8-lnk1	TTCCGATAAC	Akey-BC57-lnk1	TCTGGCAACGGC
Akey-BC9-lnk1	TGAGCGGAAC	Akey-BC58-lnk1	TCCTAGAACAC
Akey-BC10-lnk1	CTGACCGAAC	Akey-BC59-lnk1	TCCTTGATGTTC
Akey-BC11-lnk1	TCCTCGAATC	Akey-BC60-lnk1	TCTAGCTCTTC
Akey-BC12-lnk1	TAGGTGGTTC	Akey-BC61-lnk1	TCACTCGGATC
Akey-BC13-lnk1	TCTAACGGAC	Akey-BC62-lnk1	TTCCTGCTTCAC
Akey-BC14-lnk1	TTGGAGTGTC	Akey-BC63-lnk1	CCTTAGAGTTC
Akey-BC15-lnk1	TCTAGAGGTC	Akey-BC64-lnk1	CTGAGTTCCGAC
Akey-BC16-lnk1	TCTGGATGAC	Akey-BC65-lnk1	TCCTGGCACATC
Akey-BC17-lnk1	TCTATTCGTC	Akey-BC66-lnk1	CCGCAATCATC
Akey-BC18-lnk1	AGGCAATTGC	Akey-BC67-lnk1	TTCCTACCAGTC
Akey-BC19-lnk1	TTAGTCGGAC	Akey-BC68-lnk1	TCAAGAAGTTC
Akey-BC20-lnk1	CAGATCCATC	Akey-BC69-lnk1	TTCAATTGGC
Akey-BC21-lnk1	TCGCAATTAC	Akey-BC70-lnk1	CCTACTGGTC
Akey-BC22-lnk1	TTCGAGACGC	Akey-BC71-lnk1	TGAGGCTCCGAC
Akey-BC23-lnk1	TGCCACGAAC	Akey-BC72-lnk1	CGAAGGCCACAC
Akey-BC24-lnk1	AACCTCATTC	Akey-BC73-lnk1	TCTGCCTGTC
Akey-BC25-lnk1	CCTGAGATAC	Akey-BC74-lnk1	CGATCGGTTC
Akey-BC26-lnk1	TTACAACCTC	Akey-BC75-lnk1	TCAGGAATAC
Akey-BC27-lnk1	AACCATCCGC	Akey-BC76-lnk1	CGGAAGAACCTC
Akey-BC28-lnk1	ATCCGGAATC	Akey-BC77-lnk1	CGAAGCGATTC
Akey-BC29-lnk1	TCGACCACTC	Akey-BC78-lnk1	CAGCCAATTCTC
Akey-BC30-lnk1	CGAGGTTATC	Akey-BC79-lnk1	CCTGGTTGTC

Akey-BC31-lnk1	TCCAAGCTGC	Akey-BC80-lnk1	TCGAAGGCAGGC
Akey-BC32-lnk1	TCTTACACAC	Akey-BC81-lnk1	CCTGCCATTCGC
Akey-BC33-lnk1	TTCTCATTGAAC	Akey-BC82-lnk1	TTGGCATCTC
Akey-BC34-lnk1	TCGCATCGTTC	Akey-BC83-lnk1	CTAGGACATTC
Akey-BC35-lnk1	TAAGCCATTGTC	Akey-BC84-lnk1	CTTCCATAAC
Akey-BC36-lnk1	AAGGAATCGTC	Akey-BC85-lnk1	CCAGCCTCAAC
Akey-BC37-lnk1	CTTGAGAATGTC	Akey-BC86-lnk1	CTTGGTTATTC
Akey-BC38-lnk1	TGGAGGACGGAC	Akey-BC87-lnk1	TTGGCTGGAC
Akey-BC39-lnk1	TAACAATCGGC	Akey-BC88-lnk1	CCGAACACTTC
Akey-BC40-lnk1	CTGACATAATC	Akey-BC89-lnk1	TCCTGAATCTC
Akey-BC41-lnk1	TTCCACTTCGC	Akey-BC90-lnk1	CTAACCACGGC
Akey-BC43-lnk1	CTTGACACCGC	Akey-BC91-lnk1	CGGAAGGATGC
Akey-BC44-lnk1	TTGGAGGCCAGC	Akey-BC92-lnk1	CTAGGAACCGC
Akey-BC45-lnk1	TGGAGCTTCCTC	Akey-BC93-lnk1	CTTGTCCAATC
Akey-BC46-lnk1	TCAGTCCGAAC	Akey-BC94-lnk1	TCCGACAAGC
Akey-BC47-lnk1	TAAGGCAACCAC	Akey-BC95-lnk1	CGGACAGATC
Akey-BC48-lnk1	TTCTAAGAGAC	Akey-BC96-lnk1	TTAAGCGGTC
Akey-BC49-lnk1	TCCTAACATAAC		

Appendix II

Griffin library & V_HH library

The Griffin library& V_HH library is sequenced in the reverse direction so needs to be reverse complemented before running the translation script. This can easily be done by creating a new text file containing the following line in the directory where the fasta files are located.

Reverse_Complement.sh

```
seqtk seq -r split_barcode_BC01.fasta > split_barcode_BC01.rev.fasta
seqtk seq -r split_barcode_BC02.fasta > split_barcode_BC02.rev.fasta
seqtk seq -r split_barcode_BC03.fasta > split_barcode_BC03.rev.fasta
seqtk seq -r split_barcode_BC04.fasta > split_barcode_BC04.rev.fasta
seqtk seq -r split_barcode_BC05.fasta > split_barcode_BC05.rev.fasta
```

The output files generated here then become the input files for the translation.sh script, so that needs to be edited accordingly (i.e. will be in the format perl translate.pl -i **yyyBC01.rev.fasta** -s opq -f 1 >**yyyBC01.frame1)** . Save the file as “reverse.sh” then run by typing “parallel <reverse.sh” without the quotes at the prompt. The script in 2. outputs 3 frames for each sequence. Only one of these contains the correct frame (but we don't know which yet, and it could be in any one of the three), so all 3 frames are joined together and output as a single “.frames” file:

Edit each line to reflect the file names generated above:

```
cat NGS1_BC01.frame1.fasta NGS1_BC01.frame2.fasta NGS1_BC01.frame3.fasta
>NGS1_BC01.frames.fasta
cat NGS1_BC02.frame1.fasta NGS1_BC02.frame2.fasta NGS1_BC02.frame3.fasta
>NGS1_BC02.frames.fasta
cat NGS1_BC03.frame1.fasta NGS1_BC03.frame2.fasta NGS1_BC03.frame3.fasta
>NGS1_BC03.frames.fasta
cat NGS1_BC04.frame1.fasta NGS1_BC04.frame2.fasta NGS1_BC04.frame3.fasta
>NGS1_BC04.frames.fasta
cat NGS1_BC05.frame1.fasta NGS1_BC05.frame2.fasta NGS1_BC05.frame3.fasta
>NGS1_BC05.frames.fasta
```

Appendix III

The left flanking sequences text file contained:
VYYCAR
[^V]YYCAR
V[^Y]YCAR
VY[^Y]CAR
VYY[^C]AR
VYYC[^A]R
VYYCA[^R]
The right flanking sequences text file contained:
WGQGT
[^W]GQGT
W[^G]QGT
WG[^Q]GT
WGQ[^G]T
Zero changes permitted (Griffin Library)
The left flanking sequences text file contained:
• VYYCAR

The right flanking sequences text file contained:
• WGQGT

Appendix IV

Comparison.sh

function: compare frequencies in pairs of .LR.fa files defined by the user and outputs a tab delimited table file. This can be imported into excel and sorted.

Requires compare_collapsed_sequences_2a.Zscore.pl to be present.
calculates the total sequences for each fasta file
calculate Z score and output results

Each line takes the following format, with once comparison per line:

```
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC49.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >49vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC50.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >50vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC51.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >51vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC52.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >52vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC53.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >53vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC54.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >54vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC55.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >55vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC56.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >56vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC57.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >57vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC58.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >58vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC59.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >59vspool.table
perl compare.collapsed.sequences_2a.Zscore.pl NGS1_BC60.frames.fasta.LR.fa PA_R4neg.pool.LR.fa >60vspool.table
```

Appendix V

The left flanking sequences text file contained:
• VYYCAR
• [^V]YYCAR
• V[^Y]YCAR
• VY[^Y]CAR
• VYY[^C]AR
• VYYC[^A]R
• VYYCA[^R]
• [^V][^Y]YCAR
• [^V]Y[^Y]CAR
• [^V]YY[^C]AR
• [^V]YYC[^A]R
• [^V]YYCA[^R]
• V[^Y][^Y]CAR
• V[^Y]Y[^C]AR
• V[^Y]YC[^A]R
• V[^Y]YCA[^R]
• VY[^Y][^C]AR
• VY[^Y]C[^A]R
• VY[^Y]CA[^R]
• VYY[^C][^A]R
• VYY[^C]A[^R]
• VYYC[^A][^R]

The right flanking sequences text file contained:
• WGQGT
• [^W]GQGT
• W[^G]QGT
• WG[^Q]GT
• WGQ[^G]T
• WGQG[^T]
• [^W][^G]QGT
• [^W]G[^Q]GT
• [^W]GQ[^G]T
• [^W]GQG[^T]
• W[^G][^Q]GT
• W[^G]Q[^G]T
• W[^G]QG[^T]
• WG[^Q][^G]T
WG[^Q]G[^T]
WGQ[^G][^T]

One change permitted (V_HH Library)
The left flanking sequences text file contained:
• YYCAA
• [^Y]YCAA
• Y[^Y]CAA
• YY[^C]AA
• YYC[^A]A
• YYCA[^A]

The right flanking sequences text file contained:
• WGQGT

• [^W]GQGT
• W[^G]QGT
• WG[^Q]GT
• WGQ[^G]T
• WGQG[^T]

Up to two changes permitted (V_HH Library)
The left flanking sequences text file contained:
• YYCAA
• [^Y]YCAA
• Y[^Y]CAA
• YY[^C]AA
• YYC[^A]A
• YYCA[^A]
• [^Y][^Y]CAA
• [^Y]Y[^C]AA
• [^Y]YC[^A]A
• [^Y]YCA[^A]
• Y[^Y][^C]AA
• Y[^Y]C[^A]A
• Y[^Y]CA[^A]
• YY[^C][^A]A
• YY[^C]A[^A]
• YYC[^A][^A]

The right flanking sequences text file contained:
• WGQGT
• [^W]GQGT
• W[^G]QGT
• WG[^Q]GT
• WGQ[^G]T
• WGQG[^T]
• [^W][^G]QGT
• [^W]G[^Q]GT
• [^W]GQ[^G]T
• [^W]GQG[^T]
• W[^G][^Q]GT
• W[^G]Q[^G]T
• W[^G]QG[^T]
• WG[^Q][^G]T
• WG[^Q]G[^T]
• WGQ[^G][^T]

REFERENCES

ABI-GHANEM, D., WAGHELA, S. D., CALDWELL, D. J., DANFORTH, H. D. & BERGHMAN, L. R. 2008. Phage display selection and characterization of single-chain recombinant antibodies against *Eimeria tenella* sporozoites. *Veterinary immunology and immunopathology,* 121**,** 58-67.

AC'T HOEN, P., JIRKA, S. M., BRADLEY, R., SCHULTES, E. A., AGUILERA, B., PANG, K. H., HEEMSKERK, H., AARTSMA-RUS, A., VAN OMMEN, G. J. & DEN DUNNEN, J. T. 2012. Phage display screening without repetitious selection rounds. *Analytical biochemistry,* 421**,** 622-631.

ADAMS, J. J., NELSON, B. & SIDHU, S. S. 2014. Recombinant genetic libraries and human monoclonal antibodies. In *human monoclonal antibodies* (pp. 149-170).

AHMAD, T. A., EL-SAYED, B. A. & EL-SAYED, L. H. 2016. Development of immunization trials against *Eimeria* spp. *Trials in vaccinology,* 5**,** 38-47.

ALEMAYEHU, T., BRUNO, J., GETACHEW, F. & DESSIE, T. 2018. Socio-economic, marketing and gender aspects of village chicken production in the tropics: A review of literature. *ILRI Project Report. Nairobi, Kenya: International Livestock Research Institute (ILRI). Patron: Professor Peter C Doherty AC, FAA, FRS Animal scientist, Nobel Prize Laureate for Physiology or Medicine–1996*.

ALFALEH, M. A., JONES, M. L., HOWARD, C. B. & MAHLER, S. M. 2017. Strategies for selecting membrane protein-specific antibodies using phage display with cell-based panning. *Antibodies,* 6**,** 10.

AMARE, A., MENGISTU, A. & NAZIR, S. 2012. Prevalence and aetiology of poultry coccidiosis and associated risk factors in White Leghorn grower chickens at Kombolcha poultry farm Ethiopia. *J World's Poult Res,* 2**,** 54-9.

AMER, M., AWAAD, M., EL-KHATEEB, R. M., ABU-ELEZZ, N., SHEREIN-SAID, A., GHETAS, M. & KUTKAT, M. 2010. Isolation and identification of *Eimeria* from field coccidiosis in chickens. *Journal of american science,* 6**,** 1107-1114.

AMERAH, A. & RAVINDRAN, V. 2015. Effect of coccidia challenge and natural betaine supplementation on performance, nutrient utilization, and intestinal lesion scores of broiler chickens fed suboptimal level of dietary methionine. *Poultry science,* 94**,** 673-680.

AMERSDORFER, P., WONG, C., SMITH, T., CHEN, S., DESHPANDE, S., SHERIDAN, R. & MARKS, J. D. 2002. Genetic and immunological comparison of anti-botulinum type A antibodies from immune and non-immune human phage libraries. *Vaccine,* 20**,** 1640-1648.

ANDREWS, R., O'DONOGHUE, P., ADAMS, M. & PROWSE, S. 1990. Enzyme markers for the genetic characterization of avian *Eimeria* spp. *Parasitology research,* 76**,** 627-629.

ANDRIS-WIDHOPF, J., RADER, C., STEINBERGER, P., FULLER, R. & BARBAS III, C. F. 2000. Methods for the generation of chicken monoclonal antibody fragments by phage display. *Journal of immunological methods,* 242**,** 159-181.

ARABKHAZAELI, F., MODRISANEI, M., NABIAN, S., MANSOORI, B. & MADANI, A. 2013. Evaluating the resistance of *Eimeria* spp. field isolates to anticoccidial drugs using three different indices. *Iranian journal of parasitology,* 8**,** 234.

ARAKAWA, M., YAMASHIRO, T., UECHI, G. I., TADANO, M. & NISHIZONO, A. 2007. Construction of Human Fab (y1/k) Library and Identification of Human Monoclonal Fab Possessing Neutralizing Potency against Japanese Encephalitis Virus. *Microbiology and immunology,* 51**,** 617-625.

ARBABI-GHAHROUDI, M., TANHA, J. & MACKENZIE, R. 2005. Prokaryotic expression of antibodies. *Cancer and metastasis reviews,* 24**,** 501-519.

AWAIS, M. M., AKHTAR, M., IQBAL, Z., MUHAMMAD, F. & ANWAR, M. I. 2012. Seasonal prevalence of coccidiosis in industrial broiler chickens in Faisalabad, Punjab, Pakistan. *Tropical animal health and production,* 44**,** 323-328.

AZZAZY, H. M. & HIGHSMITH JR, W. E. 2002. Phage display technology: clinical applications and recent innovations. *Clinical biochemistry,* 35**,** 425-445.

BAEK, H., SUK, K.-H., KIM, Y.-H. & CHA, S. 2002. An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic acids research,* 30**,** e18-e18.

BAHARA, N. H. H., TYE, G. J., CHOONG, Y. S., ONG, E. B. B., ISMAIL, A. & LIM, T. S. 2013. Phage display antibodies for diagnostic applications. *Biologicals,* 41**,** 209-216.

BAKHSHINEJAD, B., ZADE, H. M., SHEKARABI, H. S. Z. & NEMAN, S. 2016. Phage display biopanning and isolation of target-unrelated peptides: in search of nonspecific binders hidden in a combinatorial library. *Amino acids,* 48**,** 2699-2716.

BAPTISTA, P., PEREIRA, E., EATON, P., DORIA, G., MIRANDA, A., GOMES, I., QUARESMA, P. & FRANCO, R. 2008. Gold nanoparticles for the development of clinical diagnosis methods. *Analytical and bioanalytical chemistry,* 391**,** 943-950.

BARBAS, C. F., KANG, A. S., LERNER, R. A. & BENKOVIC, S. J. 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proceedings of the national academy of sciences,* 88**,** 7978-7982.

BARDWELL, J. C., MCGOVERN, K. & BECKWITH, J. 1991. Identification of a protein required for disulfide bond formation in vivo. *Cell,* 67**,** 581-589.

BARKWAY, C. P., POCOCK, R. L., VRBA, V. & BLAKE, D. P. 2015. Loop-mediated isothermal amplification (LAMP) assays for the species-specific detection of *Eimeria* that infect chickens. *JoVE* (*Journal of visualized experiments),* 96, e52552.

BARRETO, K., MARUTHACHALAM, B. V., HILL, W., HOGAN, D., SUTHERLAND, A. R., KUSALIK, A., FONGE, H., DECOTEAU, J. F. & GEYER, C. R. 2019. Next-generation sequencing-guided identification and reconstruction of antibody CDR combinations from phage selection outputs. *Nucleic acids research,* 47**,** e50-e50.

BARUTZKI, D. & SCHAPER, R. 2011. Results of parasitological examinations of faecal samples from cats and dogs in Germany between 2003 and 2010. *Parasitology research,* 109**,** 45-60.

BASAK, S., LEE, S., BARTA, J. & FERNANDO, M. 2006. Differential display analysis of gene expression in two immunologically distinct strains of *Eimeria maxima*. *Parasitology research,* 99**,** 28.

BASS, S., GREENE, R. & WELLS, J. A. 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins: Structure, function, and bioinformatics,* 8**,** 309-314.

BATES, A. & POWER, C. A. 2019. David vs. Goliath: the structure, function, and clinical prospects of antibody fragments. *Antibodies,* 8**,** 28.

BEADLE, C., LONG, G. W., MCELROY, P., HOFFMAN, S., WEISS, W., MARET, S. & OLOO, A. 1994. Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. *The lancet,* 343**,** 564-568.

BECKER, E. R., JESSEN, R., PATTILLO, W. H. & DOORNINCK, W. M. 1956. A biometrical study of the oocyst of *Eimeria necatrix*, a parasite of the common fowl. *The journal of protozoology,* 3**,** 126-131.

BECKER, E. R., ZIMMERMANN, W. J. & PATTILLO, W. 1955. A biometrical study of the oocyst of *Eimeria brunetti*, a parasite of the common fowl. *The journal of protozoology,* 2**,** 145-150.

BELLI, S. I., FERGUSON, D. J., KATRIB, M., SLAPETOVA, I., MAI, K., SLAPETA, J., FLOWERS, S. A., MISKA, K. B., TOMLEY, F. M. & SHIRLEY, M. W. 2009. Conservation of proteins involved in oocyst wall formation in *Eimeria maxima*, *Eimeria tenella* and *Eimeria acervulina*. *International journal for parasitology,* 39**,** 1063-1070.

BELLI, S. I., LEE, M., THEBO, P., WALLACH, M. G., SCHWARTSBURD, B. & SMITH, N. C. 2002a. Biochemical characterisation of the 56 and 82 kDa immunodominant gametocyte antigens from *Eimeria maxima*. *International journal for parasitology,* 32**,** 805-816.

BELLI, S. I., MAI, K., SKENE, C. D., GLEESON, M. T., WITCOMBE, D. M., KATRIB, M., FINGER, A., WALLACH, M. G. & SMITH, N. C. 2004. Characterisation of the antigenic and immunogenic properties of bacterially expressed, sexual stage antigens of the coccidian parasite, *Eimeria maxima*. *Vaccine,* 22**,** 4316-4325.

BELLI, S. I., SMITH, N. C. & FERGUSON, D. J. 2006. The coccidian oocyst: a tough nut to crack! *Trends in parasitology,* 22**,** 416-423.

BELLI, S. I., WALLACH, M. G., LUXFORD, C., DAVIES, M. J. & SMITH, N. C. 2003. Roles of tyrosine-rich precursor glycoproteins and dityrosine-and 3, 4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. *Eukaryotic cell,* 2**,** 456-464.

BELLI, S. I., WITCOMBE, D., WALLACH, M. G. & SMITH, N. C. 2002b. Functional genomics of gam56: characterisation of the role of a 56 kilodalton sexual stage antigen in oocyst wall formation in *Eimeria maxima*. *International journal for parasitology,* 32**,** 1727-1737.

BENNETT, R. & IJPELAAR, J. 2005. Updated estimates of the costs associated with thirty four endemic livestock diseases in Great Britain: a note. *Journal of agricultural economics,* 56**,** 135-144.

BEVER, C. S., DONG, J.-X., VASYLIEVA, N., BARNYCH, B., CUI, Y., XU, Z.-L., HAMMOCK, B. D. & GEE, S. J. 2016. VHH antibodies: emerging reagents for the analysis of environmental chemicals. *Analytical and bioanalytical chemistry,* 408**,** 5985-6002.

BIRD, R. E., HARDMAN, K. D., JACOBSON, J. W., JOHNSON, S., KAUFMAN, B. M., LEE, S.-M., LEE, T., POPE, S. H., RIORDAN, G. S. & WHITLOW, M. 1988. Single-chain antigen-binding proteins. *Science,* 242**,** 423-426.

BLAKE, D. P., BILLINGTON, K. J., COPESTAKE, S. L., OAKES, R. D., QUAIL, M. A., WAN, K.-L., SHIRLEY, M. W. & SMITH, A. L. 2011. Genetic mapping identifies novel highly protective antigens for an apicomplexan parasite. *PLoS pathogens,* 7**,** e1001279.

BLAKE, D. P., CLARK, E. L., MACDONALD, S. E., THENMOZHI, V., KUNDU, K., GARG, R., JATAU, I. D., AYOADE, S., KAWAHARA, F. & MOFTAH, A. 2015. Population, genetic, and antigenic diversity of the apicomplexan *Eimeria tenella* and their relevance to vaccine development. *Proceedings of the national academy of sciences,* 112**,** E5343-E5350.

BLAKE, D. P., HESKETH, P., ARCHER, A., CARROLL, F., SHIRLEY, M. W. & SMITH, A. L. 2005. The influence of immunizing dose size and schedule on immunity to subsequent challenge with antigenically distinct strains of *Eimeria maxima*. *Avian pathology,* 34**,** 489-494.

BLAKE, D. P., HESKETH, P., ARCHER, A., CARROLL, F., SMITH, A. L. & SHIRLEY, M. W. 2004. Parasite genetics and the immune host: recombination between antigenic types of *Eimeria maxima* as an entree to the identification of protective antigens. *Molecular and biochemical parasitology,* 138**,** 143-152.

BLAKE, D. P., HESKETH, P., ARCHER, A., SHIRLEY, M. W. & SMITH, A. L. 2006. *Eimeria maxima*: the influence of host genotype on parasite reproduction as

revealed by quantitative real-time PCR. *International journal for parasitology,* 36**,** 97-105.
BLAKE, D. P., PASTOR-FERNÁNDEZ, I., NOLAN, M. J. & TOMLEY, F. M. 2017. Recombinant anticoccidial vaccines-a cup half full? *Infection, genetics and evolution,* 55**,** 358-365.
BLAKE, D. P. & TOMLEY, F. M. 2014. Securing poultry production from the ever-present *Eimeria* challenge. *Trends in parasitology,* 30**,** 12-19.
BRADBURY, A. & MARKS, J. D. 2004. Antibodies from phage antibody libraries. *Journal of immunological methods,* 290**,** 29.
BRADBURY, A. R., SIDHU, S., DÜBEL, S. & MCCAFFERTY, J. 2011. Beyond natural antibodies: the power of in vitro display technologies. *Nature biotechnology,* 29**,** 245.
BRATKOVIČ, T. 2010. Progress in phage display: evolution of the technique and its applications. *Cellular and molecular life sciences,* 67**,** 749-767.
BREMNER, A. 2018. Innate responses and biomarkers of resistance to Eimeria infection in the chicken. PhD Thesis, University of Edinburgh.
BRUIN, R. D., SPELT, K., MOL, J., KOES, R. & QUATTROCCHIO, F. 1999. Selection of high-affinity phage antibodies from phage display libraries. *Nature biotechnology,* 17**,** 397-399.
BUMSTEAD, N. & MILLARD, B. 1992. Variation in susceptibility of inbred lines of chickens to seven species of *Eimeria*. *Parasitology,* 104**,** 407-413.
CANTACESSI, C., RIDDELL, S., MORRIS, G. M., DORAN, T., WOODS, W. G., OTRANTO, D. & GASSER, R. B. 2008. Genetic characterization of three unique operational taxonomic units of *Eimeria* from chickens in Australia based on nuclear spacer ribosomal DNA. *Veterinary parasitology,* 152**,** 226-234.
CARMEN, S. & JERMUTUS, L. 2002. Concepts in antibody phage display. *Briefings in functional genomics,* 1**,** 189-203.
CARSWELL, R. 1838. *Pathological anatomy: illustrations of the elementary forms of disease*, Longman, Orme, Brown, Green and Longman.
CHALLEY, J. R. & BURNS, W. C. 1959. The invasion of the cecal mucosa by *Eimeria tenella* sporozoites and their transport by macrophages. *The journal of protozoology,* 6**,** 238-241.
CHAPMAN, H. 1978. Studies on the excystation of different species of *Eimeria* in vitro. *Zeitschrift für parasitenkunde,* 56**,** 115-121.
CHAPMAN, H. 1982. The use of enzyme electrophoresis for the identification of the species of *Eimeria* present in field isolates of coccidia. *Parasitology,* 85**,** 437-442.
CHAPMAN, H. 2014. Milestones in avian coccidiosis research: a review. *Poultry science,* 93**,** 501-511.
CHAPMAN, H. D., BARTA, J. R., BLAKE, D., GRUBER, A., JENKINS, M., SMITH, N. C., SUO, X. & TOMLEY, F. M. 2013. A selective review of advances in coccidiosis research. *Adv parasitol,* 83**,** 93-171.
CHAPMAN, H. D. & JEFFERS, T. K. 2014. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. *International journal for parasitology: Drugs and drug resistance,* 4**,** 214-217.
CHAPMAN, H. D., JEFFERS, T. K. & WILLIAMS, R. B. 2010. Forty years of monensin for the control of coccidiosis in poultry. *Poultry science,* 89**,** 1788-1801.
CHEN, G. & SIDHU, S. S. 2014. Design and generation of synthetic antibody libraries for phage display. In *monoclonal antibodies,* 96, 113-131.
CHEN, W., PRABAKARAN, P., ZHU, Z., FENG, Y., STREAKER, E. D. & DIMITROV, D. S. 2012. Characterization of human IgG repertoires in an acute HIV-1 infection. *Experimental and molecular pathology,* 93**,** 399-407.

CHOI, K. D. & LILLEHOJ, H. S. 2000. Role of chicken IL-2 on γδ T-cells and *Eimeria acervulina*-induced changes in intestinal IL-2 mRNA expression and γδ T-cells. *Veterinary immunology and immunopathology,* 73**,** 309-321.

CH'NG, A. C. W., CHOONG, Y. S. & LIM, T. S. 2016. Phage Display-Derived Antibodies: Application of Recombinant Antibodies for Diagnostics. *Proof and concepts in rapid diagnostic tests and technologies.* IntechOpen, 107-135.

CLACKSON, T., HOOGENBOOM, H. R., GRIFFITHS, A. D. & WINTER, G. 1991. Making antibody fragments using phage display libraries. *Nature,* 352**,** 624.

CLACKSON, T. & LOWMAN, H. B. 2004. *Phage display: a practical approach*, Oxford University Press, USA, 266.

CLARK, E. L., MACDONALD, S. E., THENMOZHI, V., KUNDU, K., GARG, R., KUMAR, S., AYOADE, S., FORNACE, K. M., JATAU, I. D. & MOFTAH, A. 2016. Cryptic *Eimeria* genotypes are common across the southern but not northern hemisphere. *International journal for parasitology,* 46**,** 537-544.

CLARK, E. L., TOMLEY, F. M. & BLAKE, D. P. 2017. Are *Eimeria* genetically diverse, and does it matter? *Trends in parasitology,* 33**,** 231-241.

CLEMENTI, N., MANCINI, N., SOLFOROSI, L., CASTELLI, M., CLEMENTI, M. & BURIONI, R. 2012. Phage display-based strategies for cloning and optimization of monoclonal antibodies directed against human pathogens. *International journal of molecular sciences,* 13**,** 8273-8292.

CLICK, E. M. & WEBSTER, R. E. 1998. The TolQRA proteins are required for membrane insertion of the major capsid protein of the filamentous phage f1 during infection. *Journal of bacteriology,* 180**,** 1723-1728.

CONWAY, D. P. & MCKENZIE, M. E. 2007. *Poultry coccidiosis: diagnostic and testing procedures*, John Wiley & Sons, Blackwell publishing, Oxford.

CONWAY, D. P., SASAI, K., GAAFAR, S. M. & SMOTHERS, C. D. 1993. Effects of different levels of oocyst inocula of *Eimeria acervulina*, E. *tenella*, and E. *maxima* on plasma constituents, packed cell volume, lesion scores, and performance in chickens. *Avian diseases***,** 118-123.

D'ANGELO, S., KUMAR, S., NARANJO, L., FERRARA, F., KISS, C. & BRADBURY, A. R. 2014. From deep sequencing to actual clones. *Protein engineering, design & selection,* 27**,** 301-307.

DAKPOGAN, H. B. & SALIFOU, S. 2013. Coccidiosis prevalence and intensity in litter based high stocking density layer rearing system of Benin. *Journal of animal and plant science,* 17**,** 2522-2526.

DALLOUL, R. A. & LILLEHOJ, H. S. 2006. Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert review of vaccines,* 5**,** 143-163.

DAS, P., RAJIB, D., NOOR, M. & ISLAM, M. 2004. A retrospective analysis on the proportional incidence of poultry diseases in greater Mymensingh district of Bangladesh. *Proceedings of Seminar*, 33.

DE GUSSEM, M. 2007. Coccidiosis in poultry: review on diagnosis, control, prevention and interaction with overall gut health. *Proceedings of the 16th European Symposium on Poultry Nutrition*, 2, 253-261.

DE VLIEGER, D., BALLEGEER, M., ROSSEY, I., SCHEPENS, B. & SAELENS, X. 2019. Single-Domain Antibodies and Their Formatting to Combat Viral Infections. *Antibodies,* 8**,** 1.

DERDA, R., TANG, S. K., LI, S. C., NG, S., MATOCHKO, W. & JAFARI, M. R. 2011. Diversity of phage-displayed libraries of peptides during panning and amplification. *Molecules,* 16**,** 1776-1803.

DOBELL, C. 1922. The discovery of the coccidia. *Parasitology,* 14**,** 342-348.

DOOLEY, H., FLAJNIK, M. F. & PORTER, A. J. 2003. Selection and characterization of naturally occurring single-domain (IgNAR) antibody fragments from immunized sharks by phage display. *Molecular immunology,* 40**,** 25-33.

DUFFY, C., MATHIS, G. & POWER, R. 2005. Effects of Natustat™ supplementation on performance, feed efficiency and intestinal lesion scores in broiler chickens challenged with *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella*. *Veterinary parasitology,* 130**,** 185-190.

DUONG-LY, K. C. & GABELLI, S. B. 2015. Affinity purification of a recombinant protein expressed as a fusion with the Maltose-Binding Protein (MBP) tag. *Methods in enzymology.* 559, 17-26.

DUSZYNSKI, D. W., SCOTT, D. T., ARAGON, J., LEACH, A. & PERRY, T. 1999. Six New *Eimeria* Species from Vespertilionid bats of North America. *The journal of parasitology*, 66, 496-503.

DYBALLA, N. & METZGER, S. 2009. Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. *JoVE (Journal of visualized experiments)***,** e1431.

EDGAR, S. 1954. Effect of temperature on the sporulation of oocysts of the protozoan, *Eimeria tenella*. *Transactions of the american microscopical Society,* 73**,** 237-242.

ELLIS, J. & BUMSTEAD, J. 1990. *Eimeria* species: studies using rRNA and rDNA probes. *Parasitology,* 101**,** 1-6.

EMANUEL, P. A., DANG, J., GEBHARDT, J. S., ALDRICH, J., GARBER, E. A., KULAGA, H., STOPA, P., VALDES, J. J. & DION-SCHULTZ, A. 2000. Recombinant antibodies: a new reagent for biological agent detection. *Biosensors and bioelectronics,* 14**,** 751-759.

ESCHENBACHER, K.-H., EGGLI, P., WALLACH, M. & BRAUN, R. 1996. Characterization of a 14 kDa oocyst wall protein of *Eimeria tenella* and *E. acervulina*. *Parasitology,* 112**,** 169-176.

FAGERLUND, A., MYRSET, A. H. & KULSETH, M. A. 2008. Construction and characterization of a 9-mer phage display pVIII-library with regulated peptide density. *Applied microbiology and biotechnology,* 80**,** 925-936.

FANTHAM, H. 1910. The Morphology and Life-History of *Eimeria* (Coccidium) avium: a Sporozoö causing a fatal disease among young Grouse. *Proceedings of the Zoological Society of London*, 80, 672-691.

FATOBA, A. J. & ADELEKE, M. A. 2018. Diagnosis and control of chicken coccidiosis: a recent update. *Journal of parasitic diseases,* 42**,** 483-493.

FAVER, J. C., RIEHLE, K., LANCIA JR, D. R., MILBANK, J. B., KOLLMANN, C. S., SIMMONS, N., YU, Z. & MATZUK, M. M. 2019. Quantitative Comparison of Enrichment from DNA-Encoded Chemical Library Selections. *ACS combinatorial science*, 21, 75-82.

FAYER, R. 1980. Epidemiology of protozoan infections: the coccidia. *Veterinary parasitology,* 6**,** 75-103.

FENG, M., BIAN, H., WU, X., FU, T., FU, Y., HONG, J., FLEMING, B. D., FLAJNIK, M. F. & HO, M. 2018. Construction and next-generation sequencing analysis of a large phage-displayed VNAR single-domain antibody library from six naïve nurse sharks. *Antibody therapeutics,* 2**,** 1-11.

FERGUSON, D., BELLI, S. I., SMITH, N. C. & WALLACH, M. G. 2003. The development of the macrogamete and oocyst wall in *Eimeria maxima*: immuno-light and electron microscopy. *International journal for parasitology,* 33**,** 1329-1340.

FERGUSON, D., HUTCHISON, W. & SIIM, J. C. 1975. The ultrastructural development of the macrogamete and formation of the oocyst wall of Toxoplasma gondii. *Acta pathologica microbiologica scandinavica section B microbiology,* 83**,** 491-505.

FISH, F. 1931. Quantitative and statistical analyses of infections with *Eimeria tenella* in the chicken. *American journal of epidemiology,* 14**,** 560-576.

FITZ-COY, S. & EDGAR, S. 1992. Pathogenicity and control of *Eimeria mitis* infections in broiler chickens. *Avian diseases***,** 44-48.

FLAJNIK, M. F., DESCHACHT, N. & MUYLDERMANS, S. 2011. A case of convergence: why did a simple alternative to canonical antibodies arise in sharks and camels? *PLoS biology,* 9**,** e1001120.

FORNACE, K. M., CLARK, E. L., MACDONALD, S. E., NAMANGALA, B., KARIMURIBO, E., AWUNI, J. A., THIEME, O., BLAKE, D. P. & RUSHTON, J. 2013. Occurrence of *Eimeria* species parasites on small-scale commercial chicken farms in Africa and indication of economic profitability. *PLoS One,* 8**,** e84254.

FOSTER, A. D., INGRAM, J. D., LEITCH, E. K., LENNARD, K. R., OSHER, E. L. & TAVASSOLI, A. 2015. Methods for the creation of cyclic peptide libraries for use in lead discovery. *Journal of biomolecular screening,* 20**,** 563-576.

FRY, M., HUDSON, A., RANDALL, A. & WILLIAMS, R. 1984. Potent and selective hydroxynaphthoquinone inhibitors of mitochondrial electron transport in *Eimeria tenella* (Apicomplexa: Coccidia). *Biochemical pharmacology,* 33**,** 2115-2122.

FRÖLICH, S., FARHAT, J. & WALLACH, M. 2013. Designing strategies for the control of coccidiosis in chickens on poultry farms using modern diagnostic tools. *Rep parasitol,* 3**,** 1-10.

GADELHAQ, S. M., ARAFA, W. M. & ABOLHADID, S. M. 2018. In vitro activity of natural and chemical products on sporulation of *Eimeria* species oocysts of chickens. *Veterinary parasitology,* 251**,** 12-16.

GERHOLD, R. W., MCDOUGALD, L. R. & BECKSTEAD, R. B. 2015. A Novel, Simplified Technique to Amplify *Eimeria* (Coccidia: Apicomplexa) DNA from Oocysts. *Journal of parasitology,* 101**,** 102-103.

GHAREKHANI, J., SADEGHI-DEHKORDI, Z. & BAHRAMI, M. 2014. Prevalence of coccidiosis in broiler chicken farms in Western Iran. *Journal of veterinary medicine,*10, 55-59.

GIANNENAS, I., TSALIE, E., TRIANTAFILLOU, E., HESSENBERGER, S., TEICHMANN, K., MOHNL, M. & TONTIS, D. 2014. Assessment of probiotics supplementation via feed or water on the growth performance, intestinal morphology and microflora of chickens after experimental infection with *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella*. *Avian pathology,* 43**,** 209-216.

GLANVILLE, J., ZHAI, W., BERKA, J., TELMAN, D., HUERTA, G., MEHTA, G. R., NI, I., MEI, L., SUNDAR, P. D. & DAY, G. M. 2009. Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. *Proceedings of the national academy of sciences,* 106**,** 20216-20221.

GODFRAY, H. C. J., BEDDINGTON, J. R., CRUTE, I. R., HADDAD, L., LAWRENCE, D., MUIR, J. F., PRETTY, J., ROBINSON, S., THOMAS, S. M. & TOULMIN, C. 2010. Food security: the challenge of feeding 9 billion people. *Science,* 327**,** 812-818.

GODWIN, R. M. & MORGAN, J. A. 2015. A molecular survey of *Eimeria* in chickens across Australia. *Veterinary parasitology,* 214**,** 16-21.

GOLD, D., STEIN, B. & TZIPORI, S. 2001. The utilization of sodium taurocholate in excystation of Cryptosporidium parvum and infection of tissue culture. *Journal of parasitology,* 87**,** 997-1000.

GOLETZ, S., CHRISTENSEN, P. A., KRISTENSEN, P., BLOHM, D., TOMLINSON, I., WINTER, G. & KARSTEN, U. 2002. Selection of large diversities of antiidiotypic antibody fragments by phage display. *Journal of molecular biology,* 315**,** 1087-1097.

GORE, T. C. & LONG, P. L. 1982. The biology and pathogenicity of a recent field isolate of *Eimeria praecox* Johnson, 1930. *The journal of protozoology,* 29**,** 82-85.

GOUGH, K. C., MADDISON, B. C., SHIKOTRA, A., MOISEEVA, E. P., YANG, W., JARVIS, S. & BRADDING, P. 2015. Evidence for a novel Kit adhesion domain mediating human mast cell adhesion to structural airway cells. *Respiratory research,* 16**,** 79-86.

GRAAT, E., HENKEN, A., PLOEGER, H., NOORDHUIZEN, J. & VERTOMMEN, M. 1994. Rate and course of sporulation of oocysts of *Eimeria acervulina* under different environmental conditions. *Parasitology,* 108**,** 497-502.

GRACE, D., MUTUA, F., OCHUNGO, P., KRUSKA, R., JONES, K., BRIERLEY, L., LAPAR, M., SAID, M., HERRERO, M. & PHUC, P. 2012. Mapping of poverty and likely zoonoses hotspots. Report to the UK department for international development. *International livestock research institure, Nairobi, Kenya*.

GRIEP, R. 1999. Development of recombinant antibody technology for application in plant pathogen diagnosis, PhD Thesis, University of Wageningen.

GRIFFITHS, A. D., WILLIAMS, S. C., HARTLEY, O., TOMLINSON, I., WATERHOUSE, P., CROSBY, W. L., KONTERMANN, R., JONES, P., LOW, N. & ALLISON, T. A. 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. *The EMBO journal,* 13**,** 3245-3260.

GRILLI, G., BORGONOVO, F., TULLO, E., FONTANA, I., GUARINO, M. & FERRANTE, V. 2018. A pilot study to detect coccidiosis in poultry farms at early stage from air analysis. *Biosystems engineering,* 173, 64-70.

GYÖRKE, A., POP, L. & COZMA, V. 2013. Prevalence and distribution of *Eimeria* species in broiler chicken farms of different capacities. *Parasite,* 20, 45.

HAFTU, B., ASRESIE, A. & HAYLOM, M. 2014. Assessment on Major Health Constraints of Livestock Development in Eastern Zone of Tigray: The Case of "Gantaafeshum Woreda" Northern Ethiopia. *Journal of veterinary science and. technology,* 5**,** 174.

HAKE, T. 1839. *Treatie on Varicose Capillaris, as Constitut. of Carcin. of the Hepatic Ducts*. With an account of a New form of pus golule. 22-25.

HAMERS-CASTERMAN, C., ATARHOUCH, T., MUYLDERMANS, S., ROBINSON, G., HAMMERS, C., SONGA, E. B., BENDAHMAN, N. & HAMMERS, R. 1993. Naturally occurring antibodies devoid of light chains. *Nature,* 363**,** 446.

HAMIDINEJAT, H., SHAPOURI, M. S., MAYAHI, M. & BORUJENI, M. P. 2010. Characterization of *Eimeria* species in commercial broilers by PCR based on ITS1 regions of rDNA. *Iranian journal of parasitology,* 5**,** 48.

HAMMOND, D. M. & LONG, P. L. 1973. The coccidia. *Eimeria*, *Isospora, Toxoplasma*, and related genera. *The coccidia. Eimeria, Isospora, Toxoplasma, and related genera.*https://doi.org/10.1007/978-2-8178-0847-5.

HARMSEN, M. & DE HAARD, H. 2007. Properties, production, and applications of camelid single-domain antibody fragments. *Applied microbiology and biotechnology,* 77**,** 13-22.

HAUG, A., GJEVRE, A.-G., SKJERVE, E. & KALDHUSDAL, M. 2008a. A survey of the economic impact of subclinical *Eimeria* infections in broiler chickens in Norway. *Avian pathology,* 37**,** 333-341.

HAUG, A., GJEVRE, A.-G., THEBO, P., MATTSSON, J. G. & KALDHUSDAL, M. 2008b. Coccidial infections in commercial broilers: epidemiological aspects and comparison of *Eimeria* species identification by morphometric and polymerase chain reaction techniques. *Avian pathology,* 37**,** 161-170.

HAUG, A., THEBO, P. & MATTSSON, J. G. 2007. A simplified protocol for molecular identification of *Eimeria* species in field samples. *Veterinary parasitology,* 146**,** 35-45.

HAYAJNEH, F., JALAL, M., ZAKARIA, H., ABDELQADER, A. & ABUAJAMIEH, M. 2018. Anticoccidial effect of apple cider vinegar on broiler chicken: an organic treatment to measure anti-oxidant effect. *Polish journal of veterinary sciences,* 361-369.

HILL, D. F. & PETERSEN, G. B. 1982. Nucleotide sequence of bacteriophage f1 DNA. *Journal of virology,* 44**,** 32-46.

HOLLIGER, P. & HUDSON, P. J. 2005. Engineered antibody fragments and the rise of single domains. *Nature biotechnology,* 23**,** 1126-1136.

HOOGENBOOM, H. R., DE BRUINE, A. P., HUFTON, S. E., HOET, R. M., ARENDS, J.-W. & ROOVERS, R. C. 1998. Antibody phage display technology and its applications. *Immunotechnology,* 4**,** 1-20.

HOOGENBOOM, H. R., GRIFFITHS, A. D., JOHNSON, K. S., CHISWELL, D. J., HUDSON, P. & WINTER, G. 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic acids research,* 19**,** 4133-4137.

HOSKINS, R. A., STAPLETON, M., GEORGE, R. A., YU, C., WAN, K. H., CARLSON, J. W. & CELNIKER, S. E. 2005. Rapid and efficient cDNA library screening by self-ligation of inverse PCR products (SLIP). *Nucleic acids research,* 33**,** e185-e185.

HUFTON, S. E., MOERKERK, P. T., MEULEMANS, E. V., DE BRUÏNE, A., ARENDS, J.-W. & HOOGENBOOM, H. R. 1999. Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. *Journal of immunological methods,* 231**,** 39-51.

HUNG, L.-Y., WANG, C.-H., FU, C.-Y., GOPINATHAN, P. & LEE, G.-B. 2016. Microfluidics in the selection of affinity reagents for the detection of cancer: paving a way towards future diagnostics. *Lab on a Chip,* 16**,** 2759-2774.

HUST, M. & DÜBEL, S. 2004. Mating antibody phage display with proteomics. *Trends in biotechnology,* 22**,** 8-14.

INNES, E. & VERMEULEN, A. 2006. Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma and Neospora*. *Parasitology,* 133**,** S145-S168.

JADHAV, B., NIKAM, S., BHAMRE, S. & JAID, E. 2011. Study of *Eimeria necatrix* in broiler chicken from Aurangabad District of Maharashtra state India. *International multidisciplinary research journal,* 1,11.

JATAU, I. D., SULAIMAN, N. H., MUSA, I. W., LAWAL, A. I., OKUBANJO, O. O., ISAH, I. & MAGAJI, Y. 2012. Prevalence of coccidia infection and preponderance *Eimeria* species in free range indigenous and intensively managed exotic chickens during hot-wet season. *Asian journal of poultry science,* 6**,** 79-88.

JENKINS, M., ALLEN, P., WILKINS, G., KLOPP, S. & MISKA, K. 2008. *Eimeria praecox* infection ameliorates effects of *Eimeria maxima* infection in chickens. *Veterinary parasitology,* 155**,** 10-14.

JENKINS, M. C. & DAME, J. B. 1987. Identification of immunodominant surface antigens of *Eimeria acervulina* sporozoites and merozoites. *Molecular and biochemical parasitology,* 25**,** 155-164.

JENKINS, M. C., PARKER, C. & RITTER, D. 2017. *Eimeria* Oocyst Concentrations and Species Composition in Litter from Commercial Broiler Farms During Anticoccidial Drug or Live *Eimeria* Oocyst Vaccine Control Programs. *Avian diseases,* 61**,** 214-220.

JOHNSON, J. & REID, W. M. 1970. Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. *Experimental parasitology,* 28**,** 30-36

JOHNSON, W. 1930. A Study on *Eimeria necatrix*. *Agricul Experi Station Tech Bull,* 538**,** 30-33.

JOHNSTON, D. & FERNANDO, M. 1997. Isoenzymes of *Eimeria* from the domestic fowl: electrophoretic variants among species, strains and clones. *Parasitology research,* 83**,** 464-470.

JOYNER, L. 1978. The identification and diagnosis of avian coccidiosis. *Avian coccidiosis***,** 29-49.

JOYNER, L. & LONG, P. 1974. The specific characters of the *Eimeria*, with special reference to the coccidia of the fowl. *Avian pathology,* 3**,** 145-157.
JOŃCZYK, E., KŁAK, M., MIĘDZYBRODZKI, R. & GÓRSKI, A. 2011. The influence of external factors on bacteriophages. *Folia microbiologica,* 56**,** 191-200.
KABOUDI, K., UMAR, S. & MUNIR, M. T. 2016. Prevalence of Coccidiosis in Free-Range Chicken in Sidi Thabet, Tunisia. *Scientifica,* 10, 15-22.
KADYKALO, S., ROBERTS, T., THOMPSON, M., WILSON, J., LANG, M. & ESPEISSE, O. 2018. The value of anticoccidials for sustainable global poultry production. *International journal of antimicrobial agents,* 51**,** 304-310.
KANG, A. S., BARBAS, C. F., JANDA, K. D., BENKOVIC, S. J. & LERNER, R. A. 1991. Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proceedings of the national academy of sciences,* 88**,** 4363-4366.
KARAER, Z., GUVEN, E., AKCAY, A., KAR, S., NALBANTOGLU, S. & CAKMAK, A. 2012. Prevalence of subclinical coccidiosis in broiler farms in Turkey. *Tropical animal health and production,* 44**,** 589-594.
KARIM, M., BASAK, S. C. & TREES, A. J. 1996. Characterization and immunoprotective properties of a monoclonal antibody against the major oocyst wall protein of *Eimeria tenella*. *Infection and immunity,* 64**,** 1227-1232.
KAWAHARA, F., TAIRA, K., NAGAI, S., ONAGA, H., ONUMA, M. & NUNOYA, T. 2008. Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. *Avian diseases,* 52**,** 652-656.
KOCYLOWSKI, B., ZELAZNY, J., ANTYCHOWICZ, J. & PANCZYK, J. 1976. Incidence of carp coccidiosis and its control. *Bulletin of the veterinary institute in Pulawy,* 20**,** 12-17.
KOUWENHOVEN, B. & VAN DER HORST, C. J. 1972. Disturbed intestinal absorption of vitamin A and carotenes and the effect of a low pH during *Eimeria acervulina* infection in the domestic fowl (Gallus domesticus). *Zeitschrift für parasitenkunde,* 38**,** 152-161.
KRAMER, M. F., VESEY, G., LOOK, N. L., HERBERT, B. R., SIMPSON-STROOT, J. M. & LIM, D. V. 2007. Development of a Cryptosporidium oocyst assay using an automated fiber optic-based biosensor. *Journal of biological engineering,* 1**,** 3.
KUHN, P., FÜHNER, V., UNKAUF, T., MOREIRA, G. M. S. G., FRENZEL, A., MIETHE, S. & HUST, M. 2016. Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display. *PROTEOMICS–Clinical applications,* 10**,** 922-948.
KUMAR, S., GARG, R., MOFTAH, A., CLARK, E. L., MACDONALD, S. E., CHAUDHRY, A. S., SPARAGANO, O., BANERJEE, P. S., KUNDU, K. & TOMLEY, F. M. 2014. An optimised protocol for molecular identification of *Eimeria* from chickens. *Veterinary parasitology,* 199**,** 24-31.
KUTICIC, V. & WIKERHAUSER, T. 1996. Studies of the effect of various treatments on the viability of *Toxoplasma gondii* tissue cysts and oocysts. In *Toxoplasma gondii,* 261-265.
KUČERA, J. 1990. Identification of *Eimeria* species in Czechoslovakia. *Avian pathology,* 19**,** 59-66.
LADNER, R. C., SATO, A. K., GORZELANY, J. & DE SOUZA, M. 2004. Phage display-derived peptides as therapeutic alternatives to antibodies. *Drug discovery today,* 9**,** 525-529.
LAL, K., BROMLEY, E., OAKES, R., PRIETO, J. H., SANDERSON, S. J., KURIAN, D., HUNT, L., YATES, J. R., WASTLING, J. M. & SINDEN, R. E. 2009. Proteomic comparison of four *Eimeria tenella* life-cycle stages: Unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. *Proteomics,* 9**,** 4566-4576.

LANNE, B. & PANFILOV, O. 2005. Protein staining influences the quality of mass spectra obtained by peptide mass fingerprinting after separation on 2-d gels. A comparison of staining with coomassie brilliant blue and sypro ruby. *Journal of proteome research,* 4**,** 175-179.

LAUWEREYS, M., GHAHROUDI, M. A., DESMYTER, A., KINNE, J., HÖLZER, W., DE GENST, E., WYNS, L. & MUYLDERMANS, S. 1998. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *The EMBO journal,* 17**,** 3512-3520.

LEBANI, K. 2015. Antibody discovery for development of a serotyping dengue virus NS1 capture assay. PhD Thesis, The university of Queensland, Australia.

LEBANI, K., JONES, M. L., WATTERSON, D., RANZONI, A., TRAVES, R. J., YOUNG, P. R. & MAHLER, S. M. 2017. Isolation of serotype-specific antibodies against dengue virus non-structural protein 1 using phage display and application in a multiplexed serotyping assay. *PloS one,* 12**,** e0180669.

LEDSGAARD, L., KILSTRUP, M., KARATT-VELLATT, A., MCCAFFERTY, J. & LAUSTSEN, A. 2018. Basics of antibody phage display technology. *Toxins,* 10**,** 236.

LEE, W. G., KIM, Y.-G., CHUNG, B. G., DEMIRCI, U. & KHADEMHOSSEINI, A. 2010. Nano/Microfluidics for diagnosis of infectious diseases in developing countries. *Advanced drug delivery reviews,* 62**,** 449-457.

LEOW, C. H., FISCHER, K., LEOW, C. Y., BRAET, K., CHENG, Q. & MCCARTHY, J. 2018. Isolation and characterization of malaria PfHRP2 specific V NAR antibody fragments from immunized shark phage display library. *Malaria journal,* 17**,** 383.

LEVINE, N. D. 2018. *The Coccidian Parasites of Rodents:* CRC Press.Boca Raton https://doi.org/10.1201/9781351070737.

LEVINE, P. 1942. A new coccidium pathogenic for chickens, *Eimeria brunetti* n. sp.(Protozoa: Eimeriidae). *Cornell vet,* 32**,** 430-439.

LI, G., LILLEHOJ, E. P. & LILLEHOJ, H. S. 2002. Interleukin-2 production in SC and TK chickens infected with *Eimeria tenella*. *Avian diseases,* 46**,** 2-9.

LI, Y., COCKBURN, W., KILPATRICK, J. & WHITELAM, G. C. 1999. Selection of rabbit single-chain Fv fragments against the herbicide atrazine using a new phage display system. *Food and agricultural immunology,* 11**,** 5-17.

LI, Y., COCKBURN, W., KILPATRICK, J. B. & WHITELAM, G. C. 2000a. High affinity ScFvs from a single rabbit immunized with multiple haptens. *Biochemical and biophysical research communications,* 268**,** 398-404.

LI, Y., KILPATRICK, J. & WHITELAM, G. C. 2000b. Sheep monoclonal antibody fragments generated using a phage display system. *Journal of immunological methods,* 236**,** 133-146.

LI, Z., KRIPPENDORFF, B.-F., SHARMA, S., WALZ, A. C., LAVÉ, T. & SHAH, D. K. Influence of molecular size on tissue distribution of antibody fragments. MAbs, 2016. Taylor & Francis, 113-119.

LI, Z., TANG, X., SUO, J., QIN, M., YIN, G., LIU, X. & SUO, X. 2015. Transgenic *Eimeria mitis* expressing chicken interleukin 2 stimulated higher cellular immune response in chickens compared with the wild-type parasites. *Frontiers in microbiology,* 6, 533.

LILLEHOJ, H., KIM, C., KEELER JR, C. & ZHANG, S. 2007. Immunogenomic approaches to study host immunity to enteric pathogens. *Poultry science,* 86**,** 1491-1500.

LILLEHOJ, H., MIN, W. & DALLOUL, R. 2004. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. *Poultry science,* 83**,** 611-623.

LILLEHOJ, H. & OKAMURA, M. 2003. Host immunity and vaccine development to coccidia and Salmonella infections in chickens. *The journal of poultry science,* 40**,** 151-193.

LILLEHOJ, H. S. & LILLEHOJ, E. P. 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. *Avian diseases*, 408-425.
LILLEHOJ, H. S. & TROUT, J. M. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. *Clinical microbiology reviews,* 9, 349-360.
LIM, B. N., CHIN, C. F., CHOONG, Y. S., ISMAIL, A. & LIM, T. S. 2016. Generation of a naïve human single chain variable fragment (scFv) library for the identification of monoclonal scFv against Salmonella Typhi Hemolysin E antigen. *Toxicon,* 117, 94-101.
LIM, B. N., TYE, G. J., CHOONG, Y. S., ONG, E. B. B., ISMAIL, A. & LIM, T. S. 2014. Principles and application of antibody libraries for infectious diseases. *Biotechnology letters,* 36, 2381-2392.
LIM, C. C., WOO, P. C. & LIM, T. S. 2019. Development of a Phage Display Panning Strategy Utilizing Crude Antigens: Isolation of MERS-CoV Nucleoprotein human antibodies. *Scientific reports,* 9, 6088.
LIU, G. W., LIVESAY, B. R., KACHEROVSKY, N. A., CIESLEWICZ, M., LUTZ, E., WAALKES, A., JENSEN, M. C., SALIPANTE, S. J. & PUN, S. H. 2015. Efficient identification of murine M2 macrophage peptide targeting ligands by phage display and next-generation sequencing. *Bioconjugate chemistry,* 26, 1811-1817.
LOBAGO, F., WORKU, N. & WOSSENE, A. 2005. Study on coccidiosis in Kombolcha poultry farm, Ethiopia. *Tropical animal health and production,* 37, 245-251.
LOFGREN, J. A., DHANDAPANI, S., PENNUCCI, J. J., ABBOTT, C. M., MYTYCH, D. T., KALIYAPERUMAL, A., SWANSON, S. J. & MULLENIX, M. C. 2007. Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. *The journal of immunology,* 178, 7467-7472.
LOH, B., KUHN, A. & LEPTIHN, S. 2019. The fascinating biology behind phage display: filamentous phage assembly. *Molecular microbiology,* 111, 1132-1138.
LONG, P. & JOYNER, L. 1984. Problems in the identification of species of *Eimeria*. *The journal of protozoology,* 31, 535-541.
LONG, P. L., MILLARD, B. J., JOYNER, L. P. & NORTON, C. C. 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia veterinaria latina,* 6, 201-217.
LONG, P. L. & REID, W. M. 1982. Guide for the diagnosis of coccidiosis in chickens. Iowa University press. Ames, Iowa.
LONG, P. L., TOMPKINS, R. V. & MILLARD, B. J. 1975. Coccidiosis in broilers: Evaluation of infection by the examination of broiler house litter for Oocysts. *Avian pathology,* 4, 287-294.
LUNDEN, A., THEBO, P., GUNNARSSON, S., HOOSHMAND-RAD, P., TAUSON, R. & UGGLA, A. 2000. *Eimeria* infections in litter-based, high stocking density systems for loose-housed laying hens in Sweden. *British poultry science,* 41, 440-447.
LUU, L., BETTRIDGE, J., CHRISTLEY, R. M., MELESE, K., BLAKE, D., DESSIE, T., WIGLEY, P., DESTA, T. T., HANOTTE, O. & KAISER, P. 2013. Prevalence and molecular characterisation of *Eimeria* species in Ethiopian village chickens. *BMC veterinary research,* 9, 208.
LÖVGREN, J., PURSIHEIMO, J.-P., PYYKKÖ, M., SALMI, J. & LAMMINMÄKI, U. 2016. Next generation sequencing of all variable loops of synthetic single framework scFv—Application in anti-HDL antibody selections. *New biotechnology,* 33, 790-796.
LØSET, G. Å. & SANDLIE, I. 2012. Next generation phage display by use of pVII and pIX as display scaffolds. *Methods,* 58, 40-46.
MAI, K., SHARMAN, P. A., WALKER, R. A., KATRIB, M., SOUZA, D. D., MCCONVILLE, M. J., WALLACH, M. G., BELLI, S. I., FERGUSON, D. J. &

SMITH, N. C. 2009. Oocyst wall formation and composition in coccidian parasites. *Memorias do instituto oswaldo cruz,* 104**,** 281-289.
MANEEWANNAKUL, K., MANEEWANNAKUL, S. & IPPEN-IHLER, K. 1993. Synthesis of F pilin. *Journal of bacteriology,* 175**,** 1384-1391.
MARKS, J. D., HOOGENBOOM, H. R., BONNERT, T. P., MCCAFFERTY, J., GRIFFITHS, A. D. & WINTER, G. 1991. By-passing immunization: human antibodies from V-gene libraries displayed on phage. *Journal of molecular biology,* 222**,** 581-597.
MARVIN, D. 1998. Filamentous phage structure, infection and assembly. *Current opinion in structural biology,* 8**,** 150-158.
MATHIS, G. F. & MCDOUGALD, L. R. 1987. Evaluation of interspecific hybrids of the chicken, guinea fowl, and Japanese quail for innate resistance to coccidia. *Avian diseases***,** 740-745.
MATTIELLO, R., BOVIEZ, J. & MCDOUGALD, L. 2000. *Eimeria brunetti* and *Eimeria necatrix* in chickens of Argentina and confirmation of seven species of *Eimeria*. *Avian diseases***,** 711-714.
MAYBERRY, L. F., MARQUARDT, W. C., NASH, D. J. & PLANT, B. 1982. Genetic dependent transmission of *Eimeria separata* from *Rattus* to three strains of Mus musculus, an abnormal host. *The journal of parasitology***,** 1124-1126.
MCALLISTER, C. T., UPTON, S. J. & TRAUTH, S. E. 1994. New host and geographic records for coccidia (Apicomplexa: Eimeriidae) from North American turtles. *The journal of parasitology***,** 1045-1049.
MCCAFFERTY, J., GRIFFITHS, A. D., WINTER, G. & CHISWELL, D. J. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature,* 348, 552-554.
MCDONALD, V. & SHIRLEY, M. 2009. Past and future: vaccination against *Eimeria*. *Parasitology,* 136**,** 1477-1489.
MCDOUGALD, L. 1991. Protozoa. Calnek BW. Barnes HJ. Beard CW. Reid WM. Yoder HW. eds. *Diseases of poultry. Ames: Iowa state univ pres***,** 781-797.
MCDOUGALD, L., FULLER, L. & MATTIELLO, R. 1997. A survey of coccidia on 43 poultry farms in Argentina. *Avian diseases***,** 923-929.
MCDOUGALD, L. R. & FITZ-COY, S. H. 2008. *"Coccidiosis," in Diseases of Poultry, Blackwell Publishing Professional , Ames, Iowa, USA, Blackwell publishing professional*.
MCDOUGALD, L. R. & REID, W. M. 1997. *Diseases of poultry,* Iowa University press. Ames, Iowa.
MCLOUGHLIN, D. 1969. The influence of dexamethasone on attempts to transmit *Eimeria meleagrimitis* to chickens and E. *tenella* to turkeys. *The journal of protozoology,* 16**,** 145-148.
MEAGHER, R. J., HATCH, A. V., RENZI, R. F. & SINGH, A. K. 2008. An integrated microfluidic platform for sensitive and rapid detection of biological toxins. *Lab on a Chip,* 8**,** 2046-2053.
MIN, W., KIM, W. H., LILLEHOJ, E. P. & LILLEHOJ, H. S. 2013. Recent progress in host immunity to avian coccidiosis: IL-17 family cytokines as sentinels of the intestinal mucosa. *Developmental & comparative immunology,* 41**,** 418-428.
MIYAZAKI, N., KIYOSE, N., AKAZAWA, Y., TAKASHIMA, M., HAGIHARA, Y., INOUE, N., MATSUDA, T., OGAWA, R., INOUE, S. & ITO, Y. 2015. Isolation and characterization of antigen-specific alpaca (Lama pacos) VHH antibodies by biopanning followed by high-throughput sequencing. *The journal of biochemistry,* 158**,** 205-215.
MOHAMMED, B. R. & SUNDAY, O. S. 2015. An overview of the prevalence of avian coccidiosis in poultry production and its economic importance in Nigeria. *Veterinary research international,* 3**,** 35-45.
MOLAN, A. L., LIU, Z. & DE, S. 2009. Effect of pine bark (Pious radiata) extracts on sporulation of coccidian oocysts. *Folia parasitologica (Prague),* 56**,** 1.

MOLLA, B. & ALI, A. 2015. Epidemiological study on poultry coccidiosis: Prevalence, species identification and post mortem lesions in grower chicken in Kombolcha, North-Eastern Ethiopia. *Journal of veterinary medicine and animal health,* 7**,** 1-8.

MONEGAL, A., AMI, D., MARTINELLI, C., HUANG, H., ALIPRANDI, M., CAPASSO, P., FRANCAVILLA, C., OSSOLENGO, G. & DE MARCO, A. 2009. Immunological applications of single-domain llama recombinant antibodies isolated from a naive library. *Protein engineering, design & selection,* 22**,** 273-280.

MONNÉ, L. & HÖNIG, G. 1954. On the properties of the shells of the coccidian oocysts. *Arkiv för zoologi,* 7**,** 251-256.

MOORE, R. J. 2016. Necrotic enteritis predisposing factors in broiler chickens. *Avian pathology,* 45**,** 275-281.

MORGAN, J. A., CONSTANTINOIU, C. C. & JORGENSEN, W. K. 2009. *New Diagnostic assays to improve control of coccidiosis in poultry*. Rural industries research and development corporation. James Cook university, RIRDC press, Australia.

MORRIS, G. & GASSER, R. 2006. Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in *Eimeria*. *Biotechnology advances,* 24**,** 590-603.

MORRIS, G. M., WOODS, W. G., RICHARDS, D. G. & GASSER, R. B. 2007a. Investigating a persistent coccidiosis problem on a commercial broiler–breeder farm utilising PCR-coupled capillary electrophoresis. *Parasitology research,* 101**,** 583-589.

MORRIS, G. M., WOODS, W. G., RICHARDS, D. G. & GASSER, R. B. 2007b. The application of a polymerase chain reaction (PCR)-based capillary electrophoretic technique provides detailed insights into *Eimeria* populations in intensive poultry establishments. *Molecular and cellular probes,* 21**,** 288-294.

MULLEN, L. M., NAIR, S. P., WARD, J. M., RYCROFT, A. N. & HENDERSON, B. 2006. Phage display in the study of infectious diseases. *Trends in microbiology,* 14**,** 141-147.

MUNRO, R. & SYNGE, B. 1991. Coccidiosis in seals. *Veterinary record,* 129**,** 179-180.

MUTHAMILSELVAN, T., KUO, T.-F., WU, Y.-C. & YANG, W.-C. 2016. Herbal Remedies for Coccidiosis Control: A Review of Plants, Compounds, and Anticoccidial Actions. *Evidence-based complementary and alternative medicine,* 322, 66-85.

MUYLDERMANS, S. 2013. Nanobodies: natural single-domain antibodies. *Annual review of biochemistry,* 82**,** 775-797.

NABIAN, S., ARABKHAZAELI, F., SEIFOURI, P. & FARAHANI, A. 2018. Morphometric Analysis of the Intestine in Experimental Coccidiosis in Broilers Treated with Anticoccidial Drugs. *Iranian journal of parasitology,* 13**,** 493.

NAHAVANDI, S., BARATCHI, S., SOFFE, R., TANG, S.-Y., NAHAVANDI, S., MITCHELL, A. & KHOSHMANESH, K. 2014. Microfluidic platforms for biomarker analysis. *Lab on a Chip,* 14**,** 1496-1514.

NAIDOO, V., MCGAW, L. J., BISSCHOP, S. P., DUNCAN, N. & ELOFF, J. N. 2008. The value of plant extracts with antioxidant activity in attenuating coccidiosis in broiler chickens. *Veterinary parasitology,* 153**,** 214-219.

NAKAMURA, M., TSUMOTO, K., KUMAGAI, I. & ISHIMURA, K. 2003. A morphologic study of filamentous phage infection of *Escherichia coli* using biotinylated phages. *FEBS letters,* 536**,** 167-172.

NAKAMURA, T., KONISHI, T. & KAWAGUCHI, H. 1986. Isozymes of chicken coccidia in Japan. *Nihon juigaku zasshi. The japanese journal of veterinary science,* 48**,** 587-590.

NAQID, I. A., OWEN, J. P., MADDISON, B. C., SPILIOTOPOULOS, A., EMES, R. D., WARRY, A., FLYNN, R. J., MARTELLI, F., GOSLING, R. J. & DAVIES, R. H. 2016a. Mapping B-cell responses to Salmonella enterica serovars Typhimurium and Enteritidis in chickens for the discrimination of infected from vaccinated animals. *Scientific reports,* 6, 31186.

NAQID, I. A., OWEN, J. P., MADDISON, B. C., SPILIOTOPOULOS, A., EMES, R. D., WARRY, A., TCHÓRZEWSKA, M. A., MARTELLI, F., GOSLING, R. J. & DAVIES, R. H. 2016b. Mapping polyclonal antibody responses to bacterial infection using next generation phage display. *Scientific reports,* 6, 24232.

NGUBANE, N. A., GRESH, L., IOERGER, T. R., SACCHETTINI, J. C., ZHANG, Y. J., RUBIN, E. J., PYM, A. & KHATI, M. 2013. High-throughput sequencing enhanced phage display identifies peptides that bind mycobacteria. *PLoS One,* 8**,** e77844.

NOGAREDA, C., MORENO, J. A., ANGULO, E., SANDMANN, G., PORTERO, M., CAPELL, T., ZHU, C. & CHRISTOU, P. 2016. Carotenoid-enriched transgenic corn delivers bioavailable carotenoids to poultry and protects them against coccidiosis. *Plant biotechnology journal,* 14**,** 160-168.

NOLAN, M. J., TOMLEY, F. M., KAISER, P. & BLAKE, D. P. 2015. Quantitative real-time PCR (qPCR) for *Eimeria tenella* replication—Implications for experimental refinement and animal welfare. *Parasitology international,* 64**,** 464-470.

NORTON, C. & CHARD, M. 1983. The oocyst sporulation time of *Eimeria* species from the fowl. *Parasitology,* 86**,** 193-198.

NUTTALL, S. D., KRISHNAN, U. V., DOUGHTY, L., NATHANIELSZ, A., ALLY, N., PIKE, R. N., HUDSON, P. J., KORTT, A. A. & IRVING, R. A. 2002. A naturally occurring NAR variable domain binds the Kgp protease from Porphyromonas gingivalis. *FEBS letters,* 516**,** 80-86.

OGEDENGBE, J. D., HUNTER, D. B. & BARTA, J. R. 2011. Molecular identification of *Eimeria* species infecting market-age meat chickens in commercial flocks in Ontario. *Veterinary parasitology,* 178**,** 350-354.

OH, M.-Y., JOO, H.-Y., HUR, B.-U., JEONG, Y.-H. & CHA, S.-H. 2007. Enhancing phage display of antibody fragments using gIII-amber suppression. *Gene,* 386**,** 81-89.

OJIMELUKWE, A. E., AGU, G. O. & ABAH, A. E. 2018. Molecular identification of poultry *Eimeria* species at live bird markets in River State, Nigeria. *IOSR Journal of agricultural and veterinary science,* 11**,** 45-51.

OLA-FADUNSIN, S. D. & ADEMOLA, I. O. 2014. Anticoccidial effects of Morinda lucida acetone extracts on broiler chickens naturally infected with *Eimeria* species. *Pharmaceutical biology,* 52**,** 330-334.

OLANREWAJU, C. A. & AGBOR, R. Y. 2014. Prevalence of coccidiosis among poultry birds slaughtered at gwagwalada main market, Abuja, FCT, Nigeria. *The international journal of engineering and science,* 3**,** 41-45.

OMAR, N., LOH, Q., TYE, G., CHOONG, Y., NOORDIN, R., GLÖKLER, J. & LIM, T. 2014. Development of an antigen-DNAzyme based probe for a direct antibody-antigen assay using the intrinsic DNAzyme activity of a daunomycin aptamer. *Sensors,* 14**,** 346-355.

PANDE, J., SZEWCZYK, M. M. & GROVER, A. K. 2010. Phage display: concept, innovations, applications and future. *Biotechnology advances,* 28**,** 849-858.

PARKER, J., OVIEDO-RONDÓN, E., CLACK, B., CLEMENTE-HERNANDEZ, S., OSBORNE, J., REMUS, J., KETTUNEN, H., MÄKIVUOKKO, H. & PIERSON, E. 2007. Enzymes as feed additive to aid in responses against *Eimeria* species in coccidia-vaccinated broilers fed corn-soybean meal diets with different protein levels. *Poultry science,* 86**,** 643-653.

PAROLA, C., NEUMEIER, D. & REDDY, S. T. 2018. Integrating high-throughput screening and sequencing for monoclonal antibody discovery and engineering. *Immunology,* 153**,** 31-41.
PASCHKE, M. 2006. Phage display systems and their applications. *Applied microbiology and biotechnology,* 70**,** 2-11.
PAVLICKOVA, P., SCHNEIDER, E. M. & HUG, H. 2004. Advances in recombinant antibody microarrays. *Clinica chimica acta,* 343**,** 17-35.
PEEK, H. 2010. Resistance to anticoccidial drugs: alternative strategies to control coccidiosis in broilers. PhD Thesis, Utrecht university.
PEEK, H. & LANDMAN, W. 2011. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. *Veterinary quarterly,* 31**,** 143-161.
PENNAZIO, S. 2006. The origin of phage virology. *Biology Forum/Rivista di Biologia,* 99, 1.
PETRONE, V., CONSTANTINO, C. & PRADAL-ROA, P. 2002. Identification and quantification of granulocytes in caecal mucosa and submucosa of chickens experimentally infected with *Eimeria tenella* and Salmonella enteritidis. *British poultry science,* 43**,** 653-661.
PETTY, N. K., EVANS, T. J., FINERAN, P. C. & SALMOND, G. P. 2007. Biotechnological exploitation of bacteriophage research. *Trends in biotechnology,* 25**,** 7-15.
PITTILO, R. & BALL, S. 1980. The ultrastructural development of the oocyst wall of *Eimeria maxima*. *Parasitology,* 81**,** 115-122.
PLOSS, M. & KUHN, A. 2010. Kinetics of filamentous phage assembly. *Physical biology,* 7**,** 045002.
PONSEL, D., NEUGEBAUER, J., LADETZKI-BAEHS, K. & TISSOT, K. 2011. High Affinity, Developability and Functional Size: The Holy Grail of Combinatorial Antibody Library Generation. *Molecules,* 16**,** 3675.
PORTES, L. D. S., KIOSHIMA, E., DE CAMARGO, Z., BATISTA, W. & XANDER, P. 2017. Subtractive phage display selection for screening and identification of peptide sequences with potential use in serodiagnosis of paracoccidioidomycosis caused by Paracoccidioides brasiliensis. *Letters in applied microbiology,* 65**,** 346-353.
PRAKASHBABU, B. C., THENMOZHI, V., LIMON, G., KUNDU, K., KUMAR, S., GARG, R., CLARK, E. L., RAO, A. S. R. S., RAJ, D. G. & RAMAN, M. 2017. *Eimeria* species occurrence varies between geographic regions and poultry production systems and may influence parasite genetic diversity. *Veterinary parasitology,* 233**,** 62-72.
PRESTON-MAFHAM, R. & SYKES, A. 1967. Changes in permeability of the mucosa during intestinal coccidiosis infections in the fowl. *Experientia,* 23**,** 972-973.
PRICE, K. 2012. Use of live vaccines for coccidiosis control in replacement layer pullets. *The journal of applied poultry research,* 21**,** 679-692.
PROCUNIER, J., FERNANDO, M. & BARTA, J. 1993. Species and strain differentiation of*Eimeria* spp. of the domestic fowl using DNA polymorphisms amplified by arbitrary primers. *Parasitology research,* 79**,** 98-102.
QI, H., LU, H., QIU, H.-J., PETRENKO, V. & LIU, A. 2012. Phagemid vectors for phage display: properties, characteristics and construction. *Journal of molecular biology,* 417**,** 129-143.
QUAIL, M. A., SMITH, M., COUPLAND, P., OTTO, T. D., HARRIS, S. R., CONNOR, T. R., BERTONI, A., SWERDLOW, H. P. & GU, Y. 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC genomics,* 13**,** 341.
QUINTERO-HERNÁNDEZ, V., JUÁREZ-GONZÁLEZ, V. R., ORTÍZ-LEÓN, M., SÁNCHEZ, R., POSSANI, L. D. & BECERRIL, B. 2007. The change of the scFv into the Fab format improves the stability and in vivo toxin neutralization capacity of recombinant antibodies. *Molecular immunology,* 44**,** 1307-1315.

QUIROZ-CASTAÑEDA, R. E. & DANTÁN-GONZÁLEZ, E. 2015. Control of avian coccidiosis: future and present natural alternatives. *BioMed research international,* 67, 121-133.

QURESHI, T. 2016. Studying Transmembrane Helix Interactions in SDS micelles. PhD Thesis, Université d'Ottawa/University of Ottawa.

RAFIQUE, A., SATAKE, K., KISHIMOTO, S., HASAN KHAN, K., KATO, D.-I. & ITO, Y. 2019. Efficient Screening and Design of Variable Domain of Heavy Chain Antibody Ligands Through High Throughput Sequencing for Affinity Chromatography to Purify Fab Fragments. *Monoclonal antibodies in immunodiagnosis and immunotherapy*, 38, 190-200.

RAFTERY, L. J., HOWARD, C. B., GREWAL, Y. S., VAIDYANATHAN, R., JONES, M. L., ANDERSON, W., KORBIE, D., DUARTE, T., CAO, M. D. & NGUYEN, S. H. 2019. Retooling Phage Display with Electrohydrodynamic Nanomixing and Nanopore Sequencing. *Lab on a Chip*, 19, 4083-4092.

RAILLIET, A. & LUCET, A. 1891. Development experimental des coccidie de l'epithelium intestinale du lapin et de la poule. *CR Soc. Biol,* 43**,** 820-822.

RAJ, G. D., AARTHI, S., SELVABHARATHI, R., RAMAN, M., BLAKE, D. & TOMLEY, F. 2013. Real-time PCR-based quantification of *Eimeria* genomes: a method to outweigh underestimation of genome numbers due to PCR inhibition. *Avian pathology,* 42**,** 304-308.

RASHEED, A. & SHAMEER, M. 2016. Assessment of the level of protection against coccidiosis in broiler breeders conferred by a live anticoccidial vaccine, and its influence on early growth and development. Master of science dissertation, University of Arkansas.

RAVINDRAN, V., TANCHAROENRAT, P., ZAEFARIAN, F. & RAVINDRAN, G. 2016. Fats in poultry nutrition: Digestive physiology and factors influencing their utilisation. *Animal feed science and technology,* 213**,** 1-21.

RAVN, U., DIDELOT, G., VENET, S., NG, K.-T., GUENEAU, F., ROUSSEAU, F., CALLOUD, S., KOSCO-VILBOIS, M. & FISCHER, N. 2013. Deep sequencing of phage display libraries to support antibody discovery. *Methods,* 60**,** 99-110.

RAVN, U., GUENEAU, F., BAERLOCHER, L., OSTERAS, M., DESMURS, M., MALINGE, P., MAGISTRELLI, G., FARINELLI, L., KOSCO-VILBOIS, M. & FISCHER, N. 2010b. By-passing in vitro screening—next generation sequencing technologies applied to antibody display and in silico candidate selection. *Nucleic acids research,* 38**,** e193-e193.

REID, W. M., HINES, T. K., JOHNSON, J. & STINO, K. R. 1976. Coccidiosis: effects of high environmental temperatures on anticoccidial protection. *Poultry science,* 55**,** 1436-1441.

REID, W. M. & LONG, P. L. 1979. Diagnostic chart for nine species of fowl coccidia. A chart to aid in identification of species of *Eimeria*. University of Georgia, university of Georgia press, Athens.

RENTERO REBOLLO, I., SABISZ, M., BAERISWYL, V. & HEINIS, C. 2014. Identification of target-binding peptide motifs by high-throughput sequencing of phage-selected peptides. *Nucleic acids research,* 42**,** e169-e169.

RICHARD, G., MEYERS, A. J., MCLEAN, M. D., ARBABI-GHAHROUDI, M., MACKENZIE, R. & HALL, J. C. 2013. In vivo neutralization of α-cobratoxin with high-affinity llama single-domain antibodies (VHHs) and a VHH-Fc antibody. *PLoS One,* 8**,** e69495.

ROSE, J. B., LANDEEN, L. K., RILEY, K. R. & GERBA, C. P. 1989. Evaluation of immunofluorescence techniques for detection of Cryptosporidium oocysts and Giardia cysts from environmental samples. *Appl. Environ. Microbiol.,* 55**,** 3189-3196.

ROSE, M. & LONG, P. 1962. Immunity to four species of *Eimeria* in fowls. *Immunology,* 5**,** 79.

ROSE, M. E. 1974. Protective antibodies in infections with *Eimeria maxima*: the reduction of pathogenic effects in vivo and a comparison between oral and subcutaneous administration of antiserum. *Parasitology,* 68**,** 285-292.
ROSE, M. E. & HESKETH, P. 1979. Immunity to coccidiosis: T-lymphocyte-or B-lymphocyte-deficient animals. *Infection and immunity,* 26**,** 630-637.
ROTHE, C., URLINGER, S., LÖHNING, C., PRASSLER, J., STARK, Y., JÄGER, U., HUBNER, B., BARDROFF, M., PRADEL, I. & BOSS, M. 2008. The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *Journal of molecular biology,* 376**,** 1182-1200.
ROUET, R., JACKSON, K. J., LANGLEY, D. B. & CHRIST, D. 2018. Next-generation sequencing of antibody display repertoires. *Frontiers in immunology,* 9**,** 118.
ROUET, R., LOWE, D., DUDGEON, K., ROOME, B., SCHOFIELD, P., LANGLEY, D., ANDREWS, J., WHITFELD, P., JERMUTUS, L. & CHRIST, D. 2012. Expression of high-affinity human antibody fragments in bacteria. *Nature protocols,* 7**,** 364.
RUFF, M. D. 1999. Important parasites in poultry production systems. *Veterinary parasitology,* 84**,** 337-347.
RYVKIN, A., ASHKENAZY, H., SMELYANSKI, L., KAPLAN, G., PENN, O., WEISS-OTTOLENGHI, Y., PRIVMAN, E., NGAM, P. B., WOODWARD, J. E. & MAY, G. D. 2012. Deep panning: steps towards probing the IgOme. *PloS one,* 7**,** e41469.
RÖTHLISBERGER, D., HONEGGER, A. & PLÜCKTHUN, A. 2005. Domain interactions in the Fab fragment: a comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of different stability. *Journal of molecular biology,* 347**,** 773-789.
SAGGY, I., WINE, Y., SHEFET-CARASSO, L., NAHARY, L., GEORGIOU, G. & BENHAR, I. 2012a. Antibody isolation from immunized animals: comparison of phage display and antibody discovery via V gene repertoire mining. *Protein engineering, design and selection,* 25**,** 539-549.
SAKKAS, P., SMITH, S., HILL, T. & KYRIAZAKIS, I. 2018. A reassessment of the vitamin D requirements of modern broiler genotypes. *Poultry science,* 98**,** 330-340.
SASSO, E., PACIELLO, R., D'AURIA, F., RICCIO, G., FROECHLICH, G., CORTESE, R., NICOSIA, A., DE LORENZO, C. & ZAMBRANO, N. 2015. One-step recovery of scFv clones from high-throughput sequencing-based screening of phage display libraries challenged to cells expressing native claudin-1. *BioMed research international,* https://doi.org/10.1155/2015/703213.
SCHILDT, C. S. 1955. The effect of cecal coccidiosis on the motility of the digestive tract of the domestic fowl. PhD Thesis, university of Wisconsin--Madison.
SCHMITZ, U., VERSMOLD, A., KAUFMANN, P. & FRANK, H.-G. 2000. Phage display: a molecular tool for the generation of antibodies—a review. *Placenta,* 21**,** S106-S112.
SCHNITZLER, B. E., THEBO, P. L., MATTSSON, J. G., TOMLEY, F. M. & SHIRLEY, M. W. 1998. Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic. *Eimeria* species of the chicken. *Avian pathology,* 27**,** 490-497.
SCHOLTYSECK, E., ROMMEL, A. & HELLER, G. 1969. Licht-und elektronenmikroskopische Untersuchungen zur Bildung der Oocystenhülle bei Eimerien (*Eimeria perforans, E. stiedae und E. tenella*). *Zeitschrift für parasitenkunde,* 31**,** 289-298.
SCOTT, J. K. & SMITH, G. P. 1990. Searching for peptide ligands with an epitope library. *Science,* 249**,** 386-390.

SHARMA, V. & FERNANDO, M. 1975. Effect of *Eimeria acervulina* infection on nutrient retention with special reference to fat malabsorption in chickens. *Canadian journal of comparative medicine,* 39**,** 146.

SHARMAN, P. A., SMITH, N. C., WALLACH, M. G. & KATRIB, M. 2010. Chasing the golden egg: vaccination against poultry coccidiosis. *Parasite immunology,* 32**,** 590-598.

SHARMA[1], S., IQBAL, A., AZMI[1], S. & SHAH, H. A. 2009. Study of poultry coccidiosis in organized and backyard farms of Jammu region. *Environment,* 43**,** 1601-1604.

SHEVCHENKO, A., WILM, M., VORM, O. & MANN, M. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical chemistry,* 68**,** 850-858.

SHIN, S. H., HSU, A., CHASTAIN, H. M., CRUZ, L. A., ELDER, E. S., SAPP, S. G., MCAULIFFE, I., ESPINO, A. M. & HANDALI, S. 2016. Development of Two FhSAP2 Recombinant–Based Assays for Immunodiagnosis of Human Chronic Fascioliasis. *The American journal of tropical medicine and hygiene,* 95**,** 852-855.

SHIRLEY, M. 1975. Enzyme variation in *Eimeria* species of the chicken. *Parasitology,* 71**,** 369-376.

SHIRLEY, M. 1994. Coccidial parasites from the chicken: discrimination of different populations of *Eimeria tenella* by DNA hybridisation. *Research in veterinary science,* 57**,** 10-14.

SHIRLEY, M., CHAPMAN, H., KUCERA, J., JEFFERS, T. & BEDRNIK, P. 1989. Enzyme variation and pathogenicity of recent field isolates of *Eimeria tenella*. *Research in veterinary science,* 46**,** 79-83.

SHIRLEY, M. W., SMITH, A. L. & BLAKE, D. P. 2007. Challenges in the successful control of the avian coccidia. *Vaccine,* 25**,** 5540-5547.

SHIRLEY, M. W., SMITH, A. L. & TOMLEY, F. M. 2005. The biology of avian *Eimeria* with an emphasis on their control by vaccination. *Advances in parasitology,* 60**,** 285-330.

SHIRZAD, M. R., SEIFI, S., GHEISARI, H. R., HACHESOO, B. A., HABIBI, H. & BUJMEHRANI, H. 2011. Prevalence and risk factors for subclinical coccidiosis in broiler chicken farms in Mazandaran province, Iran. *Tropical animal health and production,* 43**,** 1601-1604.

SIDHU, S. S. & FELLOUSE, F. A. 2006. Synthetic therapeutic antibodies. *Nature chemical biology,* 2**,** 682.

SIDHU, S. S. & GEYER, C. R. 2015. *Phage display in biotechnology and drug discovery*, CRC Press.

SIMABUCO, F. M., CARROMEU, C., FARINHA-ARCIERI, L. E., TAMURA, R. E. & VENTURA, A. M. 2007. Production of polyclonal antibodies against the human respiratory syncytial virus nucleoprotein and phosphoprotein expressed in *Escherichia coli*. *Protein expression and purification,* 53**,** 209-215.

SINGH, Y. D. & MUKHERJEE, S. 2018. Clinicopathology and diagnosis of Marek's disease and coccidiosis co-infection in Giriraja chicken in Manipur. *Indian journal of veterinary pathology,* 42**,** 77-80.

SINGLA, L. & GUPTA, S. 2012. Advances in diagnosis of coccidiosis in poultry. *Veterinary Diagnostics: Current Trends, Gupta RP, Garg SR, Nehra V and Lather D (Eds), Satish Serial Publishing House, Delhi***,** 615-628.

SKERRA, A. & PLUCKTHUN, A. 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science,* 240**,** 1038.

SMITH, C. K. & LEE, D. E. 1986. Monosaccharide transport by *Eimeria tenella* sporozoites. *The journal of parasitology***,** 163-169.

SMITH, G. P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science,* 228**,** 1315-1317.

SMITH, G. P. 2019. Phage Display: Simple Evolution in a Petri Dish (Nobel Lecture). *Angewandte Chemie International Edition*, 58, 14428-14437.

SONG, X., REN, Z., YAN, R., XU, L. & LI, X. 2015. Induction of protective immunity against *Eimeria tenella*, *Eimeria necatrix*, *Eimeria maxima* and *Eimeria acervulina* infections using multivalent epitope DNA vaccines. *Vaccine,* 33**,** 2764-2770.

SOULSBY, E. J. L. 1968. Helminths, arthropods and protozoa of domesticated animals. *Helminths, arthropods and protozoa of domesticated animals,* 15, 43-55.

SOUTHERN, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of molecular biology,* 98**,** 503-517.

SOUTHERN, L. & BAKER, D. 1983. *Eimeria acervulina* infection and the zinc-copper interrelationship in the chick. *Poultry science,* 62**,** 401-404.

SPILIOTOPOULOS, A., OWEN, J. P., MADDISON, B. C., DREVENY, I., REES, H. C. & GOUGH, K. C. 2015. Sensitive recovery of recombinant antibody clones after their in silico identification within NGS datasets. *Journal of immunological methods,* 420**,** 50-55.

STANFIELD, R. L., DOOLEY, H., VERDINO, P., FLAJNIK, M. F. & WILSON, I. A. 2007. Maturation of shark single-domain (IgNAR) antibodies: evidence for induced-fit binding. *Journal of molecular biology,* 367**,** 358-372.

STEELAND, S., VANDENBROUCKE, R. E. & LIBERT, C. 2016. Nanobodies as therapeutics: big opportunities for small antibodies. *Drug discovery today,* 21**,** 1076-1113.

STEPHENS, J. 1965. Some physiological effects of coccidiosis caused by *Eimeria necatrix* in the chicken. *The journal of parasitology***,** 331-335.

STEWART, C. S., MACKENZIE, C. R. & HALL, J. C. 2007. Isolation, characterization and pentamerization of α-cobrotoxin specific single-domain antibodies from a naïve phage display library: Preliminary findings for antivenom development. *Toxicon,* 49**,** 699-709.

STOTISH, R., WANG, C. & MEYENHOFER, M. 1978. Structure and composition of the oocyst wall of *Eimeria tenella*. *The journal of parasitology***,** 1074-1081.

STRACHAN, G., MCELHINEY, J., DREVER, M. R., MCINTOSH, F., LAWTON, L. & PORTER, A. J. R. 2002. Rapid selection of anti-hapten antibodies isolated from synthetic and semi-synthetic antibody phage display libraries expressed in *Escherichia coli*. *FEMS microbiology letters,* 210**,** 257-261.

SUN, X., PANG, W., JIA, T., YAN, W., HE, G., HAO, L., BENTUÉ, M. & SUO, X. 2009. Prevalence of *Eimeria* species in broilers with subclinical signs from fifty farms. *Avian diseases,* 53**,** 301-305.

SWAYNE, D. E., GLISSON, J. R., MCDOUGALD, L. R., NOLAN, L. K., SUAREZ, D. L. & NAIR, V. L. 2013. *Diseases of poultry*, 288-298.

SWINKELS, W. 2008. Host response to *Eimeria* infections. PhD Thesis, Utrecht University.

TAN, G. H. & TAN, W. S. 2015. Comparison between Phage-ELISA and Phage Dot-Blot Assay Methods for the Detection of Hepatitis B Surface Antigen and its Antibodies in Human Serum. *International journal of biotechnology for wellness industries,* 3**,** 128-135.

TAN, S. 2005. Vinegar fermentation. Master of Science thesis, Louisiana State University, Department of Food Science. Baton Rouge.

TEETER, R. G., BEKER, A., BROWN, C., BROUSSARD, C., FITZ-COY, S., RADU, J. & NEWMAN, L. 2008. Transforming coccidiosis-mediated lesion scores into production and calorific cost. *World's poultry congress*,18, 33.

TEWARI, A. & MAHARANA, B. 2011. Control of poultry coccidiosis: changing trends. *Journal of parasitic diseases,* 35**,** 10-17.

THEBO, P., LUNDEN, A., UGGLA, A. & HOOSHMAND-RAD, P. 1998. Identification of seven *Eimeria* species in Swedish domestic fowl. *Avian pathology,* 27**,** 613-617.

TIAN, G., TANG, F., YANG, C., ZHANG, W., BERGQUIST, J., WANG, B., MI, J. & ZHANG, J. 2017. Quantitative dot blot analysis (QDB), a versatile high throughput immunoblot method. *Oncotarget,* 8**,** 58553.

TIAN, Y., RAO, H., TAO, S. & XUE, W.-T. 2018. Effect of boiling on the structure and immunoreactivity of recombinant peanut protein Ara h 1. *Food and agricultural immunology,* 29**,** 845-858.

TOGTEMA, M., HUSSACK, G., DAYER, G., TEGHTMEYER, M. R., RAPHAEL, S., TANHA, J. & ZEHBE, I. 2019. Single-domain antibodies represent novel alternatives to monoclonal antibodies as targeting agents against the human papillomavirus 16 E6 protein. *International journal of molecular sciences,* 20**,** 2088.

TOLOUDI, M., PAPADIMITRIOU, M., PANTOPIKOU, K. & PAPASOTIRIOU, I. 2016. Generation of Human Antibodies Against a Specific Peptide: a Novel Strategy Based on Human Cells. *Anticancer research,* 36**,** 4613-4621.

TONG, C., XIAO, B., CHENG, S., LI, W., LIAO, X., LUO, S. & HAO, W. 2015. Generation and Identification of Peptide-Based Monoclonal Antibodies Against Vacuolar Proton Pyrophosphatase of Toxoplasma gondii. *Monoclonal antibodies in immunodiagnosis and immunotherapy,* 34**,** 191-200.

TROTT, M., WEIß, S., ANTONI, S., KOCH, J., VON BRIESEN, H., HUST, M. & DIETRICH, U. 2014. Functional characterization of two scFv-Fc antibodies from an HIV controller selected on soluble HIV-1 Env complexes: a neutralizing V3-and a trimer-specific gp41 antibody. *PLoS One,* 9**,** e97478.

TU, Z., XU, Y., HE, Q., FU, J., LIU, X. & TAO, Y. 2012. Isolation and characterisation of deoxynivalenol affinity binders from a phage display library based on single-domain camelid heavy chain antibodies (VHHs). *Food and agricultural immunology,* 23**,** 123-131.

TURK, D. 1973. Calcium absorption during coccidial infections in chicks. *Poultry science,* 52**,** 854-857.

TURNER, K. B., NACIRI, J., LIU, J. L., ANDERSON, G. P., GOLDMAN, E. R. & ZABETAKIS, D. 2016. Next-generation sequencing of a single domain antibody repertoire reveals quality of phage display selected candidates. *PLoS One,* 11**,** e0149393.

TYZZER, E. E. 1929. Coccidiosis in gallinaceous birds. *American journal of epidemiology,* 10**,** 269-383.

TYZZER, E. E., THEILER, H. & JONES, E. E. 1932. Coccidiosis in gallinaceous birds: II. A comparative study of species of *Eimeria* of the chicken. *American journal of epidemiology,* 15**,** 319-393.

VANLANDSCHOOT, P., STORTELERS, C., BEIRNAERT, E., IBAÑEZ, L. I., SCHEPENS, B., DEPLA, E. & SAELENS, X. 2011. Nanobodies®: new ammunition to battle viruses. *Antiviral research,* 92**,** 389-407.

VAUGHAN, T. J., WILLIAMS, A. J., PRITCHARD, K., OSBOURN, J. K., POPE, A. R., EARNSHAW, J. C., MCCAFFERTY, J., HODITS, R. A., WILTON, J. & JOHNSON, K. S. 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature biotechnology,* 14**,** 309.

VENET, S., RAVN, U., BUATOIS, V., GUENEAU, F., CALLOUD, S., KOSCO-VILBOIS, M. & FISCHER, N. 2012. Transferring the characteristics of naturally occurring and biased antibody repertoires to human antibody libraries by trapping CDRH3 sequences. *PLoS One,* 7**,** e43471.

VERMEULEN, A., SCHAAP, D. & SCHETTERS, T. P. 2001. Control of coccidiosis in chickens by vaccination. *Veterinary parasitology,* 100**,** 13-20.

VERVELDE, L., VERMEULEN, A. N. & JEURISSEN, S. H. 1996. In situ characterization of leucocyte subpopulations after infection with *Eimeria tenella* in chickens. *Parasite immunology,* 18**,** 247-256.
VRBA, V., BLAKE, D. P. & POPLSTEIN, M. 2010. Quantitative real-time PCR assays for detection and quantification of all seven *Eimeria* species that infect the chicken. *Veterinary parasitology,* 174**,** 183-190.
WAKELIN, D., ROSE, M. & LONG, P. 1990. Immunity to coccidiosis. *Coccidiosis of man and domestic animals.***,** 281-306.
WALDENSTEDT, L., ELWINGER, K., LUNDÉN, A., THEBO, P. & UGGLA, A. 2001. Sporulation of *Eimeria maxima* oocysts in litter with different moisture contents. *Poultry science,* 80**,** 1412-1415.
WALLACH, M. 2010. Role of antibody in immunity and control of chicken coccidiosis. *Trends in parasitology,* 26**,** 382-387.
WALLACH, M., PILLEMER, G., YARUS, S., HALABI, A., PUGATSCH, T. & MENCHER, D. 1990. Passive immunization of chickens against *Eimeria maxima* infection with a monoclonal antibody developed against a gametocyte antigen. *Infection and immunity,* 58**,** 557-562.
WALLACH, M. G., ASHASH, U., MICHAEL, A. & SMITH, N. C. 2008. Field application of a subunit vaccine against an enteric protozoan disease. *PLoS One,* 3**,** e3948.
WAN, N., NUR, S., NIK, K., NORLIDA, O. & SAIPUL, B. 2017. Coccidiosis in village chicken: a preliminary survey in Pasir Putih District, Kelantan, West Malaysia. *Malaysian journal of veterinary research,* 8**,** 23-28.
WANG, C. & STOTISH, R. 1975. Changes of nucleic acids and proteins in the oocysts of *Eimeria tenella* during sporulation. *The journal of protozoology,* 22**,** 438-443.
WANG, Y., ZHANG, X., ZHANG, C., LIU, Y. & LIU, X. 2012. Isolation of single chain variable fragment (scFv) specific for Cry1C toxin from human single fold scFv libraries. *Toxicon,* 60**,** 1290-1297.
WHITE, B., HUH, I. & BROOKS, C. L. 2019. Structure of a VHH isolated from a naïve phage display library. *BMC research notes,* 12**,** 154.
WILLATS, W. G. 2002. Phage display: practicalities and prospects. *Plant molecular biology,* 50**,** 837-854.
WILLIAMS, R. 1995. Epidemiological studies of coccidiosis in the domesticated fowl (Gallus gallus): II. Physical condition and survival of *Eimeria acervulina* oocysts in poultry-house litter. *Applied parasitology,* 36**,** 90-96.
WILLIAMS, R. 1998. Epidemiological aspects of the use of live anticoccidial vaccines for chickens. *International journal for parasitology,* 28**,** 1089-1098.
WILLIAMS, R. 1999. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. *International journal for parasitology,* 29**,** 1209-1229.
WILLIAMS, R. 2001. Quantification of the crowding effect during infections with the seven *Eimeria* species of the domesticated fowl: its importance for experimental designs and the production of oocyst stocks. *International journal for parasitology,* 31**,** 1056-1069.
WILLIAMS, R. 2002. Anticoccidial vaccines for broiler chickens: pathways to success. *Avian pathology,* 31**,** 317-353.
WILLIAMS, R., BUSHELL, A., REPERANT, J., DOY, T., MORGAN, J., SHIRLEY, M., YVORE, P., CARR, M. M. & FREMONT, Y. 1996. A survey of *Eimeria* species in commercially-reared chickens in France during 1994. *Avian pathology,* 25**,** 113-130.
WILLIAMS, R., MARSHALL, R., PAGES, M., DARDI, M. & DEL CACHO, E. 2009. Pathogenesis of *Eimeria praecox* in chickens: virulence of field strains compared with laboratory strains of *E*. *praecox* and *E. acervulina*. *Avian pathology,* 38**,** 359-366.

WILSON, R. 2008. The use of gold nanoparticles in diagnostics and detection. *Chemical society reviews,* 37**,** 2028-2045.
WINKLER, K., KRAMER, A., KÜTTNER, G., SEIFERT, M., SCHOLZ, C., WESSNER, H., SCHNEIDER-MERGENER, J. & HÖHNE, W. 2000. Changing the antigen binding specificity by single point mutations of an anti-p24 (HIV-1) antibody. *The journal of immunology,* 165**,** 4505-4514.
WOODS, J. 2019. Selection of Functional Intracellular Nanobodies. *SLAS DISCOVERY: Advancing life sciences R&D***,** 24, 703-713.
WOODS, W., WHITHEAR, K., RICHARDS, D., ANDERSON, G., JORGENSEN, W. & GASSER, R. 2000. Single-strand restriction fragment length polymorphism analysis of the second internal transcribed spacer (ribosomal DNA) for six species of *Eimeria* from chickens in Australia. *International journal for parasitology,* 30**,** 1019-1023.
XU, J., JIANG, H., TAO, J., PENG, H., ZHANG, Y. & SUN, W. 2009. Protective efficacy of recombinant Gam56 gene expressed in *E. coli*. *Chin J Prev Vet Med,* 31**,** 892-897.
XU, J., ZHANG, Y. & TAO, J. 2013. Efficacy of a DNA vaccine carrying *Eimeria maxima* Gam56 antigen gene against coccidiosis in chickens. *The Korean journal of parasitology,* 51**,** 147.
YADAV, A. & GUPTA, S. 2001. Study of resistance against some ionophores in *Eimeria tenella* field isolates. *Veterinary parasitology,* 102**,** 69-75.
YANG, W., YOON, A., LEE, S., KIM, S., HAN, J. & CHUNG, J. 2017. Next-generation sequencing enables the discovery of more diverse positive clones from a phage-displayed antibody library. *Experimental & molecular medicine,* 49**,** e308.
YOHE, S. & THYAGARAJAN, B. 2017. Review of clinical next-generation sequencing. *Archives of pathology & laboratory medicine,* 141**,** 1544-1557.
YUN, C., LILLEHOJ, H. & LILLEHOJ, E. 2000. Intestinal immune responses to coccidiosis. *Developmental & comparative immunology,* 24**,** 303-324.
ZHANG, H., TORKAMANI, A., JONES, T. M., RUIZ, D. I., PONS, J. & LERNER, R. A. 2011. Phenotype-information-phenotype cycle for deconvolution of combinatorial antibody libraries selected against complex systems. *Proceedings of the national academy of sciences,* 108**,** 13456-13461.
ZHANG, J. J., WANG, L. X., RUAN, W. K. & AN, J. 2013. Investigation into the prevalence of coccidiosis and maduramycin drug resistance in chickens in China. *Veterinary parasitology,* 191**,** 29-34.
ZHANG, Z.-F., SHAN, X., WANG, Y.-X., WANG, W., FENG, S.-Y. & CUI, Y.-B. 2014. Screening and selection of peptides specific for esophageal cancer cells from a phage display peptide library. *Journal of cardiothoracic surgery,* 9**,** 76.
ZHU, Z. & DIMITROV, D. S. 2009. Construction of a large naive human phage-displayed Fab library through one-step cloning. *Therapeutic antibodies.* 129-142.
ZIELONKA, S., EMPTING, M., GRZESCHIK, J., KÖNNING, D., BARELLE, C. J. & KOLMAR, H. Structural insights and biomedical potential of IgNAR scaffolds from sharks. MAbs, 2015. *Taylor & Francis,* 7, 15-25.