Development of a DNA-Based Microarray for the Detection of Zoonotic Pathogens in Rodent Species

Timothy Andrew Giles, BSc MSc.

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ABSTRACT

Emerging infectious diseases are, and have always been, an important threat to human health, animal health and the global economy. Emerging and re-emerging pathogens are opportunists, responding to changes in the host or environment. Therefore, diagnostic tests used for the detection of pathogens either of animal or human origin, must be just as adaptable in utilising new technology. In complex biological situations where multiple pathogens may be present or when using wildlife samples that may be rare or precious, DNA microarray technology is particularly valuable as hundreds or thousands of targets can be screened simultaneously in a single sample.

This thesis investigated the potential of a DNA microarray as a screening tool for over 20 pathogens as part of the Novel Technologies for Surveillance of Emerging and Re-emerging Infections of Wildlife project (WildTech), an FP7 EU funded project (www.wildtechproject.com). The pathogens included zoonotic viruses, bacteria and parasites. Publicly available software was used for design of oligonucleotides for the array. Evaluation of the oligonucleotides was carried out using reference samples from a variety of sources.

Different rodent species were screened including the principal commensal species *Rattus rattus*, *Rattus norvegicus*, and *Mus musculus*. Nucleic acid extracted from samples of liver, kidney and lung was screened for the presence of pathogens. The array successfully identified *Leptospira* and Seoul hantavirus positive samples from animals trapped in the United Kingdom, France and Canada. These results were confirmed using previously established methods. It is likely that, over the next few years, diagnostic microarrays will become relatively inexpensive research tools. Molecular testing for emerging pathogens is increasingly being utilised and use of this technology will result in timely, accurate and inexpensive diagnosis to enable effective control of these infectious diseases with important implications for human health. The data contributed here can aid in the steps required to design microarrays for screening purposes.

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DECLARATION

Unless otherwise acknowledged, the work presented in this thesis is original. No part has been submitted for another degree at the University of Nottingham or elsewhere. Any views expressed in the dissertation are those of the author.

Signed…….

Date……..

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List of Abbreviations

AHVLA	Animal Health Veterinary Laboratories Agency
AS	ArrayStrip
AT	ArrayTube
ATR	ArrayTube Reader
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
CDC	Centres for Disease Control
cDNA	Complimentary DNA
CPXV	Cowpox Virus
C_T	Threshold Cycle
DNA	Deoxyribose nucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleotide
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EU	European Union
HEV	Hepatitis E Virus
HFRS	Haemorrhagic fever with renal syndrome
HPLC	High-performance liquid chromatography
HPS	Hantavirus Pulmonary Syndrome
HRP	Horse-radish peroxidase
H_2O_2	Hydrogen peroxide

IBV	Infectious Bronchitis Virus
kbp	Kilo-base pair
LAMP	Loop-mediated isothermal amplification
LCMV	Lymphocytic choriomeningitis Virus
ml	Millilitre
mM	Millimolar
M	Molar
MRSA	Methicillin-resistant *Staphylococcus aureus*
µg	Microgram
µl	Microlitre
µM	Micromolar
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NGS	Next-generation sequencing
OIE	World Organisation for Animal Health
OPV	Orthopoxvirus
OR	Odds Ratio
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PUUV	Puumala Hantavirus
qPCR	Quantitative (Real-time) PCR
RBF	Rat Bite Fever
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SEOV	Seoul Hantavirus
SOP	Standard Operating Procedue
SSC	Sodium chloride-Sodium citrate
STEC	Shiga toxin-producing E. coli
SVMS	School of Veterinary Medicine and Science
TMB	Tetramethylbenzidine
UPS	Unique-probe selector
WHO	World Health Organisation
WT	WildTech

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Wildlife populations have been known to have had a role in zoonotic pathogen emergence for many years (Daszak et al., 2001). Entire regional ecosystems have been transformed because of urbanisation during the past 50 years. Global urbanisation has the potential to concentrate human, animal and vector populations, which can contribute to an increase in pathogen transmission between these populations (Wilcox and Gubler, 2005). In addition, it has been predicted that because of climate change the distribution and prevalence of infectious diseases will change. It has been suggested that more emphasis is placed on developing effective surveillance systems in order detect new areas in which these pathogens may have spread (Lafferty, 2009).

One priority for managing emerging and re-emerging infectious diseases is to further develop and maintain the capacity for effective monitoring of infection and disease prevalence in wildlife (Polley, 2005). Despite a recognition of the requirements needed for effective monitoring, it is predicted that attempts to address this will be challenging (Halliday et al., 2012). Improving future surveillance efforts could focus on new technologies that could be utilised in disease discovery and screening for existing pathogens.

There has been an increase in the number of human diseases that have been linked to rodent reservoirs, and this has renewed interest in reservoir ecology research (Mills and Childs, 1998). A clearer understanding of the pathogens that urban rodents can carry would be beneficial, as knowledge of the risk posed to public health may be improved. A number of rodent-borne zoonotic pathogens were selected for inclusion on the array, and a short review of each of these can be found in this chapter.

The limitations of PCR technology are causing researchers to consider alternative methods as demands on quantity and rapidity of testing methods are increasing (Sachse et al., 2005). Likewise, other diagnostic tests are in need of improvement and advances in technology should be utilised fully, to improve international disease surveillance. Serological methods provide an indication of exposure to a pathogen and are best used for screening populations, although they often have a lower sensitivity than PCR. They also require an adequate time post-infection/exposure for antibodies to develop and may be unable to distinguish between different strains or antigenic types of pathogen. Microarray technology offers a tool which has the advantage of screening for a large number of pathogens directly. Nucleic acid microarrays detect the nucleic acid of pathogens that may be co-infecting a single host (Cannon et al., 2010). Early detection of pathogens in wild animals would be useful in identifying risk factors associated with disease transmission to humans or domestic animals, and this could help to prevent a possible outbreak. It has also been suggested that prevention of disease, which could be aided by an effective surveillance system, is better than a cure or reacting to an outbreak (Russell, 1986).

1.2 EMERGING DISEASES

Emerging and re-emerging diseases can have a significant effect not only on public health, but also on animal health and the global economy. The Director General of the World Organisation for Animal Health (OIE), Bernard Vallat, has been quoted as saying "As a result of globalisation and climate change we are currently facing an unprecedented worldwide impact of emerging and re-emerging animal diseases and zoonoses (an infectious disease transmitted from animals to humans or from humans to animals)" (Vallat, 2007). Emerging infectious diseases (EIDs) have been defined as either: "Those diseases which have appeared in a population for the first time, or those

diseases which have existed but are rapidly increasing in prevalence or geographic range" (Morse, 1995).

However, the principle of EIDs is nothing new to human history. Several examples include the Black Death sweeping across Europe, Spanish Conquistadors bringing measles and smallpox to indigenous populations in the Americas and rinderpest to Africa being introduced by numerous European armies (Gummow, 2010). Translocation is defined as the movement of infected people or animals into new geographic areas and is considered to be an important factor in the emergence of disease (Daszak et al., 2000). However there are numerous other factors involved in the complex issue of disease emergence, and these will be discussed in this review.

There are over 1400 known microbial diseases affecting humans, recognised by the National Academy of Sciences, Institute of Medicine, and 60% are of zoonotic origin. Zoonotic pathogens make up the majority of EID's (75%), and wildlife are one of the main sources of infection (Bidaisee and Macpherson, 2014). The emergence of zoonotic pathogens has been ordered into a set of three tiers: Tier 1 pathogens are only transmitted from animal to animal, Tier 2 pathogens are able to opportunistically infect humans, while Tier 3 pathogens are zoonotic pathogens that have acquired the ability to be transmitted from human to human without the involvement of an animal host (Rosenberg, 2014). It is at this point that the pathogen becomes a true human pathogen, some of the most widespread pandemics have resulted from a Tier 3 pathogen. The 1918 Influenza Epidemic is the worst influenza pandemic known to have occurred, with an estimated 50 million people killed (Johnson and Mueller, 2002). Another zoonotic pandemic of worldwide importance is HIV/AIDS, which is thought to have originated in chimpanzees. In 2012 it was estimated that around 35.3 million people were living with HIV (WHO, 2014). This disease has also been implicated in the

re-emergence of several infectious diseases, as secondary infections, due to the immuno-compromised status of the people infected.

The number of annual deaths attributed to infectious diseases globally is around 12 million (23%) (WHO, 2014b). With the increasing use of antimicrobials and the consequent development of increasingly resistant pathogens in some situations, such as multiple drug resistant *Mycobacterium tuberculosis*, infectious diseases are still a very real and present danger to human and animal health across the world.

Emerging and re-emerging pathogens are opportunists, and respond to changes in both the host and the environment (Woolhouse, 2002). Anthropogenic activities are the major drivers that can affect the emergence of zoonotic infectious diseases from reservoir host populations (Murphy, 2008). For example, the re-emergence of vampire bat rabies in the Amazon basin, is thought to be a result of deforestation and the development of human habitats which bring humans into closer contact with bats that carry the pathogen (Chomel et al., 2007). Bats may also have had a role in the spread of the recent Ebola outbreak from Central Africa to West Africa, which has maintained a steady human-human cycle (Vogel, 2014).

Increasing global urbanisation can lead to an increased threat to human health from emerging and re-emerging infectious diseases, particularly in cities of the developing world but also occurring more commonly in developed ones (Gratz, 1999). By 2025 it has been estimated that over 65% of the human population will reside in a city, and land coverage will double over the same time frame (Bradley and Altizer, 2006). The human population by 2025 is estimated to be around 8 billion (DeLong et al., 2010). An increasing number of people living in close proximity will aid in the transmission of density dependent pathogens. As one author puts it “cities are a perfect distribution centre for pathogens” (Morse, 1995). It has been suggested that increased antimicrobial use, which ultimately leads to resistance of pathogens to the

antimicrobials, is likely to be more common in cities due to the larger numbers of people and the greater access to hospitals; this could constitute a further risk to human health from infectious agents (Jones et al., 2008). A rise in global urbanisation will require more land use for human habitation, which in many cases will mean encroachment into the natural environment, for example with Nipah and Hendra viruses or the Ebola virus. This is likely to lead to humans coming into increased contact with wildlife and a greater risk of zoonotic pathogen transmission. It is often overlooked, but there is the possibility of humans acting as a source of new infections in wildlife (Thompson et al., 2009).

Changes in agricultural methods are also likely to represent a risk to human health. The practice of using farm land for multiple species, and areas in which domestic animals graze in the same location as wildlife, also aids pathogen transmission between the two groups. Furthermore, if the agent is zoonotic, there is a risk of transmission to humans (Woodford, 2009). The emergence of human Nipah virus, in Malysia in 1999, is a good example of this and has been attributed to an initial transmission from bats to pigs. This is thought to have occured through the pigs' consumption of contaminated food dropped by infected bats in trees bordering pig farms. The saliva present on the food dropped by the bats contained the virus which was the route of transmission to the pigs. Humans who were exposed to these infected pigs were also affected and there were 105 fatalities from a total of 265 cases (Chua et al., 2002). Bats are thought to be the primary reservoir for Nipah virus in other countries such as Bangladesh. One study noted that bats preferred to roost in areas closer to human habitation because there were a variety of fruit trees nearby from which the bats could feed, this may increase the risk of further outbreaks (Hahn et al., 2014). Human expansion into the bat's natural territory has likely led to the emergence of this pathogen.

The speed at which humans can now travel to different parts of the world has created opportunities for pathogens to rapidly expand their geographic host range, not to mention the potential for a pandemic to occur, as occurred during a recent swine flu pandemic. Four weeks after initial case reports of flu in the US, 41 countries reported swine flu infections (Wang and Palese, 2009). The global spread was possibly due to the fact that it is possible to travel half way around the world before developing symptoms, giving the pathogen time to be transmitted to others. Another example of a pathogen successfully expanding its geographic distribution is West Nile virus. The virus appeared in New York in 1999 and subsequently spread across most of the United States in the space of a few years. It is thought to have originated however, in the Middle East (Williams et al., 2002). There are many countries in which an outbreak might occur, but there may not be any further spread, if a suitable reservoir species is not present. However, with West Nile Virus, there was a suitable mosquito vector species already present in the United State, and this allowed further spread until the virus became endemic.

Susceptible wildlife populations also present a risk to pathogen emergence and spread and the introduction of African swine fever to Europe is one such example. The pathogen was introduced into continental Europe after an outbreak in Georgia in 2007 (Sánchez-Vizcaíno et al., 2013). The pathogen quickly spread among domestic pigs as well as wild boar within the country. It then spread into Armenia, Russia, Ukraine and more recently into the European Union in 2014, with the first cases reported in Lithuania, Poland, Latvia and Estonia (Gavier-Widén et al., 2015).

Another complication that can affect infectious disease is climate change. The effect that climate change may have on pathogens is difficult to estimate with the current data. It is highly likely that vector-borne diseases will be affected by a change in the environment, as these poikilothermic vectors are more sensitive to environmental

change (Lafferty, 2009). Other major disease reservoir populations such as rodents will be indirectly affected by climate change, as this would have an impact on food availability for such species (Gubler 2001). There are several changes that may be associated with climate change amongst wildlife species and on the pathogens that they may be carrying. The host or vector range could change, as could the population density. The prevalence of a certain pathogen could be affected in either the host or vector population. Finally, the pathogen rate or reproduction and development in the host could also be affected (Mills, 2010).

It is well known that certain climatic conditions can influence the prevalence of pathogens. The El Niño/Southern Oscillation (ENSO) event causes warmer environmental conditions every few years around the eastern Pacific. Associated increases in human cases of hantaviruses, for example, have since been reported, and this was thought to be due to milder winters and greater contact between humans and rodents (Zell et al., 2008). On the other hand, periods of drought can reduce food levels and force rodents into human housing areas, resulting in increased human contact (Gubler et al., 2001). If, as a result of climate change, extictions occurred in specialist rodent species, more opportunistic species such as rats would not only survive but would thrive in the absence of competition from other species. As they expanded their range they would thus potentially expand the distribution of any pathogens that they were carrying (Mills, 2010).

The trade in animals and animal related products may also have a role in pathogen emergence, though the importance of this as a factor in pathogen emergence is unknown. Through a worldwide trade in animal products, unprecedented volumes are distributed to large numbers of markets. Between 2000 and 2004 a total of 37,858,179 animals were legally imported into the United States (and it is likely that animals are also transported illegally) (Marano et al., 2007). The growing global market has also

seen the emergence and spread of new infections in human populations, such as variant Cruetzfeldt-Jacob disease (vCJD) (Kimball et al., 2005). This disease was first reported in the UK in 1996; since then a total of 229 patients with the invariably fatal disease have been reported from 12 countries (CDC, 2014). As a result of the outbreak, changes were made in livestock feeding practices, including a ban on feeding ruminants with ruminant products.

Other, deliberate, human activities can serve to intentionally cause disease outbreaks. The rise of the threat of bioterrorism, where a pathogen is deliberately released into an environment, is another possible source for emerging or re-emerging infectious diseases (Murphy, 2008).

1.3 IMPORTANCE OF SURVEILLANCE

Predicting exactly when and where the next important zoonotic pathogen will emerge is highly unlikely (Murphy, 2008). It is therefore essential that we recognise the many benefits offered by an effective surveillance system. The complexities of the relationships between pathogens, their hosts and the environment have led to an all-encompassing approach which is termed disease ecology. Disease ecology examines all aspects of any potential contributors to disease behaviour within an ecosystem (Polley, 2005). A multidisciplinary team is required for an effective surveillance system which can include: ecologists, mammalogists, ornithologists, entomologists, doctors, epidemiologists, biologists, microbiologists, geneticists, geographers and mathematicians (Childs et al., 1998). An understanding of the pathogen may be developed from cooperation between these different disciplines, leading to increased knowledge of its molecular biology and its interaction with any host species. This can then lead to effective control strategies which may act to break the transmission cycle.

One option which has been suggested for use in a surveillance system, is the study of reservoir hosts, which is conducted on a worldwide scale (Mills and Childs, 1998). This assumes that the primary reservoir host has already been identified and can enable an understanding of the reservoir ecology as it relates to human disease. This information could include the geographic distribution of the host and pathogen, the prevalence among the host population and at a more advanced level, an integrative time and place-specific model.

1.4 RODENT ECOLOGY AND THE RELATIONSHIP TO ZOONOTIC DISEASE

Among mammals, the order Rodentia has the largest number of members, with more than 2000 species which comprises 44% of all mammals (Wolff and Sherman, 2007). Having colonised every continent apart from Antarctica, rodents have had a long and often troubled association with humans. Many peoples' natural dislike of rodents is often attributed to rodents' ability to act as carriers of harmful pathogens (Webster and Macdonald, 1995).

Rodents can facilitate the infection of humans (and their domestic animals) with viruses, bacteria and parasites by acting as liaison and maintenance hosts. Liasion hosts can be defined as incidental hosts that transmit pathogens from a reservoir species to another incidental host (Garnham, 1971). A maintenance host can be defined as a host species that a pathogen depends upon for its survival (Ashford, 2003). The vast majority of zoonotic reservoir species are mammals, of which the majority are rodents (Ostfeld and Holt, 2004). In many places, rodents live in close contact with humans, their livestock, or their pets. In other places, rodents can act as a link between wildlife communities and humans, potentially exposing humans to zoonoses (Meerburg et al., 2009). It has been reported that the prevalence of zoonoses in rodents in urban areas is lower than in rural areas. However, because of the huge population densities of

rodents in urban locations, the risk that urban rodents pose to human health should not be discounted (Traweger and Slotta-Bachmayr, 2005).

The success of commensal rodents has been attributed to their ability to adapt to a wide variety of habitats (Pocock et al., 2004). Where conditions are favourable, a commensal rodent population will grow until it eventually reaches the carrying capacity (the maximum number of rodents the habitat can support). The carrying capacity is dependent on several limiting factors, primarily water, food, and the availability of shelter (Channon et al., 2006). In environments where food is abundant, commensal rodent populations are generally characterised by high densities, small home ranges, and rapid sexual maturity (Wolff and Sherman, 2007). Reliable estimates of rodent abundance in urban habitats in Europe are not readily available; estimates can range from 10 rats per person to one rat per 100 people, although these figures are not based on scientific studies (Traweger et al., 2006).

The three most common and generally important commensal rodents to European public health are the Norway rat (*Rattus norvegicus*), the black rat (*Rattus rattus*) and the house mouse (*Mus musculus domesticus*) (Masi et al., 2010). These three species are found in both urban and rural environments. As these species are globally distributed it has been suggested that some of the pathogens that they carry may also have a global distribution (Blasdell et al., 2008a). Due to the widespread distribution of these species and their capacity to harbour pathogens of zoonotic importance, these species were the main focus of this PhD project.

In urban areas these species are typically found in less affluent inner-city neighbourhoods where the majority of human and rodent interaction occurs (Childs et al., 1998b). In favourable conditions, large populations can become established and need to be managed to protect public health (Lambert et al., 2008). These three species infest human dwellings, food stores, waste areas and other locations where people live.

Infestations in both rural and urban areas represent a risk to public and animal health, and can cause significant economic losses through contamination of food (Gomez et al., 2008b). Populations may reach very high densities, particularly in urban environments (up to $7m^{-2}$), where poor hygiene or other anthropogenic factors may provide the necessary resources to thrive (Gomez et al., 2008a).

Pathogens may be transmitted from rodents to humans via a variety of routes, both direct and indirect. The risk of any type of infection following a rat bite can be as high as 10% (Elliott, 2007). It seems logical to assume that the greater the abundance of rodent reservoirs, the higher the potential delivery rates of pathogens to humans and other animals (Ostfeld and Holt, 2004).

Understanding the host population structure can be of use for both pest control efforts, and in helping to characterize and control the spread of pathogens by commensal rodents (Gardner-Santana et al., 2009). Furthemore, it has been suggested that rodent species could be used as sentinels for zoonotic disease. Animal sentinels can be used to detect pathogen emergence in new geographic areas. They may also be of use in measuring the prevalence or incidence of a pathogen and can aid in calculating the rate and direction of pathogen spread (McCluskey, 2003). Rodents are particularly useful as disease sentinels as tissue samples and post mortem material is relatively easy to obtain (Psaroulaki et al., 2010). Urban settings represent an increasing portion of the ecosystem experienced by human populations; however, the interactions of vertebrate populations in urban areas, including that of rodents and their natural mammalian predators (including domestic cats and foxes) remains strikingly under-studied (Glass et al., 2009). It has been suggested that a better surveillance of rodent populations is important in order to predict future zoonotic disease emergence (Meerburg et al., 2009).

1.5 PATHOGEN REVIEW

A number of pathogens were identified that can be regarded as emerging or re-emerging zoonotic pathogens that have been identified in rodent species. This section will provide a brief review of each pathogen and the role rodents can have in the transmission to humans.

1.5.1 *Bartonella*

There are over 30 species and subspecies of *Bartonella* which are Gram-negative bacilli or coccobacilli (Kosoy et al., 2010). Seven of these are pathogenic for humans (Just et al., 2008), and whilst many species have already been characterised, more are being discovered each year (Mogollon-Pasapera et al., 2009). *Bartonella* organisms are highly adapted to long-term survival within red blood cells and endothelial cells in a wide variety of animals, including human beings (Breitschwerdt and Kordick, 2000).

Fleas, lice, mites and ticks can act as vectors for the pathogen (Breitschwerdt and Kordick, 2000). Ectoparasite vectors are considered essential for transmission; when uninfected cats were kept with *B. henselae*-infected bacteremic cats in an ectoparasite-free environment, no *Bartonella* transmission was observed (Chomel et al., 1996). The same observation has been made in rodents for the *Bartonella* species *B. clarridgeiae* (Kordick et al., 1997).

There have been *Bartonella* species in rodents which were indistinguishable from those infecting human; this suggests that transmission may occur between the two species (Ellis et al., 1999). A study carried out in two urban locations in the U.S.A. (Los Angeles and Baltimore) detected an overall prevalence of *Bartonella* bacteraemia in *R. rattus* and *R. norvegicus* of 18% (Ellis et al., 1999). A higher prevalence (25%) has been recorded in *R. rattus* and *R. norvegicus* from an inner-city location in Vancouver, Canada from a study of over 400 rats (Himsworth et al., 2015). The results indicated that

geographical location was an important factor in pathogen presence. Multiple *Bartonella* types, including *B. tribocorum*, *B. rochalimae* and *B elizabethae* have also been identified in fleas carried by rodents in New York (Frye et al., 2015). Reported seroprevalence can reach high levels in other animal species, and more than 80% of cats can have antibodies to *Bartonella* (Chomel et al., 1995).

Human exposure to rodent-borne *Bartonella* spp. has been reported in urban areas, particularly amongst homeless people and drug users (Comer et al., 1996) and people involved in activities in more rural locations (such as orienteering), where the vector is likely to be more important (McGill et al., 2001). Of all the *Bartonella* species that can cause infection in humans, only a limited number have been caused by rodent-borne *Bartonella* species or subspecies (Boulouis et al., 2005).

Seroprevalence to *Bartonella* spp. has been reported to be 4% in healthy human blood in Sweden (Holmberg et al., 1999), and up to 30% in homeless patients in Marseilles, France (Brouqui et al., 1999). Furthermore in peridomestic and periagricultural environments in many areas of the world, human contact with rodents can pose a heightened risk from acquiring zoonotic *Bartonella* infections (Colton et al., 2012), indicated by levels of infection of up to 48% in several commensal rodent species worldwide (Kato et al., 2013, Ashford, 2003, McCluskey, 2003).

Human infection can lead to a wide range of illnesses, including cat scratch disease in immuno-competent patients and endocarditis and angiomatosis (proliferation of blood vessels, resulting in tumour-like masses under the skin and other organs) in immuno-compromised patients (Anderson and Neuman, 1997, Boulouis et al., 2005, Koehler, 1996).

Clinical diagnosis of emerging syndromes associated with *Bartonella* spp. is not easy and often requires access to laboratory diagnostic equipment (Boulouis et al., 2005). Due to the fastidious nature of the organism, culture can take 5 to 15 days for visible

colonies to form (Chomel, 2004). The most common diagnostic methods are serology (to detect antibodies) and PCR (to detect bacterial DNA); both methods are quick, sensitive and reliable. From serology however, conclusive identification of the *Bartonella* species is not always possible, because of the apparent cross-reactivity between different *Bartonella* species (Kosoy et al., 2010). PCR analysis can often be of further assistance in identifying the specific causative *Bartonella* species. However, because of the significant genetic diversity within the genus even real-time PCR is not always reliably able to detect all species of *Bartonella*.

1.5.2 *Campylobacter*

Campylobacter are Gram-negative bacteria and include around 20 species; of these, *Campylobacter jejuni* is probably the most important food-borne zoonotic bacterial pathogen (Vandamme et al., 2005). There is a high degree of genetic diversity in the *Campylobacter* genus, making many epidemiological questions difficult to answer (Acke et al., 2010).

Campylobacter spp. are thought to be primarily commensal in many livestock animal species including cattle, sheep, swine and avian species, as they are often detected in the absence of clinical disease. In humans however, the bacterium causes an enteritis of varying severity with infrequent neurological sequelae (Silva et al., 2011, Tauxe, 2002); it is frequently associated with poultry meat consumption (Berrang et al., 2001). The role of reservoirs and routes of transmission have been poorly understood for a long time because *Campylobacter* transmission routes can be complex (Devane et al., 2005). Multiple animal species, including those that are often in close contact with humans, can shed *Campylobacter* spp. in their faeces, and the bacteria have also been found in water sources (Devane et al., 2005). Many *Campylobacter* spp have been discovered in wild rodents (*M. musculus* and *R. norvegicus*) on livestock and poultry

farms, and horizontal transmission from rodents to pigs and poultry is thus a possibility, resulting from, for example, consumption of an infected rodent by a pig (Meerburg et al., 2006) or through water or feed contaminated with faeces (Biggins and Kosoy, 2001). In rodents, *Campylobacter* infection does not result in disease (Janssen et al., 2008).

The WHO has predicted that every year, 1% of the population of developed nations will suffer from campylobacteriosis (WHO, 2000). There were over 200,000 confirmed cases of human campylobacteriosis reported in the EU in 2007, with a notification rate of 45 cases per 100,000 inhabitants (Silva et al., 2011). Over a ten year period, the mean annual incidence of *Campylobacter* infections increased in Germany; in 2014 there were 72 cases per 100,000 inhabitants (Schielke et al., 2014). In the UK, 82% of people admitted to hospital with food poisoning were diagnosed with a *Campylobacter* infection, most commonly associated with undercooked chicken (Adak et al., 2002). However, it is highly likely that *Campylobacter* infections are underreported, as it has been calculated that only one out of every 9.3 cases in the UK and one out of 12 in the Netherlands are reported to national surveillance bodies (WHO, 2012).

Many culture methods are available for isolating *Campylobacter* from clinical specimens. However, the time required for these confirmatory tests can exceed 5 days and the bacteria require fastidious growth conditions (Wang et al., 2002b). One study discovered that culture had a sensitivity of 65% compared to positive results found simultaneously for at least one molecular method (conventional and real-time PCR) and one serological one (Eppinger et al., 2009). However, culture offers the advantage of testing for antimicrobial susceptibility. ELISA tests, which have a sensitivity of 82-96% (Eisen et al., 2008), based on anti-Campylobacter IgA, IgM and IgG antibodies have been used to determine seroprevalence in populations (Tatineni et al., 2008).

1.5.3 *Escherichia coli*

There are five distinct clinical groups of the Gram-negative, facultative anaerobic bacteria *E. coli* that have been identified; entero-aggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) (Rasko et al., 2008). A major virulence factor, the shiga-like toxin (Stx), is produced by EHEC isolates. Isolates that contain this virulence factor are also known as Shiga toxin producing *E. coli* (STEC). The pathogenesis is determined by the combination of a range of virulence determinants encoding adhesins, invasins and toxins such as Stx, which are also vital to their molecular identification.

E. coli has been shown to have three principal routes of transmission in humans: Contaminated food or contaminated drinking or swimming water, person-person transmission, and contact with animal faeces (Karch et al., 1999). A total of 40 outbreaks of *E. coli* O157:H7 were confirmed in Minnesota (USA) between 2000 and 2008. Of these, 18 (45%) outbreaks were caused by food-borne transmission, 15 (37.5%) were attributed to person-to-person transmission, five (12.5%) were associated with animal contact and two (5%) were due to waterborne transmission (Rounds et al., 2011). Around 100,000 cases of *E. coli* O157:H7 are reported annually in the USA (Scallan et al., 2011), and the number of cases of acute illness caused by STEC strains alone has been estimated at over 2.8 million annually worldwide (Majowicz et al., 2014).

Numerous isolates of *E. coli* have been discovered in rodents (*R. norvegicus*) living on farms and horizontal transmission is thought to occur between animal species, as the same isolates have been identified in other animal species on the same farm (Nielsen et al., 2004). The most important reservoir of STEC is considered to be ruminants, particularly cattle. However, STEC strains have also been identified from a broad range of domestic and wild animals (Nielsen et al., 2004).

The majority of *E. coli* infections (about 70%) are asymptomatic (Ludwig et al., 2002). Intestinal infections may lead to bacteraemia with the organism detectable in blood, tissues and faeces (Le Moine et al., 1987). *E. coli* of any type causing diarrhoea in humans of all ages is a global health concern, and over 2 million deaths occur each year (Chen and Frankel, 2004). The public health impact of EHEC infections in particular is high because of their potential to cause systemic complications and to cause large outbreaks (Karch et al., 2005). An outbreak of EHEC in Germany in 2011 was characterised by an unusually high number of haemolytic uraemic syndrome cases. There were 3,842 cases reported, which included 855 cases with severe haemolytic uraemic syndrome and 53 fatalities (Manitz et al., 2014). Epidemiological studies have established EHEC as one of the main causes of bloody diarrhoea in temperate climates (Karch et al., 1999). *E. coli* O157:H7 has been recognised as an emerging pathogen since 1982; prior to that date very few isolates were detected by studies in the USA (Riley et al., 1983), the UK (Day et al., 1983) or Canada (Johnson et al., 1983).

Many studies have targeted the *stx* genes as a means to detect EHEC infections (Karch et al., 1999). However, testing stools for either Stx protein by ELISA, for the *stx* genes by PCR, or culturing of the pathogen, may not identify all possible causes of diarrhoea. An alternative is to also use targets that are conserved in both for stx-negative and stx-positive strains (Friedrich et al., 2007).

1.5.4 *Francisella*

Francisella is a Gram-negative bacterium and is the only genus in the *Francisellaceae* family (Larsson et al., 2005). There are three widely recognised *Francisella* species: *F. tularensis*, *F. novicida*, and *F. philomiragia* (Keim et al., 2007). *F. tularensis* is further divided into three subspecies based on virulence in rabbits and other characteristics. *F. tularensis* subsp. *tularensis* is highly virulent in rabbits and the cause of Type A

tularemia; *F. tularensis* subsp. *Holarctica* has a lower virulence and causes Type B tularemia; *F. mediasiatica* exhibits virulence in rabbits similar to Type B organisms (Keim et al., 2007). Type A tularemia can progress into a severe septic syndrome in humans, whereas type B tularemia is rarely life-threatening.

Type A strains are distributed throughout North America, whereas type B strains are more prevalent in Europe (Desvars et al., 2015). Tularemia is widely distributed in both humans and wildlife in Europe from North to South and East to West (Hestvik et al., 2014). In Sweden it exhibits a marked seasonality; most cases in humans occur from late summer to early autumn (Eliasson and Bäck, 2007), which coincides with the peak in mosquito population.

F. tularensis subsp. *tularensis* occasionally causes severe disease in humans, whereas *F. novicida* and *F. mediasiatica* are rarely pathogenic in humans (Celli and Zahrt, 2013). Type A tularemia can cause high mortality in humans, up to 30% in untreated cases (Dennis et al., 2001). In the USA a total of 1,208 human cases of tularemia were reported between 2001-2010 (CDC, 2013).

Various *F. tularensis* subspecies have been documented in a wide range of hosts including mammals, birds, amphibians, fish, and invertebrates (Ellis et al., 2002). A number of wild animals in Germany were screened for antibodies to *F. tularensis* and 171/928 foxes, 44/345 raccoon dogs and 6/80 wild boars were seropositive (Kuehn et al., 2013). Rodents, including *R. rattus* and *M. musculus* have also been found to carry *F. tularensis* (Christova and Gladnishka, 2005). However, there is still a general lack of knowledge and understanding of the role of animal hosts on the mechanisms of maintenance, and the different routes of transmission (Hestvik et al., 2014). The main route of entry of *F. tularensis* in humans occurs after exposure to infected wildlife species, the most frequent of which are rodents, hares or rabbits. Transmission to humans can occur via direct contact, via arthropod or insect vectors, by ingestion of

contaminated materials or by direct inhalation of aerosolised organisms (Celli and Zahrt, 2013).

A variety of molecular typing methods are available for *F. tularensis* detection and diagnosis, however there are limitations due to high costs or a lack of discrimination between *F. tularensis* subspecies (Birdsell et al., 2014). Examples of molecular typing methods include pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and multi-locus variable-number tandem repeat (VNTR) analysis (MLVA). These are able to differentiate between *F. tularensis* subspecies, but these methods are more expensive (Keim et al., 2007). PCR-based assays are useful at confirming *F. tularensis* from clinical or environmental samples, and are able to discriminate between subspecies. The sensitivity can be further increased when real-time PCR is used instead (Michelet et al., 2013).

The most frequently used serological assays for tularemia are microagglutination, ELISA, and Western blotting (Sharma et al., 2013). However, it is difficult to prepare antibodies specific to the immunoglobulins of each animal species; this is especially the case for wildlife surveillance. A combination of western blotting and ELISA is often used to confirm serodiagnosis of tularemia (Schmitt et al., 2005).

1.5.5 *Leptospira*

There are around 20 species of *Leptospira* spirochaetes, with both pathogenic and non-pathogenic members, and there are over 200 pathogenic *Leptospira* serotypes (Levett, 2001). It is thought that each serotype has adapted to certain mammalian hosts; rodents, dogs, pigs and cattle are the most common. The specificity of *Leptospira* serotypes to hosts is thought to be low, as serotypes can adapt to numerous hosts, or one host might carry several distinct serotypes (Hartskeerl et al., 2011).

There are approximately 0.1-1 per 100,000 human cases per year in temperate climates, and 10-100 per 100,000 in more tropical regions (World Health Organisation, 2003). A more recent figure from the European Centre for Disease Prevention and Control (ECDC) states that in 2010 there were 588 confirmed leptospirosis cases reported in 25 European countries (ECDC, 2013). There has also been an increase in the overall incidence of leptospirosis in Ireland between 1995 and 2009, rising from 1.8 notifications/million to 5.2 notifications/million (Garvey et al., 2014). This increase in incidence may also have occurred in other European countries where, traditionally, leptospirosis was more uncommon. It has also been hypothesised that the global burden of *Leptospira* will increase due to a rise in the number of people in poorer urban areas in tropical regions which are more likely to be subject to storms and urban flooding due to climate change (Haake and Levett, 2015).

Leptospira infection is generally rarer in developed countries, however steady rates of non-travel related illness and death are known to occur (Jansen et al., 2005, Demers et al., 1983, Cacciapouti et al., 1994, Childs et al., 1992b) and the disease is becoming increasingly associated with leisure rather than professional activities, such as water sports (Nardonne et al., 2004). There are variable clinical manifestations of leptospirosis, which can range from mild febrile illness to a more severe icteric-haemorrhagic form (Weil's disease) which can cause death, primarily as a result of renal failure (Kobayashi, 2001). Severe disease is estimated to occur in 5-15% of all human infections (Spichler et al., 2008).

Almost every mammal worldwide has been shown to be a potential carrier of leptospires (Adler and de la Peña Moctezuma, 2010). The most frequent rodent host is *R. norvegicus* (Dutta and Christopher, 2005). Many surveys worldwide have shown that several rat species are serologically positive, ranging from 50 to 90% (Aviat et al., 2009). Reservoir animals, such as rats and mice, usually do not show signs of disease, but

harbour leptospires in their kidneys, and become an important source of infection for humans or other animals as the bacteria can be shed in large numbers in the urine (Adler and de la Peña Moctezuma, 2010).

Leptospirosis ecology involves a complex interaction between humans, animal reservoirs, leptospires and the environment in which they coexist (Lau et al., 2010). The role of the rat in the transmission of leptospirosis to humans has been known for many years, having been first recognised almost a century ago (Ido et al., 1917). Shedding of leptospires into the environment, and especially into fresh water, can lead to human infection following recreational activities on or in water, such as canoeing or rowing (Aviat et al., 2009). Leptospires can penetrate skin abrasions or mucous membranes and establish systemic infection by crossing tissue barriers (Faine, 1994). Infection can also be caused by inhalation of water or aerosols which then penetrate the mucous membranes of the respiratory tract (Levett, 2004).

Bacterial culture and identification is the reference method for identifying the pathogen in a diagnostic sample, but this is time-consuming, and can take weeks (Ooteman et al., 2006). PCR offers a low cost, sensitive assay which can be completed in around five hours using serum samples. PCR primers are available that only amplify pathogenic *Leptospira*, allowing for differentiation between pathogenic and non-pathogenic strains, and having been designed in 1993 are still in use today (Gravekamp et al., 1993). Point of care rapid diagnostic tests are in development, however with sensitivity no greater than 80% compared with other methods including culture (Goris et al., 2013), confirmatory tests with other methods are still required.

1.5.6 Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic, Gram-positive bacterium. Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains have emerged worldwide and have become resistant to a variety of antibiotics as a result of extensive chemotherapy over many years (WHO, 2000). Despite the global spread of MRSA, epidemiological studies have indicated that the number of strains is few in number, and the pathogen is thought to have spread clonally (Oliveira et al., 2002).

MRSA infections cause fatalities in around 19,000 hospitalised patients in the USA annually; this is roughly equivalent to the number of deaths from both AIDS and tuberculosis combined (CDC, 2005, CDC, 2006). Young, healthy and immunocompetent people can be infected with community-acquired MRSA, usually through close contact with someone who has a skin infection (Ferry and Etienne, 2007). The number of patients with hospital-acquired MRSA however, is generally higher in older individuals perhaps because they are more likely to visit hospital facilities than younger inividuals (Klein et al., 2013). There has been recent evidence that MRSA is becoming increasingly resistant to newer therapies and antimicrobials (Stryjewski and Corey, 2014). This highlights the constant need for developments in the treatment of this pathogen.

MRSA has been isolated from domestic animals including cats, dogs, pet rats and horses (Scott et al., 1988, Van Duijkeren et al., 2004, Weese et al., 2005). Human infections with some strains of MRSA have also been associated with exposure to pigs and cattle (Van Loo et al., 2007). MRSA was detected in *R. rattus* captured on pig farms in the Netherlands; these rats could easily have become infected by direct contact with contaminated faeces or dust, or even by inhalation of contaminated air (van de Giessen et al., 2009). Recently, urban rodents in Hong Kong, including *R. rattus* and *R. norvegicus* were found to be carrying MRSA, although their precise role in transmission to humans is unknown (Ho et al., 2012).

Because active screening is a critical part of any effective control strategy, there is a need for a rapid and relatively simple method of detection. The standard method for detection of MRSA is by culturing the pathogen; however, this takes up to five days. This delay in obtaining culture results leads to a suboptimal control strategy. In some cases carriers may not be kept in protective isolation for a number of days, or non-carriers are unnecessarily kept in isolation, until the results of the culture become available (Kluytmans, 2007). A PCR assay was developed which can rapidly and reliably identify MRSA carriers, and improve the efficacy of MRSA surveillance programs (Huletsky et al., 2005).

1.5.7 Mycobacteria

The genus *Mycobacterium* is one of the most numerous in the Actinobacteria phylum, with over 140 different species and a genome size around 4.4 million bp (Garcia-Betancur et al., 2012). The way in which Mycobacterial species are defined has changed after developments in genotyping technologies including alignments of conserved gene sequences such as the 16SrRNA gene (Tortoli, 2003). However, with >99% identity between some Mycobacterial species, this highly conserved gene is of limited use in the differentiation between these species (Garcia-Betancur et al., 2012). Other genes have been used as an alternative to differentiate between species, for example, the *rpoB* or the *recA* gene (Adékambi and Drancourt, 2004).

Over 50 of the species within the *Mycobacterium* genus have been categorised as potential human pathogens. The *Mycobacterium tuberculosis* complex (MTC) are some of the most closely studied, and include the human pathogens *M. tuberculosis*, *M. africanum*, and *M. cannetti* and the predominantly animal pathogens *M. bovis*, *M. microti*, and *M. caprae* (Incandela et al., 2013). In spite of the different diseases and the differences in geographic distribution, host preference, virulence traits and

antimicrobial susceptibility patterns, the interspecies genetic identity of MTC species is 99.9%, and the 16S ribosomal gene sequence is identical (Mostowy et al., 2005). This makes it very difficult to differentiate between individual species. Consequently, further work is needed in order to improve our understanding of mycobacterial species and to develop methods to differentiate between infectious species.

Some of these zoonotic pathogens also have economic importance, for example *M. bovis* infections in the UK cattle herd. These can also have political consequences due to the presence of a wildlife reservoir (badgers) and conservationist groups seeking to prevent any wildlife culling to prevent further spread. Whilst rodents are unlikely to be a main source of infection to other species (Mathews et al., 2006), they have been found to be capable of carrying and excreting infectious MTC species (Clarke et al., 2007).

It is thought that around one-third of the world's population is infected, either latently or actively, with tuberculosis (TB), the vast majority of which are caused by *M. tuberculosis*. Zoonotic transmission of TB is a rare occurrence. Recent reports indicate that TB is slowly declining but in 2013 an estimated 9 million people developed TB, and 1.5 million died from the disease (WHO, 2014c).

PCR and real-time PCR have been used for MTC detection and both have a similar sensitivity (75-77%) and specificity (99%) compared with culture methods (Tortoli et al., 2012). The poor sensitivity however, limits the effectiveness of PCR. After their introduction in 2001, IFN-γ release assays were widely regarded as a breakthrough in tuberculosis identification and control. These assays are thought to be more specific than the tuberculin skin test (in BCG vaccinated populations) as they do not use the same antigens as those on the BCG vaccine or environmental bacteria (de Souza et al., 2014). However, both of these tests are poor indicators in predicting progression from subclinical to clinical TB (Trajman et al., 2013).

1.5.8 *Rickettsia typhi*

Bacteria in the genus *Rickettsia* are Gram-negative bacteria of vertebrate hosts (Raoult and Roux, 1997) with over thirty recognised species (Merhej and Raoult, 2011). The bacteria are divided into two main groups according to the type of disease produced: The spotted fever group, and the typhus group (Botelho-Nevers and Raoult, 2011). They have a wide host range which can include vertebrates, arthropods, annelids, amoebae and plants (Perlman et al., 2006).

In human disease the incubation period can vary between 3 and 15 days, and is dependent on the route of entry, be it inhalation, ingestion, or bite of infected arthropods and the pathogen load (Azad and Radulovic, 2003). Whilst most cases are mild (symptoms include headache, fever and muscle pain), a mortality rate of 1% has been observed despite appropriate antimicrobial treatment. The incidence of murine typhus appears to have decreased in developed countries, and it is believed that pest control has been one of the major factors contributing to its decline (Gikas et al., 2002). Over a 3 year period from 2008-2010, a study in France found that there were 32 confirmed cases of murine typhus out of a total 42, 276 sera samples, and that all of these confirmed cases were acquired abroad (Walter et al., 2012). Whilst the incidence may be decreasing in developed countries the risk to holiday makers is still present.

A study in Seville, Spain, found that over a 12 year period 104 cases were reported to a hospital. The majority of cases (63.5%) were from an urban setting with the remainder from more rural settings (Bernabeau-Wittel et al., 1999). Murine typhus can also cause sporadic cases in areas which had been thought free of the disease, as was the case in southern Italy recently where a 75-year-old woman was infected with *R. typhi* (Luciani et al., 2014). Many of the endemic foci are often international ports, because of the large populations of *R. rattus* and *R. norvegicus* commonly seen there,

and the disease is usually reported in people whose occupation brings them into close contact with rats and their fleas (Traub et al., 1978).

R. typhi is a zoonotic pathogen maintained in rats (*R. rattus* and *R. norvegicus*) and transmitted to humans through abrasions in the skin or contamination of the conjunctiva or inhalation of infected faeces from the oriental rat flea, *Xenopsylla cheopis* (Azad, 1990). Vertical transmission by the transovarian route has also been demonstrated in fleas, allowing the pathogen to be maintained in the flea population (Merhej and Raoult, 2011).

Infections in rats and fleas are commensal (Azad, 1990). Rats serve as amplifying hosts that make *R. typhi* accessible to vector species such as fleas. The pathogen has been isolated from rats worldwide, and fleas may experimentally acquire infection through feeding on infected rats (Arango-Jaramillo et al., 1988). A low seroprevalence (0.36%) in *R. norvegicus* has been reported in Vancouver, Canada recently (Himsworth et al., 2015). There was also no evidence of urban rats in New York harbouring the pathogen as flea hosts found on rats were tested by PCR for *R. typhi* and were all found to be negative (Frye et al., 2015). A higher prevalence was however, observed in an urban area of the Democratic Republic of Congo (Kisangani) where 20% of *R. rattus* (20 sampled) and 11% of *R. norvegicus* (106 sampled) were identified (Laudisoit et al., 2014). Urban rodents therefore, do have the potential to act as a reservoir species for this pathogen in some geographical locations. *M. musculus* has also been reported to harbour *R. typhi* (Azad, 1990).

Traditionally, *Rickettsia* species are difficult to isolate and maintain. The isolation of rickettsiae from blood cultures is rarely successful and is usually performed in specialised research laboratories (Civen and Ngo, 2008). Serological tests are the most frequent and widely available methods for diagnosis. Because of the antigenic cross-reactivity among rickettsiae, it is recommended that serologic tests are complemented

by PCR (Azad et al., 1997), which can have an analytical sensitivity range from 10 to 10^4 femtograms (Kato et al., 2013). Molecular tests face difficulties when the pathogen burden is low, and this has resulted in a low clinical sensitivity (48%) recorded in recent studies using Loop-mediated isothermal amplification (LAMP) assays (Butler, 1983).

1.5.9 *Salmonella*

Salmonella enterica is a Gram-negative pathogen which infects and produces disease in a wide range of animal species. The species is divided into more than 2500 serotypes on the basis of two surface antigens, both of which show substantial diversity (CDC, 2008). A small number of the serotypes cause typhoid-like infections in humans and animals. The remainder colonise the gastrointestinal tract of animals, including livestock though not always causing disease, and enter the human food chain resulting in gastroenteritis in humans (Coburn et al., 2007).

Salmonella is one of the most prevalent causes of human gastroenteritis in industrialised countries (Skov et al., 2008); whereas, in developing countries, it is associated with more severe gastroenteritis and a high incidence of invasive illness, often resulting in septicaemia and fever, with consequently high mortality. Exact estimates of infection vary due to a lack of consistency in diagnosis and reporting. However, it is estimated that worldwide each year there are over 200 million cases of *Salmonella*-related gastroenteritis and 3 million deaths from non-typhoidal *Salmonella* (Coburn et al., 2007, World Health Organisation, 2005). Two of the most important non-typhoid serotypes in Europe and the USA are *S.enterica* serotype Enteritidis (*S*. Enteritidis) and *S.* Typhimurium (Threlfall, 2002). These two serotypes are common causes of bacterial gastroenteritis in the EU (EFSA, 2010). There are over 100,000 human cases reported each year in Europe, and the overall economic burden in the EU could be as high as EUR 3 billion a year (EFSA, 2015). There have been significant

increases in the occurrence of antimicrobial drug resistance in non-typhoid *Salmonella* spp. in developed countries, and it has been suggested that this is a consequence of the prophylactic use of antimicrobial drugs in food-producing animals (Adesiji et al., 2014).

Similar to other enteric bacteria, *Salmonella* infection is spread through the faecal-oral route of contamination. The bacterium can enter the environment directly from the faeces of infected humans or animals (Levantesi et al., 2012). This can then enter the human food chain either by contamination of water sources or infected animals can pass the bacteria to humans. The presence of *Salmonella* in eggs is also a very common infection route, with outbreaks occurring in the UK in 2003, 2006, 2008, 2009, 2010 and 2011 having been traced back to eggs (Harker, 2014).

The epidemiology of *Salmonella*-associated infections varies widely depending on the type of *Salmonella* involved (Sánchez-Vargas et al., 2011). Some serotypes are restricted to certain species, for example *S.* Typhi in humans, whereas other serotypes such as *S*. Typhimurium have a broader host range which can include humans, chickens, pigs, cats, cattle and rodents. There have been many reports of the detection of the organism in the faeces of rodents (Yokoyama et al., 2007), and in some countries it has been suggested that a rodent reservoir was one of the contributing factors in frequent outbreaks of the disease (Yim et al., 2010). However, it is unlikely that rodents contribute directly to human infection, but they may instead have a role as a reservoir host on chicken or pig farms and help maintain infection in food-producing animals. The pathogen has been identified in high numbers within wild urban rodent populations sampled in Yokohama city, Japan, with both *R. rattus* (13.0%-15.5%) and *R. norvegicus* (18.0%-21.9%) positive by PCR; *S.* Typhimurium was identified most frequently (Maruyama et al., 2000).

The gold standard test for diagnosing *Salmonella* infection is isolation of the organism from a bacteriological culture, usually made from infected faecal samples. However,

this is not suitable for routine testing of large numbers of samples as it can take up to five days to obtain results (Uyttendaele et al., 2003). In clinical settings, where a more rapid diagnosis is required, methods such as PCR, microarrays and LAMP are available and are used more frequently. These have a sensitivity and specificity comparable to the gold standard (Li and Ruan, 2009). However, bacteria may only be present for a few days which can limit the effectiveness of molecular methods. In these circumstances, serological antibody assays can be used to provide evidence of exposure, though the sensitivity and specificity of some of these assays is low. Whilst standardised assays have been developed for the veterinary field, there is no universal ELISA for the human non-typhoid *Salmonella* (Kuhn et al., 2012).

1.5.10 *Streptobacillus monoliformis*

The only species in the genus *Streptobacillus*, *Streptobacillus moniliformis* is a Gram-negative non-motile and non-acid fast rod (Levaditi et al., 1925). The majority of human infections with *S. moniliformis* occur in people who come into close contact with pet rats (Rat Bite Fever) (Graves and Janda, 2001). Other animal species including mice, cats, and dogs can be infected by *S. moniliformis*, but they are not considered to present a significant risk of infection to humans as they are not considered to be natural hosts (Gaastra et al., 2009). Most of the information regarding *S. moniliformis* infection is obtained from from individual case reports. Oral infection via contaminated food or water leads to a disease known as Haverhill Fever (McEvoy et al., 1987, Place and Sutton, 1934). In cases in which a bite was not reported, it was concluded that close contact with rats caused transmission of the pathogen (Albedwawi et al., 2006, Madhubashini et al., 2013). Homeless people, farmers, sewage workers, hunters, trappers and tourists are also thought to be of higher risk, due to a higher probability of contact with rodent species (Gaastra et al., 2009).

There is a mortality rate of 13% in cases of untreated Rat Bite Fever (Elliott, 2007), which is often caused by endocarditis (Chen et al., 2007). Infections are usually subclinial and as such are not reported (Gaastra et al., 2009). The majority of reported infections occur in the USA, and most European reports come from the UK and France, although there are sporadic reports from other countries within the EU. Human to human transmission has never been documented (McKee and Pewarchuk, 2013).

It is generally assumed that both *R. rattus* and *R. norvegicus* are natural hosts and asymptomatic carriers of *S. moniliformis*. A Japanese study in an urban area of Tokyo revealed that 92% of wild *R. norvegicus* and 58% of *R. rattus* species were positive for *S. moniliformis* DNA by PCR (Kimura et al., 2008). This is higher than that found in a more recent study in New York where 17% of *R. norvegicus* were PCR positive (Firth et al., 2014). Whilst most rats are colonised asymptomatically, they may occasionally demonstrate disease (Elliott, 2007). It has been suggested that different species of the genus *Rattus* could harbour different types of bacterial strains (Kimura et al., 2008). Whether the difference in strains results in a higher degree of pathogenicity is unknown.

Clinical diagnosis of Rat Bite Fever (RBF) is often based on bacterial cultures (Chen et al., 2007). However, *S. moniliformis* is an extremely fastidious organism, which may take as long as seven days, and can sometimes fail to produce culturable bacteria (Elliott, 2007). Despite this, clinical diagnosis of RBF is often based on bacterial cultures. PCR, which has a similar sensitivity, can be used as an alternative to culture (Boot et al., 2002). There are currently no validated serological tests available for humans, but assays which have a good sensitivity are available for rodents, and these are frequently used to monitor laboratory animals for antibodies to *S. moniliformis.* Any serologically positive animals are subsequently tested by PCR (Gaastra et al., 2009). A recent study demonstrated the use of a PCR and electrospray ionisation followed by mass spectrometry using a sample of human synovial fluid to successfully detect the bacteria

when culture appeared negative (sensitivity of synovial fluid culture is approximately 80%) (Handke et al., 2010). Despite the increased sensitivity shown by this method, the costs involved could prohibit widespread use.

1.5.11 *Yersinia*

The genus *Yersinia* is comprised of Gram-negative coccobacilli and contains three well documented human bacterial pathogens: *Yersinia pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica.*

There are three recognised forms of plague in humans, which are caused by *Y. pestis*. Bubonic plague is the most common, and arises following a bite from a flea which has fed on an infected animal. Septicaemic plague occurs when bacteraemia occurs without the development of buboes (swollen lymph nodes). Pneumonic plague develops when there is colonisation of the alveolar spaces in the lung leading to pneumonia (Poland and Barnes, 1979). In the absence of treatment, the mortality rate of bubonic plague caused by *Y. pestis* ranges from 40-70% (Galimand et al., 2006). If the pathogen reaches the lungs and successfully replicates, it can be transmitted between humans via aerosol (Eppinger et al., 2010). *Y. enterocolitica* and *Y. pseudotuberculosis* can cause Yersiniosis in humans (symptoms include abdominal pain, diarrhoea and fever) (Backhans et al., 2010). *Y. pseudotuberculosis* and *Y. enterocolitica* have a broad host range and are capable of infecting a wide variety of animals, including humans (de Barcellos and de Castro, 1981, Rosner et al., 2010).

Y. pestis can infect most mammalian species however, this is misleading as many species of mammal fail to develop the high bacteraemia required to infect fleas. The majority of mammalian species are likely, therefore, to be dead end hosts (Eisen and Gage, 2009). Both animals and the environment (soil) can act as reservoirs for *Y. pseudotuberculosis* and *Y. enterocolitica*, from which epizootic and human infection may

arise. Transmission usually occurs via contaminated food (unwashed fruit or vegetables) or drinking water (Zhou and Yang, 2009).

R. rattus has been closely associated with *Y. pestis* throughout history due to rats' role in plague pandemics; although they are susceptible to infection, these rodents can be resistant to the disease, acting instead as reservoir hosts for other species (Dennis et al., 1999). They can also act as amplifying hosts, where the bacteria multiply into large numbers before being transmitted to a flea for further dissemination. On some pig farms, up to 20% of rodents tested positive for *Y. enterocolitica* strains, though most of these were in close proximity to infected pigs. This has led some authors to classify them as carriers of bacteria they contract from infected pigs or the environment rather than reservoirs of infection (Backhans et al., 2010). Similar to *Y. enterocolitica*, wild rodents can also act as reservoirs of *Y. pseudotuberculosis* on pig farms, particularly on organic farms where pigs have greater contact with other species as they are not housed indoors (Laukkenen et al., 2008).

Future pandemics of *Y. pestis* are unlikely, thanks to improved living conditions, public health and antibiotics. However, a plague outbreak following release of a biological weapon is a plausible threat, particularly if the pneumonic type is weaponised (Inglesby et al., 2000a). A recent outbreak of pneumonic plague in Madagascar affected 20 people (Richard et al., 2015). Health services managed to deliver antimicrobials to five people, and these all survived. The remaining 15, untreated patients all died. This emphasises the importance of antimicrobial treatment and how deadly the pathogen is when appropriate treatment is unavailable. Considered endemic in the US, due to the prevalence in praire dogs, there have been 1006 human cases of plague over a period of 113 years, with larger outbreaks occurring in the early part of the last century (Kugeler et al., 2015).

The wide-scale distribution of *Y. enterocolitica* appears to reflect its large host range, which includes domestic and wild animals, in addition to humans (Rosner et al., 2010). However, the definitive animal host for most pathogenic serogroups appears to be the pig (Bottone, 1997). *Y. enterocolitica* has been identified on all continents except Antarctica, and has been frequently isolated in Europe (Fredrikkson-Ahomaa and Stolle, 2006). The highly pathogenic *Y. enterocolitica* type 1B/O8 has traditionally been geographicaly restricted to North America, although it has emerged recently in Japan and Europe and has been found in wild rodents (Oda et al., 2015, Rastawicki et al., 2013). Recent studies in Europe have focused on the role of companion animals, particularly dogs, as carriers of *Y. enterocolitica* (Wojciechowska et al., 2010). Faecal samples (4,325) from dogs from 14 European countries were screened for *Y. enterocolitica* and the pathogen was isolated from 4.6% of these, which shared common genotypes with strains isolated from humans (Stamm et al., 2013). It is also possible that rodents could come into contact with faecal matter from infected pigs and dogs, which could create an epizootic cycle, and would represent a potential threat to public health. Indeed, food contaminated by bird or rodent faeces has been suspected as the original source of the bacteria in some epidemics (Williams and Barker, 2001).

Y. pseudotuberculosis infections are distributed worldwide, although the epidemiology can be complex and the incidence in many countries is unknown (Long et al., 2010). It has a broad host range, including wild and domestic animals and birds (Schuenemann et al., 2011). Similar to *Y. enterocolitica*, the pig is thought to be the primary reservoir of human pathogenic *Y. pseudotuberculosis* and can often be found in healthy pigs at slaughter (Vanantwerpen et al., 2014). The European wild boar population is steadily increasing and, like the domestic pig, these animals can act as a reservoir for both *Y. enterocolitica* and *Y. pseudotuberculosis*. A total of 20% of wild boars, from a study population of 80, were found to be PCR positive to *Y. enterocolitica*

and *Y. pseudotuberculosis* (Sannö et al., 2014). As with *Y. enterocolitica* the principal mode of transmission is thought to be via contaminated food or water (Vogel, 2014). Routine surveillance of human *Y. pseudotuberculosis* is rarely performed, and as a result any trends in human *Y. pseudotuberculosis* infection are difficult to establish (Fleige and Pfaffi, 2006). Yersiniosis, which is caused by either *Y. enterocolitica* or *Y. pseudotuberculosis* decreased over a five-year period from 2008-2012 in the EU (ECDC, 2014). In 2012 the confirmed case rate of yersiniosis was 1.96 cases per 100,000 inhabitants in EU countries, with Germany showing the highest proportion (41%) of all reported cases.

The existing reference tests for *Y. pestis*, which can only be performed in a laboratory, are the isolation of *Y. pestis* and the detection of F1 antigen (Fraction 1 capsular antigen) by ELISA. These tests are specific, but were found to lack sensitivity when used in countries where plague is endemic. This may have been because there were low levels of the pathogen in the samples tested or because of sample deterioration, contamination of specimens during their transport time to the laboratory or due to antimicrobial treatment of patients before sampling. An alternative to these tests is an immunochromatographic lateral flow dipstick-assay (ICT) that detects the F1 antigen and has proven to be a specific, sensitive and rapid tool in countries with endemic *Y. pestis* (Chanteau et al., 2003). As useful as this test can be, there have been occasions when the F1 antigen was recorded as missing in *Y. pestis*, rendering the test ineffective in some cases (Leal-Balbino et al., 2004).

The isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* from clinical, food and environmental samples can be difficult as culture can have low sensitivity, a long incubation time, an inability to differentiate between species and a lack of discrimination between pathogenic and non-pathogenic strains (Fredrikkson-Ahomaa and Stolle, 2006). The usual method of identifying Yersiniosis-causing pathogens is to

diagnose the clinical symptoms and use immunoassays, such as ELISA, to find serological evidence of infection (Rosner et al., 2010). Real-time PCR assays are highly specific in detecting the pathogen however, cultivation is still required to assess antibiotic susceptibility and to acquire an isolate of the organism for genotyping (Riehm et al., 2011).

1.5.12 Cowpox

Poxviruses comprise a large family of linear dsDNA viruses (Alzhanova and Früh, 2010). Members of the genus *Orthopoxvirus* (OPV) such as cowpox (CPXV) and monkeypox are pathogenic for animals and humans (Meyer et al., 1994). Among OPV species, CPXV has the largest genome size averaging about 220 kbp (Carroll et al., 2011).

Long-term ecological studies of CPXV indicate that whilst CPXV has a wide host range, rodents are the main reservoir host despite its infrequent isolation of the pathogen from them (Alzhanova and Früh, 2010). A mean seroprevalence of 20-30% was found in several wild rodent species across Europe, though this varied depending on the location as in some cases it was 0% and in others it was as high as 69% (Kinnunen et al., 2011). CPXV in rodent populations in Germany is believed to be the cause of sporadic infections in cats, due to their hunting behaviour, and they may subsequently infect humans (Kaysser et al., 2010). For immunocompetent people, CPXV is not very contagious and mild cases may often be missed (Hönlinger et al., 2005); it is likely that only the more severe cases are reported (Pahlitzsch et al., 2006). However, fatal cases have been reported in immunocompromised people (Czerny et al., 1991).

The source of infection in most human cases is domestic cats. Transmission usually occurs through a skin lesion, but in some cases, it is transferred via the mucosa of the eye (Pahlitzsch et al., 2006). Rodent to human transmission has been known to occur from direct contact with pet rodents, as happened recently in France; this is of

increasing concern given the rising popularity of pet rats (Duocournau et al., 2013). Some authors have indicated that domestic cats can be regarded as a sentinel species for CPXV prevalence among rodents, due to the higher probability of a cat coming into contact with an infected rat and then becoming infected. However, the infection is often misdiagnosed as veterinarians often suspect other, more common, dermatologic diseases when CPXV is present (Kaysser et al., 2010).

The termination of routine vaccination with the vaccinia virus (because of the eradication of smallpox), which had a broad cross-protectivity, led to a susceptible population for various orthopoxviruses (Mohamed et al., 2009). The last few decades have witnessed a rise in the number of reports of human and animal CPXV infection in Europe (Carroll et al., 2011). In European countries (Vorou et al., 2008), cases of human CPXV are mainly recorded only in countries where the disease is notifiable, such as in Germany and France (European Centre for Disease Prevention and Control, 2009). In the UK, serological testing has been conducted in a number of rodent species; antibodies to CPXV have been found occasionally in *M. musculus,* but the highest seroprevalence was found in bank voles, where it was found to be around 10% for much of the year but increased to 80% in late summer and early autumn (Chantrey et al., 1999). This seasonality has also been observed in other, more recent studies (Begon et al., 2009).

Numerous identification and differentiation methods for CPXV have existed for a number of years, including serological and PCR methods (Ropp et al., 1995). In the acute phase of the disease, CPXV can be differentiated from other OPV by PCR from exudates, contents of pustules, scabs of skin lesions, and other clinical materials (Becker et al., 2009). Quantitative PCR is currently used for rapid diagnosis of CPXV as it combines simple analysis and high sensitivity, albeit at a higher cost (Maksyutov et al., 2015). In Europe most detection in wildlife is based on serological methods, with one of

the limitations being that it only detect exposure to an OPV and cannot differentiate to the species level (Pahlitzsch et al., 2006).

1.5.13 Hantavirus

Hantavirus is a genus of enveloped, negative sense, segmented ssRNA viruses of around 80 to 120 nm in diameter that belongs to the Bunyaviridae family (Arikawa et al., 2007). There are three primary clades associated with the different rodent species: *Arvicolinae* (voles, lemmings, muskrats), *Sigmodontinae* (New world rats and mice) and *Murinae* (Old world rats and mice) (Ramsden et al., 2008).

There are over 40 known hantavirus species and 22 of these are considered pathogenic for humans (Manigold and Vial, 2014). Hantaviruses can cause two human zoonotic diseases: Haemorrhagic fever with renal syndrome (HFRS) and Hantavirus pulmonary syndrome (HPS). Depending on the species, HFRS has a range of mortality rates in humans from 0.1% to 12% (Dearing and Dizney, 2010). Hantaviruses occur worldwide, with the majority of cases originating from Asia. Infection in humans annually results in over 200,000 hospitalisations and thousands of deaths. The majority of these cases however, are not reported because the symptoms are not recognised by clinicians (Dearing and Dizney, 2010). In 2012 there were 4440 cases of Hantavirus infection in the EU which was an increase of 51.7% from 2011, though it was similar to the number of cases reported in 2010 (ECDC, 2014a). The all time peak incidence recorded in Germany was also reported in 2012 with 2800 reported cases (Kruger et al., 2013). In the UK, it has recently been reported that a seroprevalence of 7.6% was recorded in a region where a human case of HFRS was confirmed (Jameson et al., 2014). HFRS-causing hantaviruses are present in many European countries; these viruses include Puumala virus, Dobrava-Belgrade virus, Saarema virus, Seoul virus, Tula virus, Kurkino virus and Sochi virus (Reusken and Heyman, 2013). Due to the extensive

geographic distribution of some host species, it is not uncommon to find numerous hantavirus species circulating in the same region (Sibold et al., 1999).

Transmission of the virus to humans and to other potential hosts is via inhalation of aerosolised virus or by bites from infected rodents (Zeier et al., 2005). Hantavirus infection is thought to involve both direct and indirect transmission. The importance of direct or indirect transmission can vary depending on hantavirus species, host-population dynamics, and season; it can also vary in different environments (Kallio et al., 2006). In rats, it is thought that infection has an age-dependent pathology; infection of neonates causes fatal disease, whereas infection of adult rodents has no apparent effect (Kariwa et al., 1996). A study on the effects of SEOV on *R. rattus* suggested that there were no observable fitness consequences to infection with the virus in adult animals (Klein et al., 2004).

There are several existing methods for the detection of hantaviruses; the standard diagnostic methods are based on serological and immunological techniques (Kramski et al., 2007). The current gold standard for typing of hantavirus antibody responses is the focus reaction neutralisation test, which is able to differentiate between different hantavirus species (Mertens et al., 2011). A disadvantage of the focus reaction neutralisation test is the time taken for results to be obtained, which is approximately 11 days (Handke et al., 2010). ELISA and the focus reaction neutralisation test have high sensitivity and specificity, which is around 99%. Sensitive and specific detection of hantavirus in patients can be monitored by reverse transcriptase PCR, which is useful in determining the level and duration of viraemia (Evander et al., 2007).

1.5.14 Hepatitis E Virus

The only member of the Hepeviridae family and Hepevirus genus, Hepatitis E Virus (HEV) is a non-enveloped virus with a positive-sense, ssRNA genome (Reuter et al., 2006). HEV has been divided into two species: a mammalian one which only infects mammals and an avian one which only infects birds. There are thought to be four genotypes of mammalian HEV, all of which can infect humans (Aggarwal and Nail, 2009). Results from a study in Germany suggest that there may be a fifth genotype of mammalian HEV, which principally infects rats (Johne et al., 2010). A recent study characterised the full genome of an additional rat HEV strain detected in Vietnam which was sufficiently different to the one found in Germany that it can be considered a new genotype of rat HEV (Li et al., 2013a).

HEV infection in humans manifests as subclinical to fulminant liver disease; clinical symptoms appear to be dose dependent, and a low dose usually results in a mild infection. The most commonly affected group is young to middle aged adults 15-40 years of age, and infection can have a mortality rate of 0.2-1% (Chandra et al., 2008). In the past, HEV was rarely identified in industrialised countries, with the few reported cases of infection, usually in someone who had recently travelled to an endemic region. In recent years however, cases of autochthonous HEV have been diagnosed with increasing frequency in individuals who have no history of travelling abroad (Purcell and Emerson, 2010). Europe is considered a non-endemic region for human HEV, with few clinical cases and the seroprevalence in the general population is thought to be 1-3% (Bihl and Negro, 2010). However, a recent study in Spain on over a thousand routine blood donor samples found a seroprevalence to HEV IgG of up to 20% (Sauleda et al., 2014). There are occupational groups that are considered more at risk as they are more likely to come into contact with HEV positive animals (e.g. farmers, veterinarians,

butchers or persons handling animal meat or consumers of undercooked swine/wild deer meat), and can have a seroprevalence of 16% (Peralta et al., 2009).

There have been numerous cases of people contracting HEV after consumption of undercooked swine or deer meat (Wang, 2013, Long et al., 2004). The rat-borne genotype has been detected in rodents in the USA, Germany and Vietnam. This indicates that rat HEV infection could be globally distributed (Johne et al., 2010, Johne et al., 2010a, Purcell et al., 2011, Li et al., 2013a).

Domestic pigs and wild boar are known to be reservoirs for the virus and people regularly coming into contact with these animals are considered to be more at risk than the general population, as the virus is usually transmitted via the faecal-oral route (Reuter et al., 2009).

In urban areas in particular, rodents are attracting increasing attention as potential reservoirs of the virus. One study found high seroprevalence in commensal rodent species; the highest levels were in *R. rattus* (59.7%) and *R. norvegicus* (68.5%) (Favorov et al., 2000). A recent study in Zhanjiang, China found only rat HEV; no other mammalian genotypes were detected. The authors concluded that wild rats are exclusively susceptible to rat HEV (Li et al., 2013b). However, the susceptibility of rats to human HEV and their possible role in transmission to humans remains unknown. Furthermore, the susceptibility of humans to rat HEV is currently unknown, though experiments with rhesus monkeys have indicated that humans are not thought to be susceptible to rat HEV (Purcell et al., 2011). Nonetheless a recent study has shown that rat HEV can propagate efficiently in some human cell lines (Jirintai et al., 2014), suggesting that further study is warranted to determine the zoonotic potential of rat HEV.

Acute or chronic HEV infection is commonly identified by detection of antibodies to HEV (Reuter et al., 2009). However one study found serological assays were much more

sensitive in detecting symptomatic HEV infections rather than subclinical ones; this may be particularly problematic in countries with low rates of endemicity (Myint et al., 2006). HEV RNA can be detected in both serum and faecal samples by qPCR, which offers a greater sensitivity than serological methods (Cooper et al., 2005). However, HEV RNA may be short-lived in serum, and after the onset of symptoms may only last 10-30 days, while faecal shedding of the virus may last for up to 2-3 months (Bihl and Negro, 2010). This suggests that faecal samples may be of greater use for the detection of HEV.

1.5.15 *Lymphocytic choriomeningitis* virus

Lymphocytic choriomeningitis Virus (LCMV) is the original member of the family *Arenaviridae* (Tagliapietra et al., 2009). It is an enveloped virus with a segmented, negative-strand RNA genome about 10 kbp in length (Salvato and Shimomaye, 1989). There are considerable levels of genetic diversity within and between the three lineages of LCMV (Albariño et al., 2010), and perhaps because of this, variations in the pathogenicity of different LCMV strains have been observed (Riviere et al., 1985).

LCMV is globally distributed and can be particularly severe in congenital infections. One study reported that over one-third of infants with congenital LCMV died, and the majority of the survivors suffered severe neurodevelopmental sequelae (Wright et al., 1997). In immunocompromised adults, LCMV infections can result in severe disease and death (Ortiz-Riaño et al., 2011). Severe LCMV infection has been detected in organ transplant recipients; patients had more severe clinical features and a case mortality >90% (Fischer et al., 2006, Schafer et al., 2014).

However, in most cases of LCMV, the symptoms are mild (Peters, 2006). LCMV is the only member of the Arenaviridae family which has been detected on every continent, except Antarctica; this has been attributed to the ubiquity of its reservoir, *M. musculus*

(Howard, 1986). One epidemiological study in Madrid, Spain, reported that around 9% of wild mice can be infected with LCMV (Lledó et al., 2003). A study on wild rats in the UK found a prevalence of 25% in 35 animals sampled from livestock farms (Stuart et al., 2011). Although LCMV is the only arenavirus which has been reported in Europe, data on its incidence and epidemiological features are insufficient to determine an accurate level of prevalence (Tagliapietra et al., 2009). The same study performed in Madrid found LCMV specific antibodies in 1-2% of 400 human serum samples from both men and women who visited a hospital for reasons unrelated to infectious diseases (Lledó et al., 2003). There may also be a greater risk for those who work closely with rodents, be it in pet shops or in breeding facilities (Knust et al., 2014).

Infected populations of *M. musculus* maintain the virus via congenital transmission, though it readily infects other rodents (Cordey et al., 2011). The virus is shed in nasal secretions, saliva, milk, semen, urine and faeces (Barton and Mets, 2001). Male rodents are prone to fighting, and horizontal transmission via saliva has been known to occur (Mills et al., 1999). Humans can become infected with LCMV by inadvertently inhaling aerosolised rodent excreta (Childs et al., 1992a). Few isolates of wild rodent LCMV have been characterised, so relatively little is known about their genetic diversity (Blasdell et al., 2008).

The method of choice for detection of arenavirus infections in humans is indirect immunofluorescence tests as these are relatively simple and have a good sensitivity (Charrel and de Lamballerie, 2003). However, the host seroconversion time may prevent rapid definitive diagnosis during an epizootic. In these situations qPCR is used for the detection of LCMV (Besselsen et al., 2003). There are frequently two or more arenaviruses present in the same geographic area, and this has necessitated the development of a test that can distinguish between arenaviruses. Viral RNA can be detected in serum, plasma, urine, throat wash and other tissues in one day by qPCR

(Charrel and de Lamballerie, 2003). A qPCR assay which was developed to detect LCMV in mice was reported to be approximately 1000-fold more sensitive than a previously used method, the plaque assay (McCausland and Crotty, 2007).

1.5.16 *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular parasite, with a genome of approximately 65 Mb (Bahl et al., 2010) and is the only known species in the genus *Toxoplasma*. *T. gondii* occurs globally, and can infect virtually all mammalian and avian species. Because of the wide range of potential host species, and in the percentage of globally infected animals, *T. gondii* is considered one of the most successful eukaryotic pathogens (Su et al., 2010). Despite this, the genetic diversity of the pathogen is very low, with an identity of approximately 99% (Laliberté and Carruthers, 2008).

T. gondii is one of the most common parasites found in humans and other homeothermic animals; nearly one-third of the human population have antibodies to *T. gondii* indicating exposure at some point (Dubey, 2004). It has also been described as the most prevalent parasitic zoonotic disease in the world (Tenter et al., 2000). The symptoms of toxoplasmosis are similar to mild flu or glandular fever. Despite being the most commonly reported zoonotic parasite in humans in the EU, routine monitoring is considered inadequate; thus, the incidence of human disease and infection is likely to be underestimated (EFSA, 2007a). Prevalence can reach very high levels across species; for example, it can reach 45% in domestic and feral cats, 20-60% in rodents, 13-66% in birds and 22-84% in humans (Webster, 2001).

T. gondii has developed a broad range of potential transmission routes (Tenter et al., 2000). It can be transmitted from cats, the definitive host (in which sexual replication occurs), to intermediate hosts and vice versa (Tenter, 2009). Upon infection in any host, *T. gondii* is disseminated throughout various tissues. Tissue cysts (bradyzoites) are then

formed, which can remain active throughout life (Owen and Trees, 1998). The pathogen can be transmitted via the faecal-oral route, transplacental route or by consumption of infected tissues in all potential host species (Dubey, 2008). The faeces from an infected cat can contain millions of oocysts, which may remain viable for over a year, thereby contaminating the environment (Webster, 2001). Congenital transmission in humans can occur either through primary exposure of the female during pregnancy, or upon reactivation of a chronic infection during pregnancy; either result in infection of the foetus (Williams et al., 2005). A recent study based on mathematical modelling published in France has suggested that congential Toxoplasmosis in France is decreasing and that by 2020 it will have a seropravelence in women aged 30 of 26.5%, down from 76.3% in 1980 and 48.9% in 2000 (Nogareda et al., 2014).

Studies have found that rodents with a *T. gondii* infection lose their natural aversion to cat urine, and may in fact find the smell more appealing (Vyas et al., 2007). This would clearly aid the pathogen's transmission to its definitive host. Adult-acquired infection in immune-competent humans is likely to result in lifelong infection; complications such as chronic infections which can lead to fatal encephalitis or systemic disease may arise from the infection, and immune-compromised individuals are at greater risk of developing severe disease (Jones and Dubey, 2010). Congenital infections, which may initially be asymptomatic, tend to relapse and can result in progressive damage, most commonly to the retina and in some cases the brain (Jongert et al., 2009). Toxoplasmosis can have a high impact socially and economically, in terms of human suffering and in the cost of care of sick children (Hill and Dubey, 2002). There have also been recent studies which have found a positive correlation between *T. gondii* infection and a greater disease burden caused by other pathogens in some countries (Flegr et al., 2014). Latent *T. gondii* infection therefore could be acting as a driver of other infectious diseases. This hypothesis has not been thoroughly studied, and

therefore further work is needed to clarify and fully understand the role of *T. gondii* in other infections.

Serological assays are used primarily for the chronic detection of *T. gondii* and methods such as the Sabin Deldman dye test and immunofluorescence test are available as effective diagnostic tools to detect *T. gondii*. However, there is no gold standard test which can be applied to all possible host species (Basso et al., 2013). Using PCR as a detection tool in animals can be difficult, this is because the available sample size tends to be small, and the distribution of tissue cysts can be variable. Additionally the number of parasites affecting a particular tissue can be low (Howe et al., 1997). Direct methods such as culture or PCR are often used in potentially immune-compromised individuals or congenital infections (Wiechmann and Grupe, 2004).

1.6 DIAGNOSTIC TEST TECHNOLOGIES

1.6.1 Assay types; uses and limitations

There are roles for immunoassay methods both in identification and in seroepidemiology of emerging diseases. However, nucleic acid methods have been preferred for identification of novel pathogens for many years and particularly in virus discovery (Chiu, 2013). An immunoassay, in a very basic sense, is the hybridisation of an antibody to an antigen, which then relies on the ability of the detection system to correctly detect the resulting hybridisation (Gosling, 1990). The widespread use of enzyme immunoassays and the enzyme-linked immunosorbent assay (ELISA) is shown by the number of times it has been used as a keyword in the literature since the 1970's (Lequin, 2005).

1.6.1.1 Immuno-assays

Perhaps one of the greatest advantages of immunoassay technology is that it is so widespread, laboratories worldwide have access to the equipment necessary for the required diagnostic tests. It is also much faster than gold standard methods such as culture or virus neutralisation tests which can only be carried out with stocks of live pathogen and tissue cultures. (Safiullah et al., 2009). Some of these can be highly pathogenic and therefore they need to be carried out in specialised high bio-containment facilities which can increase costs (Paweska et al., 2008).

More recent developments in immunoassays include high-throughput approaches such as the flow-through membrane immunoassay platform. This is performed on a 96-well vacuum plate spotted with capture molcules; gold nanoparticle-labeled secondary antibodies are used to provide visible assay signals (Ramachandran et al., 2013). Used as a possible alternative to ELISA, this test offers the advantage of being more high-throughput; however it has the same issues with antibody cross-reactivity. New methods for the rapid identification of viral haemorrhagic fevers are now being developed; one study compared a multiplexed Luminex-based immunoassay to the traditionally-used ELISA for the detection of the virus (Wu et al., 2014). This study again utilised a flow-through immunoassay platform; the platform had a good sensitivity for Hantavirus (98.04%), but showed a lower specificity (66.67%-100.00%). This lower specificity is of concern, because it is important to be able to distinguish between false positives from cross-reacting antibodies, and true positives, for accurate pathogen detection.

The disadvantages in using tests such as the more widely used ELISA and other immunoassays are that to detect an immune response to a pathogen, antibodies to the antigen(s) on the pathogen must be formed. In an outbreak scenario, it may take too long for the host to sero-convert, and the pathogen may be able to spread due to slow

host seroconversion. Furthermore, in some biological situations, multiple pathogens may be present. In addition, constantly evolving viral serotypes can render antibody-based detection nearly impossible (Wang et al., 2002a). Only a single antigen is used in some tests, and if this evolves sufficiently to be undetectable by antibody-based detection, determination of the infecting serotype is unlikely (Safiullah et al., 2009).

Despite the limitations, serological methods have proven invaluable for many epidemiological investigations and can act as a complement to nucleic acid detection methods. Serology can aid in the identification of the source of infection, clarify disease epidemiology, and aid in the identification of latent infections (Meyer et al., 2014). However, this does require that properly validated assays are available which are both sensitive and specific to the pathogen of interest.

1.6.1.2 Molecular assays

Molecular techniques can have a high sensitivity and specificity, be rapid to perform, and have the potential for automation which can make standardisation easier. Molecular assays can also be of use in the identification of novel, nonculturable or very slowly growing organisms (Dong et al., 2008). A very important development in the rapid detection of microorganisms was the development of nucleic acid amplification techniques, notably PCR (Schena, 2003).

There is an increasing demand on the quality, quantity and rapidity of microbial diagnosis, and the limitations of PCR are becoming more apparent. This is especially apparent when the identification of strain or species type is required and there may only be single nucleotide polymorphisms (SNPs) or other minor sequence alterations (Sachse et al., 2005). Some PCR methods can also be complex and expensive to carry out (Safiullah et al., 2009). Novel diagnostic tools are needed that are able to accurately detect a broad range of pathogens that are both highly sensitive and specific. This could

assist public health measures by improving patient management, reducing potentially inappropriate antibiotic use, and aid infection control measures in hospitals and other health care facilities (Cannon et al., 2010). One recently developed approach combines multiplex PCR with a suspension array which utilises microscopic beads attached to a fluorescent marker, and enables the simultaneous detection of six different foodborne pathogens such as *E. coli* O157:H7 and *Salmonella* (Sun et al., 2014). The genes targeted with this approach were specific to the pathogens of interest. However, this new method has only been tested with experimental bacterial strains, and has not yet been tested with clinical samples.

Use of real-time PCR or quantitative PCR is increasingly widespread as the cost of reagents has decreased. This approach has successfully been used for the detection of *Leptospira* in human blood samples before antibody has been produced, and when there were a low number of leptospires present in the blood (Bourhy et al., 2011). However, the cost of running real-time PCR is still comparatively expensive for some laboratories and the required equipment is not always available, which limits its use. Another of the drawbacks to both standard PCR and real-time PCR is that they cannot distinguish between DNA from live or from dead cells (Elizaquível et al., 2012). This can cause false positive results in some cases, if the goal is detection of active infection. However, efforts are being made to develop PCR methods which are able to differentiate between DNA from live and dead cells (Codony et al., 2015). Kits such as the Qiagen BLU-V viability kit are available that allow this differentiation using propidium monoazide which selectively enters dead cells and upon photo-activation intercalates and binds covalently to DNA. This inhibits amplification of nucleic acid from dead cells, and allows differentiation as only nucleic acid from live cells will be amplified.

1.6.1.3 Next-generation sequencing

There are two approaches to explore selected regions of the genome with next-generation sequencing (NGS) technology. The first is by PCR, which uses multiplex primer pairs in a mixture that are combined with genomic DNA of interest in a multiplex approach to preserve DNA. The second approach involves hybridisation capture, which takes advantage of the hybridisation of DNA fragments from a whole-genome library to complementary sequences that were synthesised and combined into a mixture of probes designed with high specificity for the matching regions in the genome (Palacios et al., 2007). Both of these amplification approaches are then subjected to NGS.

The main advantage of NGS is the ability to generate large quantities of highly detailed sequence data which, in some cases, can be in excess of one billion reads of sequence per run (Vayssier-Taussat et al., 2013). Any nucleic acid in a sample will be sequenced, be it host or pathogen. This makes NGS useful in identifying pathogens as it is very sensitive and can detect pathogenic nucleic acid at very low levels. However, the sheer amount of data collected from NGS means that trained personnel, such as bioinformaticians, are required to correctly analyse the data. Data analysis pipelines are well established for microarrays, and as a result, is an easier process than analysing the data produced by NGS. Among the main disadvantages of NGS however, are the cost and the requirement for specialised laboratory equipment which many laboratories do not have the capacity to operate (Barzon et al., 2013).

Whilst the pathogens studied in this project are zoonotic, the chances of further spread between humans are limited in many cases. NGS is a useful tool for novel pathogen discovery, due to the capacity to sequence any nucleic acid within a sample, and provides a good opportunity to identify undiscovered pathogens that may pose a threat to human health (Chiu, 2013). However, while periodic sampling of animals can

lead to discovery of novel pathogens, it is too expensive to be of use as a large-scale surveillance strategy (Rosenberg, 2014).

The most commonly used NGS methods are Illumina, Roche 454 and SOLiD technology. More recent developments include Oxford Technologies Nanopore which became available in 2012 (Barba et al., 2014). The services offered by these companies are constantly improving and the Illumina HiSeq 2500 platform for example is able to sequence the full human genome in around 24 hours compared to the years it took to sequence the first human genome.

It has been suggested that NGS methods can be complementary to conventional molecular methods such as microarrays and qPCR (Barzon et al., 2013). The unbiased sequencing used in NGS is particularly useful for novel pathogen discovery in clinical samples, and as the cost of NGS is decreasing and as more laboratories have the capacity to utilise the technology, these techniques are likely to become more useful as diagnostic tools in a clinical setting. One of the most common NGS techniques used in pathogen discovery is Illumina, and an Illumina Hi-Seq2000 sequencer was recently used to successfully identify unanticipated bacterial species in ticks in France (Vayssier-Taussat et al., 2013).

1.6.2 Microarrays

1.6.2.1 Overview

DNA microarray technology is a useful tool for studying the interaction between a pathogen and it's host, and identification of multiple strains because it is able to analyse thousands of targets simultaneously (Bryant et al., 2004). Microarrays consist of a solid surface upon which a collection of probes are spotted that contain a specific DNA sequence of varying length, which can be used to hybridise with a target sequence in a sample. Similar to immunoassays, microarrays rely upon hybridisation of a labelled

target sample with a probe fixed on a solid support. The main difference between the two is that immunoassays are used to detect antibodies and the majority of microarrays are used to detect nucleic acid. Using sequence-specific probes as a principle has been used in a variety of methods including northern blot, southern blot, and in situ hybridisation. Microarrays are named because there can be many thousands of spots on a single microarray platform, and these are ordered into a collection of analytical elements configured in rows and columns (Schena, 2003). The ability to simultaneously detect multiple analytes in one sample by hybridisation between the probe and target is one of the main advantages of microarray technology (Seidel and Niessner, 2008).

Nucleic acid abundance is an important facor in the detection of pathogens, which is also reliant on the sensitivity of the probes on the array. The hybridisation between nucleic acid and sample presents a digital readout after analysis with an array reader, which is used to interpret the result. Therefore, the sensitivty of a probe directly relates to the reliability of the experiment (Russell et al., 2009). Probes on a microarray should be designed so that they are specific for their target i.e. no cross-hybridisation should occur (specificity). They should also bind efficiently to their respective targets, when the target sequence is low in abundance targets (sensitivity). Finally, probes should display a consistent hybridisation behaviour over repeated experiments (Loy and Bodrossy, 2006). Binding specificity allows the sample to be analysed semi-quantitatively. Greater specificity reduces the chance of cross hybridisation, which occurs when a target binds to, a non-specific sequence (Schena, 2003). Using short (15-30 bases) oligonucleotide sequences which act as the probes that are highly specific to one species can allow strain identification. Using longer oligonucleotides (50-70 bases) can decrease the specificity of the array, but it may allow detection of a number of related species, which may be beneficial in some circumstances, for example in detecting a novel pathogen (Wang et al., 2003b). Unwanted cross hybridisation with both long and short

oligonucleotides however, results in a background signal, termed noise, which can mask the real data. This can be tested electronically as there are numerous software programmes that allow the user to create oligonucleotides and see if they have significant homology with other species (Burton et al., 2005).

At the most basic level, there are two ways to produce a microarray: either premade nucleic acid probes are deposited on the probe surface or the probe sequences are generated in situ by oligonucleotide synthesis on the array. The laboratory protocol for probe hybridisation is further optimised and miniaturised into microarray format for a multitude of purposes (Schena, 2003). Producing a good probe set can be difficult since there are several criteria that need to be balanced when designing probe sequences for an array: these are sensitivity, specificity, noise and bias.

Microarrays are utilised for the detection of a wide range of infectious diseases. A small selection of these include the CLART® Pneumovir array, the GreeneChip and the DEFRA BioChip. The Pneumovir array detects and characterises 17 common human viruses causing respiratory infections such as influenza, adenovirus and coronavirus subtypes (Kouni et al., 2013). This array, whilst correctly identifying numerous co-infections, was unable to detect all coronaviruses present in a sample and it had not been fully validated. Another type of array has been designed with 281 markers to characterise different *Salmonella* species for traits including antimicrobial resistance and virulence. Using this array 21 strains of *S*. Typhimurium from human clinical cases were analysed and compared for similarities in pathogenicity, phages, antimicrobial resistance, mobility, serotype and metabolism (Litrup et al., 2010). Whilst useful in determining *Samonella* types, the array is limited to *Salmonella*, and other arrays must be used for different pathogens. By contrast, the GreeneChip is a pan-microbial array which detects a wide range of viruses, as well as bacteria, fungi and protozoa on the basis of conserved 16S/18S sequences (Palacios et al., 2007). It was designed to detect

thousands of different pathogens, however a limitation of the array is that the use of 16S and 18S rRNA for probe sequences makes it more difficult to identify specific bacterial, fungal or parasitic species within a sample, because the sequences within the 16S and 18S rRNA can be highly conserved across different species. Cross-hybridisation is particularly likely to happen with different bacterial species that may have a highly conserved 16S rRNA sequence. The DEFRA BioChip was designed for the identification and characterisation of around 580 viruses responsible for diseases in humans, animals, plants and fish (A. Abu-Median, personal communication). The array was successfully used during an outbreak of equine encephalosis virus in horses in Israel (Mildenberg et al., 2009). Methods including virus isolation, serology and PCR failed to identify a candidate virus. The BioChip indicated the presence of equine encephalosis virus which was later confirmed with PCR and DNA sequencing.

1.6.2.2 Array specificity, sensitivity and noise

Specificity of a probe on an array is determined from the likelihood that the probe will bind to target sequences in a sample. A major source of non-specificity is cross-hybridisation. Probes with reduced specificity will generate less accurate hybridisation signals. There is however one application where lower specificity is preferred, cross-species/strain hybridisation, where arrays designed for one species/strain are hybridised with samples derived from a related species. This type of analysis is important when screening for novel species or there is not an existing array available for a specific pathogen. In such cases, longer probes are frequently preferred (Bar-Or et al., 2007) as in general it is considered that targets with an overall sequence identity of around 70% are likely to cross-hybridise with a long cDNA probe (Russell et al., 2009, Xu et al., 2001). The probes used in this project were designed so that there was no similarity greater than 75% with any non-specific sequence.

Sensitivity on microarrays is determined by the intensity with which the probe binds to its target sequence. This affects the signal that is read from the microarray and further analysis of the data (Lemoine et al., 2009). Unlike specificity, which can be manipulated by altering the probe sequence, probe sensitivity is more complex and is reliant upon several factors including: The thermodynamic properties of the probe sequence, the availability of the target sequence under hybridisation conditions, and on the concentration of the probe on an array (Draghici et al., 2006). The hybridisation stringency is also an important factor to consider. This determines the annealing of the probe with the target sequence and is affected by the temperature and salt concentration used in the hybridisation step. At a higher stringency, hybridisation only occurs between strands with a high degree of complementarity. Lower stringency allows annealing between strands with some degree of base mismatch between the sequences. Buffers such as sodium chloride and sodium citrate (SSC), sodium dodecyl sulphate (SDS) and Denhardt's solution are used which act to optimise the interactions between the probes and target sequence, and reduces the chance of non-specific binding. Following hybridisation, the array surface needs to be washed. This is done in order to remove any non-specifically bound DNA from the probes and unbound probes. This is commonly performed by a series of wash steps which removes the non-specifically bound DNA. This is generally peformed by stringent washes being carried out first followed by lower stringent washes. The volume of wash buffer used is generally larger than that used in the hybridisation step as any impurities and contaminants that may contribute to the background noise on the array are effectively diluted and the signal caused by these is reduced (Russell et al., 2009).

Noise is the random signal variation and is often expressed as the coefficient of variation. Finally, bias is caused by probe-specific or technical effects that can cause a systematic deviation of the measurement from the true signal. Noise and bias can be

reduced by having probes of greater length and having multiple probes of the same sequence on the array (Kreil et al., 2006). Balancing these factors is far from easy and inevitably the selection of good probe sequences involves trade-offs between each of these variables.

Variability in microarray experiments can occur for several reasons. Sample processing and labelling can be variable although performing experimental replicates can reduce this. The probes on a microarray can also introduce a considerable source of variation. This type of variation can be substantially reduced by optimising the array printing and hybridisation process (Russell et al., 2009).

It is thought that over time reliable microarrays will become relatively inexpensive, similar to the way that DNA sequencing changed from a largely in-house technique to a commercial technique. However, there has been some speculation that the next generation of high-throughput sequencing technologies will replace microarray-based analysis (Everett et al., 2010). Considered below are some advantages and disadvantages of using microarrays for pathogen detection.

1.6.3 Microarray advantages

Microarray technology has many potential applications which can include: genotyping and determination of disease-relevant genes, mutation analysis, screening of SNPs, and detection of food-borne pathogenic bacteria, viruses and parasites (Kumar, 2009). Another advantage is that when performing large scale screening one may be able to identify co-infections, which may not be possible with other methods such as PCR (Cannon et al., 2010). Microarray technology can detect novel genes in a sequence and from this, and past sequences, an evolutionary trend can be observed that may lead to alterations in the pathogenicity (Kadlec et al., 2009). Detecting emerging pathogens could become an easier prospect with the advances in molecular testing, and could

result in a quick and accurate diagnosis (Dong et al., 2008). When rapid diagnosis is required, sequence-independent PCR followed by hybridisation with microarrays offers a particularly effective approach for detection, generating results in 2-6 hours once arrays are optimised (Uttamchandani et al., 2008). This may be of tremendous use in an outbreak scenario, when rapid point of care diagnostics are required (Seidel and Niessner, 2008). The use of arrays in gene transcription studies has indicated that they are semi-quantitative in nature and there has been good correlation between results by qPCR and arrays (Kouni et al., 2013, Martínez et al., 2014)

1.6.4 Microarray disadvantages

Some criticisms of microarray technology are that; the fluorescence based glass slide arrays are relatively expensive and limited to laboratory use. An alternative to the glass slide microarray is the ArrayTube™ (AT) platform, which reduces costs and can be used with less specialised equipment (Ehricht et al., 2006). The AT platform consists of a small array surface placed on the bottom of a plastic vial. Hybridisation signals are detected by an enzyme-catalysed precipitation reaction (Müller et al., 2009). Probe selection can be a complicated and time consuming process and, because of the vast number of organisms in nature, it can be difficult to design an oligonucleotide sequence that is specific to just one (Watson et al., 2007). As the number of databases that hold sequence information for these species increases, it is easier to design probes that are specific to individual organisms (Kumar, 2009). The hybridisation process in a microarray experiment can be affected by multiple factors and optimisation of the protocol needs to take sequence composition, target preparation, probe length, and hybridisation temperature amongst others into account (Schena, 2003). The variation observed during hybridisation can be assessed by using replicates of the same assay. Another challenge in microarray experiments is the presence of host nucleic acid in a

sample, which can lower the sensitivity. A general approach to improve the sensitivity of the array may be the removal of host nucleic acid i.e. enriching pathogen-derived nucleic acid using specified methods and kits prior to PCR amplification (Gurrala et al., 2009).

1.7 CONCLUSION

It has been suggested that a decrease in the number of cases of some infectious diseases led to a sense of complacency, which resulted in a weaker disease surveillance network (Berkelman and Hughes, 1993). As pathogens continue to adapt to the changing conditions in human society and the environment, for example increasing urbanisation, humans remain at risk to a wide variety of threats in the form of emerging, re-emerging and drug-resistant infections (Berkelman et al., 1994). While surveillance is vital in measuring trends in infectious disease prevalence, it is relatively poorly supported worldwide, with inadequate training and resources being devoted to it (McNabb, 2010).

As the speed at which travel and trade increases, events at a local level can quickly acquire global significance and reports of disease outbreaks can be rapidly disseminated thanks to improvements in communication technology. However, this can prove to be a double-edged sword as rumours that may later prove to be false, could lead to a response that could cause a disruption in travel and trade and economic losses to affected countries. This highlights the necessity for a reliable, fast and accurate surveillance system (Grein et al., 2000). Infectious disease control and infection can be best addressed by maintaining strong surveillance systems and by international cooperation, as pathogens do not respect national borders (Heymann and Rodier, 1998). Among governments worldwide, it has been recognised that there are mutual benefits to global surveillance (McNabb, 2010).

Currently there is no EU level co-ordination of wildlife disease surveillance, which is surprising given the importance of zoonotic diseases, with approximately 861 human pathogens zoonotic in origin (Taylor et al., 2001). There is also a lack of diagnostic tests available for large scale screening of samples for multiple pathogens. In wildlife, it is common to have co-infections and this can cause differing symptoms compared to when a single pathogen is present. Long term proactive wildlife population and disease monitoring is vital to provide baseline data for epidemiological analyses and design of surveillance programmes, and is lacking in many EU countries (Böhm et al., 2006). A coordinated EU surveillance strategy coupled with risk analysis that uses new technologies to better identify, characterise and quantify current and future disease threats within and without the EU are needed (Hutchings, 2006).

The EU-funded Novel Technologies for Surveillance of Emerging and Re-emerging Infections of Wildlife (WildTech) project (http://www.wildtechproject.com/wildtech/), of which this work was part, was established to create a technology centre that could be used in Europe and elsewhere as a basis for high throughput disease diagnosis in wildlife.

1.8 AIMS

The principle aim of this project was to design and develop a diagnostic tool, which was then used to screen for a variety of pathogens of zoonotic importance originating from rodents, using samples from within and outside of Europe. The design and evaluation of the microarray used to screen the samples was a major theme in this work. This information should allow a better understanding of the challenges faced in microarray design and on the prevalence of zoonotic pathogens in urban rodent populations.

1.9 OBJECTIVES

The objectives of this study were to:

1. Design and evaluate primers and probes which would be used on the microarrays to screen rodent samples.
2. Screen a large number of rodent kidney, liver and lung samples for zoonotic pathogens using microarrays.
3. Perform confirmatory tests, using previously validated methods, on all array positive results.

CHAPTER TWO

EXPERIMENTAL DESIGN

2.1 PROBE DESIGN

For each pathogen a literature review was carried out to identify genes of interest for which to design unique probes. Multiple probes were then designed to target these identified regions of the genome for each pathogen. Once a potential target gene was identified, the FASTA sequence for each target gene was aligned with genes from similar species to identify the most conserved regions.

60-mer oligonucleotides were chosen, which offer a good compromise between sensitivity and specificity and are regularly used on different microarray platforms (Russell et al., 2009, Wang et al., 2003a). Oligonucleotide probe lengths commonly vary from 25-mer to 70-mer (Wang et al., 2003a, Tiquia et al., 2004). In order to achieve specificity comparable to other molecular techniques, such as PCR, non-target transcripts must have an identity <75% in order to prevent significant cross-hybridisation. In addition, stretches of contiguous complementary sequence (100% similarity) >15 bp must be avoided (Kane et al., 2000). The algorithms used in probe design have been written to design probes without these contiguous sequences. Probes were designed in several ways:

- A visual inspection of a gene alignment produced in Clustal X2 from multiple isolates was used to identify some probes. 60-mer nucleotide sequences were then selected for BLAST analysis from within these regions for use as probes. This is explained in further detail in Chapter 3.
- In addition to visual inspection and design of probes, two free software packages were also used for probe design:

- Unique Probe Selector (UPS) (http://array.iis.sinica.edu.tw/ups/) (Chen et al., 2010).
- OligoWiz (http://www.cbs.dtu.dk/services/OligoWiz/) (Wernersson and Nielsen, 2005, Wernersson et al., 2007).

UPS is a programme that uses an algorithm which includes parameters such as GC content, the secondary structure of probes, and a low-complexity mask to produce a probe sequence. These parameters all contribute to the design of probes that are specific to the sequence of interest, reducing the chance of cross-hybridisation or background noise. Probe candidates that were designed after these parameters had been run were ranked by E-value of the probe-target duplex and presented in a FASTA output (Chen et al., 2010).

OligoWiz is a graphical user interface which the user interacts with. OligoWiz uses a scoring-scheme that works as follows: At each position along the input sequence, the suitability of placing a probe is evaluated according to five criteria. These include cross-hybridisation, melting temperature, folding (self-annealing), position and low complexity. Each individual score ranges from 0.0 (poorly suited) to 1.0 (well suited). The individual scores are combined to form a total score, which is used for the selection of the probes (Wernersson and Nielsen, 2005, Wernersson et al., 2007). The weight of these criteria can be modified by the user if, for example, the melting temperature is the most important factor to consider. OligoWiz has a database of sequences which can be compared against the uploaded sequence to determine the suitability of the probe and to predict if there will be any cross-hybridisation. During the design stage the default settings used were for Prokaryotic long-mers (45-55). Other options include long-mers for eukaryotic organisms and short-mers (24-26) for both eukaryotic and prokaryotic organisms. The settings for probe length were modified from the default settings so that the optimal oligonucleotide length was set at 60 bp, with the minimum

and maximum lengths set at 57 and 60 bp respectively. The default OligoWiz setting was used for Tm and Cross-hybridisation. The probe position was changed from the default Poly-T primed to Random primed as Poly-T primed is used for eukaryotic organisms. Other options for position included a 5' preference, a 3' preference and Mid preference. A cut-off point of 0.8 was used to score probes. Probes that have a total score above this figure have been found to hybridise with greater signal intensity than probes which scored 0.75 and below (Ermini et al., 2011). This score was used as a threshold so that the most effective probes would be designed by the software.

At present, this database does not include any viruses, so OligoWiz was not used when designing probes for the virus species. UPS and manual inspection of the aligned sequences were used to design the virus probes. Probes for bacteria and parasites were designed using all three methods.

In addition to *in silico* analyses performed by UPS and OligoWiz during the probe design process, a further confirmation by BLAST analysis was carried out on all the probes to determine if there was any predicted cross-hybridisation with any other sequences on the NCBI database. The selected probe sequences were synthesised commercially by Metabion International (Jena, Germany) each at 15 µM with an NH_2 modification at the 3' end, no modification at the 5' end, purification with HPLC at 0.004 µmole scale and absolutely biotin-free for subsequent spotting on the arrays.

2.2 PRIMER DESIGN

Oligonucleotide primers were designed using Primer3 (http://frodo.wi.mit.edu/). The amplicon size was set between 250 and 750 bases with an optimum of 500 bases. Primers were designed to flank the sequences for which probes had been designed. Primers were synthesised desalted at a scale of 0.05 µmoles. A subset of primers were synthesised with biotin labelling at their 5' end to determine how effective biotinylated

primers would be when used in combination with microarrays. The use of biotinylated primers would negate the need for a biotin-labelling step and thus would decrease the overall laboratory time required. Table 2.1 shows the primers designed in the project.

2.3 MICROARRAY FABRICATION

The synthesised probes were spotted by touch printing onto arrays by Alere Technologies GmbH (Jena, Germany). A number of probes were initially spotted onto the ArrayTube format and additional probes were then spotted on the ArrayStrips at a later date (See Chapter 3). For each array, the probes were printed in duplicate and there were also spots used for biotin markers (positive assay control) and spotting buffers (negative control). The probe position was not randomised during printing; instead probes for each pathogen were clustered together by Alere. The ArrayTube has a 3 x 3 mm chip fitted at the bottom of a 1.5 ml micro-reaction tube and accommodates up to 225 spots, whereas a single ArrayStrip is made up of eight individual wells with a 4 x 4 mm chip at the bottom of each well which accommodates over 600 spots. The ArrayStrip was used for screening samples as there were a larger number of probes so a larger number of pathogens could be screened for. An additional advantage in using the ArrayStrip was that 12 strips could be assembled into a microplate frame which allowed the parallel testing of up to 96 samples.

The concentration of each spot was 15 µM. Figure 2.1 shows an example of an ArrayTube layout for *T. gondii*. Three ArrayTube types were produced: Hanta_HEV_01, Yersinia_01 and Toxoplasma_01. The ArrayTube Hanta_HEV_01 had probes specific for HEV, SEOV, PUUV, as well as a set of more generic probes to detect multiple hantavirus species. The ArrayTube Yersinia_01 had probes for *Y. pestis, Y. enterocolitica, Y. pseudotuberculosis* and a set of generic probes to detect multiple *Yersinia* types. The third ArrayTube, Toxoplasma_01, only had probes for *T. gondii*. The other pathogens

had not been added to the project at this point so these were not included on the ArrayTubes. A pilot ArrayStrip (WT_Rodent_Chip_02) was synthesised after the ArrayTubes had been tested, and used for optimisation procedures before the final screening chip was synthesised. This ArrayStrip had probes for all pathogens listed in Table 2.1 except *Mycobacteria* and *Fransicella*. The final screening chip (Rodent_Chip_03) also included probes for *Mycobateria* and *Francisella* species, which had been designed and tested in a parallel project in the same laboratory. The probes for these pathogens had already been tested in ArrayTubes, during cross-hybridisation testing. PCR products from reference material from both these genera (*M. bovis*, *M. avium* subspecies *paratuberculosis* and *F. tularensis* subspecies *tularensis*) were hybridised with all three ArrayTubes and the ArrayStrip during the evaluation stage.

		87	88	89	90	91	92	93	94	95	96	97		
			77	78	79	80	81	82	83	84	85	86		
40			67	68	69	70	71	72	73	74	75	76		
41			57	58	59	60	61	62	63	64	65	66		
42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
92	93	94	95	96	97	1	2	3	4	5	6	7	8	9
77	78	79	80	81	82	83	84	85	86	87	88	89	90	91
62	63	64	65	66	67	68	69	70	71	72	73	74	75	76
47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
30			21	22	23	24	25	26	27	28	29			46
			12	13	14	15	16	17	18	19	20			
		1	2	3	4	5	6	7	8	9	10	11		

Figure 2.1. A colour coded layout of the ArrayTube Toxoplasma_01.
Each numbered spot was printed in duplicate. Spots 1-95 show probes for *T. gondii*, and spots 96 and 97 were the spotting buffer and biotin markers, respectively. The black areas were the orientation markers.

Table 2.1. Primers used in this study

Pathogen	Primer name	Primer sequence (5'-3')	Target region	Melting temperature (°C)	GC content (%)	Amplicon size (bp)	Accession number	Reference
Y. enterocolitica	Y.ent/YE4072/4447336/F	AATTGCAACAATTGGCACCT	YE4072	60.37	40	499	AM286415.1	This study
	Y.ent/YE4072/4447834/R	ATATCTGCTTGTGGGCCAGT		59.58	50			
Y. enterocolitica	Y.ent/YE4078/4456680/F	TTTCTGGCCTTTCTGCTGTT	YE4078	59.99	45	510	AM286415.1	This study
	Y.ent/YE4078/4457189/R	AGATGCTGGGAATGTCGTGT		60.54	50			
Y. pseudotuberculosis	Y.pse/YPTB0154/178134/F	GGTGTTTGGGCCAGAGATAA	YPTB0154	59.93	50	303	CP001048.1	This study
	Y.pse/YPTB0154/178436/R	AGATTGCGTGATGCATCCTT		60.63	45			
Y. pestis	Y.pes/PcP/8532/F	ATCTTACTTTCCGTGAGAAG	pPcP	55.00	40	480	CP000310	This study
	Y.pes/PcP/9011/R	CTTGGATGTTGAGCTTCCTA		58.00	45			
Y. pestis	Y.pes/pPCP/8374/F	CCCGAAAGGAGTGCGGGTAA	pPCP	66.51	60	529	CP000310	This study
	Y.pes/pPCP/8902/R	CGCCCCGTCATTATGGTGAA		66.11	55			
Generic *Yersinia*	Y.gen/YOPH/62151/F	AAAGCCATTTCCGTATGCTG	YOPH	60.10	45	514	CP002182.1	This study
	Y.gen/YOPH/62664/R	TGTACTCGCATTTGGCTGAC		59.87	50			
T. gondii	T.gon/B1/12/F	CTCCGTCGTCCGTCGTAATA	B1	61.04	55	499	EU340880.1	This study
	T.gon/B1/510/R	TCGACAATACGCTGCTTGAA		60.54	45			
T. gondii	T.gon/SAG2/10/F	TCAAAGACCACGAGCCTAGC	SAG2	60.54	55	491	AY187279.1	This study
	T.gon/SAG2/500/R	ACAGGGTCAAACCCAGATGT		59.28	50			
T. gondii	T.gon/BSR4/96/F	CAGTGGAGCAGTTGTTGCAT	BSR4	59.90	50	447	XM_002371647.1	This study
	T.gon/BSR4/542/R	CATCCGACGACGAATGATTT		60.86	45			
T. gondii	T.gon/GRA6/372/F	GTAGCGTGCTTGTTGGCGAC	GRA6	61.00	60	790	XM_002371898.1	This study
	T.gon/GRA6/1162/R	TACAAGACATAGAGTGCCCC		54.00	50			
T. gondii	T.gon/ROP16/440/F	GATGGTTTCCAACAGAGTCAA	ROP16	57.63	43	476	GQ249090.1	This study
	T.gon/ROP16/915/R	CAGCTGCTGAACTTCTTCCA		59.31	50			

C. jejuni	Camp/hipO/1662/F	ACTTCTTTATTGCTTGCTGC	*HipO*	53.00	40	323	Z36940	(Wang et al., 2002b)
	Camp/hipO/1984/R	GCCACAACAAGTAAAGAAGC		54.00	45			
C. jejuni	Camp/tonB1/1273/F	AGCCACAGAAAGCAAATGGA	tonB1	56.00	45	550	AB562904	(Cornelius et al., 2010, Rozynek et al., 2005)
	Camp/tonB1/1822/R	TGCTTGAGTTGCGCTAGTTG		57.00	50			
C. jejuni	Camp/flgS/776650/F	CTTGCAAGCATGGGTAGTGT	*flgS*	57.00	50	404	CP001960	(Cornelius et al., 2010, Wösten et al., 2004)
	Camp/flgS/777053/R	GGCTTTTCACATTCGCTTTC		54.00	45			
C. jejuni	Camp/neuA3/1316412/F	CGCAAGTGTAGCCCAAAAAT	*neuA3*	55.00	45	447	CP001960	(Cornelius et al., 2010, Guerry et al., 1996)
	Camp/neuA3/1316858/R	TCCATTACAAAAAGCCCTGTG		55.00	43			
Cowpox	Cow/HA/178301/F	ATGACACGATTGCCAATACTTC	HA	55.00	41	629	HQ420900	(Stephenson and Warnes, 1998)
	Cow/HA/178929/R	CTTACTGTAGTGTATGAGACAGC		54.00	43			
LCMV	LCMV/S/1742/F	ATGATGCAGTCCATGAGTGCACA	S segment	60.00	48	650	AB627951	This study
	LCMV/S/2391/R	TCAGGTGAAGGATGGCCATACAT		59.00	48			
LCMV	LCMV/NP/779/F	CCTGGGAAAACCACTGCACA	Nucleoprotein	64.35	55	670	DQ361065	This study
	LCMV/NP/1448/R	TTGACAAGGTGCAGGAAGATGC		64.75	50			
Leptospira	Lept/hemo/3184769/F	ACGCGGATCCTTCTACTCCT	Haemolysin	58.00	55	477	AE016823.1	This study
	Lept/hemo/3185246/R	GGCTGATCGAATCTTTCCAA		54.00	45			
S. Typhimurium	Salm/CDP/2167279/F	CCAGCACCAGTTCCAACTTGATAC	CDP	59.00	50	663	CP003386	(Lim et al., 2003)
	Salm/CDP/2005357/R	GGCTTCCGGCTTTATTGGTAAGCA		61.00	50			
Generic *Salmonella*	Salm/flag/1366055/F	GGTAGAAATTCCCAGCGGGTACTGG	Flagellin	62.00	56	428	CP003386	(Lim et al., 2003)
	Salm/flag/1366482/R	GCCAACCATTGCTAAATTGGCGCA		63.00	50			
E. coli	E.coliStxF	CCTGTGAGCTATACGGAAAGTAC	*stx*	55.00	49	629	BA000007	(David Gally and Sean McAteer Personal Communication 2013)
	E.coliStxR	TCAGATGCCATTCTGGCAACTCG		60.00	52			
SEOV	SEOV/S/472/F	AGAGGCAGGCAGACTTCAAA	S segment	56.00	50	499	KC626089	This study

	SEOV/S/971/R	CCAGCAAACACCCATATTGA		53.00	45			
PUUV	PUUV/S/29/F	CGAGAAAGACTGGAATGAGTGA	S segment	45.45	55	446	HE801633	This study
	PUUV/S/475/R	CACGCGTTGAAAGCATGTA		47.37	54			
Generic hantavirus	HAN-L-F1	ATGTAYGTBAGTGCWGATGC	L segment	49.00	47	412	DQ268652	(Klempa et al., 2006)
	HAN-L-R1	AACCADTCWGTYCCRTCATC		47.00	50			
HEV	HEV/ORF1/4109/F	GTTGAGGCCATGGTGGAG	ORF1	55.00	61	615	KC618403	This study
	HEV/ORF1/4724/R	AGAGGACCACCGAATCATCA		55.00	50			
Bartonella	Bart/gltA/362/F	GGGGACCAGCTCATGGTGG	*gltA*	60.00	68	378	HG969191	This study
	Bart/gltA/740/R	AATGCAAAAAGAACAGTAAACA		50.00	28			
MRSA	MRSA/mecA/2343/F	GGCCAATACAGGAACAGCAT	*mecA*	55.00	50	582	AB781450	This study
	MRSA/mecA/2925/R	TTCACCTGTTTGAGGGTTGA		54.00	45			
Rickettsia	Rick/17kD/1047487/F	GCTCTTGCAGCTTCTATGTT	17kD	45.00	53	434	CP003397	This study
	Rick/17kD/1047921/R	CCGCCAACCTGACGGGCAATGG		65.00	68			
S. monilliformis	S.monil/asp/F	ACAGGAACTCTTACACATGTTGC	*asp*	56.00	43	499	CP001779	This study
	S.monil/asp/R	GACTTTGCCCTATTTTCAGCA		54.00	42			
Francisella	FT RD-F	TTTATATAGGTAAATGTTTTACCTGTACCA	RD1	62.60	26	1521	CP010290	(Broekhuijsen et al., 2003)
	FT RD-R	GCCGAGTTTGATGCTGAAAA		56.40	45			
Mycobacteria	TB 16S-F	TGCACTTCGGGATAAGCCTG	16S	60.50	55	1055	CP009427	(Abu-Bakr Abu-Median Personal Communication 2013)
	TB 16S-R	TAGCATGTGTGAAGCCCTGG		60.50	55			

The selected pathogens, and the source of nucleic acids used in the evaluation steps are listed in Table 2.2. An in-depth literature search on the genomes, and methods for diagnosis of these pathogens was conducted, which aided identification of appropriate regions of the genome of each pathogen. These regions were frequently whole genes, including virulence genes, antibiotic resistance genes and species-specific genes. Where available the strain information has been given. However, for MRSA and *M. bovis* the strain details were not supplied. The only information that was received was that the samples were from chromosomal DNA.

Table 2.2. A list of all the pathogens and reference material, where available, that were selected for inclusion on the array.
** indicates samples for which further information regarding strain identify was not made available.

Pathogen	Source	Sample type
Yersinia pestis	Konrad Sachse (FLI)	*Y. pestis* NCTC5923 Type strain DNA
Yersinia enterocolitica	Brendan Wren (LSHTM)	*Y. enterocolitica* 8081 DNA
Yersinia pseudotuberculosis	Karen Kempsall (PHE)	*Y. pseudotuberculosis* YpIII DNA
Leptospira interrogans	Zoree Djelouadji (VetAgro Sup Campus Veterinaire de Lyon)	DNA from culture and clinically infected animals *L. interrogans* Icterohaemorrhagiae RGA *L. interrogans* Icterohaemorrhagiae M20
Rickettsia typhi	Unavailable	N/A
Bartonella spp	Unavailable	N/A
Streptobacillus monilliformis	Unavailable	N/A
Campylobacter jejuni	Michael Jones (UNott)	*C. jejuni* strain NCTC11168t DNA
E. coli	Sean McAteer, David Gally (UEd)	Stx negative TUV93-0 DNA Stx positive Sakai DNA
Salmonella enterica	Paul Barrow, Margaret Lovell (UNott)	DNA from culture *S.* Typhimurium LT2 *S.* Enteritidis P125109 (PT4)
Methicillin resistant *Staphylococcus aureus*	HPA	Chromosomal DNA**
Hepatitis E Virus	Sylvia Grierson (AHVLA) Nicole Pavio (UMR 1161 ANSES-INRA-ENVA Virologie) Charalambos Billinis (University of Thessaly)	RNA from clinical samples Genotype 3e and 3f
Seoul hantavirus	EVA	RNA of strain R22VP30 from infected *R. norvegicus*
Puumala hantavirus	EVA	RNA of strain CG18-20 from infected *Myodes glareolus*
Cowpox virus	EVA	RNA of strain Compiegne 2009 isolate KP/LP from culture
Lymphocytic choriomeningitis virus	EVA	Entry clone LCMV L1 domain (EVA catalogue number 231) DNA
Toxoplasma gondii	Fimme van der Wal (CVI)	*T. gondii* RH-type DNA
M. bovis	Konrad Sachse (FLI)	Chromosomal DNA**

Mycobacterium avium subspecies *paratuberculosis*	Konrad Sachse (FLI)	44133 chromosomal DNA
F. tularensis subspecies *tularensis*	Konrad Sachse (FLI)	*F. tularensis* ATCC 6223 chromosomal DNA

Institute Abbreviations

Friedrich-Loeffler-Institute (FLI)
London School of Hygiene and Tropical Medicine (LSHTM)
Public Health England (PHE)
The University of Nottingham (UNott)
The University of Edinburgh (UEd)
European Virus Archive (EVA)
Central Veterinary Institute (CVI)

2.4 SEQUENCE-INDEPENDENT AMPLIFICATION

The goal of sequence-independent PCR amplification is to randomly amplify any nucleic acid present in a sample (Froussard, 1992). If RNA is present in a sample, then reverse-transcription must be carried out first so that a cDNA product is obtained, which can be used for subsequent testing. The protocol consists of two sets of enzymatic reactions. In Round A, Sequenase or Klenow polymerase (Promega) was used to extend randomly annealed primer A (a tagged pentadecamer random primer) to generate templates for subsequent PCR. During Round B, the specific primer B (with a tag sequence identical to that of primer A) was used to amplify the templates previously generated. A third reaction step was carried out for biotin-labelling of the product from Round B. The concentration of nucleic acid in each of the samples was measured spectrophotometrically using a Thermo Scientific^{TM} NanoDrop8000.

2.4.1 Round A

2.4.1.1 Reverse Transcription of RNA random priming and primer extension

Any RNA present in the sample was reverse transcribed into cDNA with 1.0 µl of primer A (GTT TCC CAG TCA CGA TCN NNN NNN NNN NNN NN) (40 µM), 1.0 µl of 10mM dNTP mix (Invitrogen), and variable amounts of water and template (minimum 50 ng/µl) were mixed in a PCR tube to a total volume of 13µl. The volume of water was variable to allow for different concentrations of template. This was then heated to 65°C for five min using a thermal cycler. The mixture was placed on ice for at least one minute. A separate mixture containing 4.0 µl of 5x Reverse Transcriptase Buffer (Invitrogen), 1.0 µl of 0.1M Dithiothreitol (DTT) (Invitrogen), 1.0 µl of RNase inhibitor, RNaseOUT (Invitrogen), and 1.0 µl of SuperScript III Reverse Transcriptase (Invitrogen) was added to the PCR tube contents, and mixed by pipetting. The 20 µl reaction was incubated using a thermal cycler at 25°C for five min, then at 50°C for one hour and finally at 70°C

for 15 min to inactivate the reaction. The reaction was left at room temperature for five min, followed by one minute on ice. The mix was then heated to 94 °C for two min, and rapidly cooled to 10°C in the thermal cycler for five min. 10 µl of Klenow mix (1.0 µl 10x Klenow buffer (Promega), 8.7 µl water, 0.3 µl Klenow polymerase (Promega)) was then added. The mixture was gradually heated to 37°C over 8 min, and then held at 37°C for a further 8 min. This was followed by a rapid increase to 94°C for two min after which the mix was cooled to 10°C for five min, during which 1.2 µl of diluted Klenow (1:4) was added. The temperature was again increased to 37°C over 8 minfollowed by a hold of 8 min at 37°C, and then the reaction was terminated by putting the mixture on ice for 5 min. The product could then either be kept at -20°C or used immediately in Round B.

2.4.1.2 Round A (for DNA)

Primer extension was effected with 1.0 µl Primer A, 1.0 µl 10x Klenow buffer and variable amounts of water and template (minimum 50ng/µl) to make a total volume of 10 µl. The volume of water was variable to allow for different concentrations of template. This sample mixture was then heated to 94°C for two min and then allowed to cool to 10°C in a thermal cycler for five min. The following 5.05 µl reaction mix was added to the sample mixture during its incubation at 10°C: 0.5 µl 10x Klenow buffer, 1.5 µl 3mM dNTPs, 0.75 µl 0.1M DTT, 1.5 µl 500 µg/ml BSA, 0.3 µl Klenow polymerase, 0.5 µl water. The mix was thensubjected to the same conditions as for RNA/cDNA with Klenow polymerase detailed in 2.4.1.1.

2.4.2 Round B

Standard PCR was then conducted using Primer B (GTT TCC CAG TCA CGA TC) (100 µM) to amplify the round A product, using the protocol shown in Table 2.3. A 50 µl reaction mix was made from the following: 39.0 µl water, 1.5 µl 50mM Magnesium chloride ($MgCl_2$) (Invitrogen), 5.0 µl 10x Mg-free buffer (Invitrogen), 0.5 µl 25mM dNTP mix, 0.5 µl Primer B, 0.5 µl *Taq* polymerase (5 U/µl) (Invitrogen), 3.0 µl Round A product.

Table 2.3. Round B PCR protocol

Step	Temperature(°C)	Time	
Initial denaturation	94°C	10 s	
Denaturation	94°C	30 s	35 cycles
Annealing	50°C	30 s	
Extension	72°C	2 min	
Final extension	72°C	2 min	
Final hold	4°C	Indefinitely	

2.5 SEQUENCE-SPECIFIC AMPLIFICATION

A sequence-specific PCR was performed using a 50-µl reaction containing 35.5 µl of nuclease-free water, 4.0 µl of 25mM $MgCl_2$ (Invitrogen), 5.0 µl of 10x Mg-free Buffer, 1.0 µl of 25mM dNTP mix, 1.0 µl of 10µM forward primer, 1.0 µl of 10µM reverse primer, 0.5 µl of *Taq* DNA polymerase (5 U/µl) and 2.0 µl of cDNA or DNA (optimal concentration 50ng/µl). Amplification was performed using the protocol listed below in Table 2.4. The primers were either designed using dedicated primer design software (Primer3) or were taken from the literature.

Table 2.4. Sequence-specific PCR protocol

Step	Temperature(°C)	Time	
Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	30 cycles
Annealing	60°C	60 s	
Extension	72°C	60 s	
Final extension	72°C	10 min	
Final hold	4°C	Indefinitely	

2.5.1 Multiplex PCR

A specially formulated multiplex PCR kit (Qiagen® Multiplex PCR *Plus*) was trialled during the project. The 50 µl reaction mix contained 25 µl 2x Multiplex PCR Master Mix, 5 µl 10x primer mix (2 µM each primer), and variable volumes of water and template (which was dependent on the concentration of the template). The primer mix (Table 2.5) contained 10 µl of each primer from 100 µM stock solutions. After 10 µl of each primer was added, the total volume was brought up to 500 µl with water. A multiplex PCR approach with a primer mix containing 24 primer pairs (Table 2.5). Each of the primers was biotinylated at the 5' end. The protocol used for this work is shown in Table 2.6. An aliquot of extracted nucleic acid was taken from a sample of rodent liver, lung and kidney tissue. This was then pooled to get a single sample. For the multiplex test, 20 µl of pooled rodent nucleic acid was spiked with 5 µl of the pathogen mix (Table 2.7), which contained nucleic acid from each of the reference samples of pathogens which was available.

Table 2.5. Multiplex PCR primer

Pathogen	Primer	Pathogen	Primer
Y. enterocolitica	Y.ent/YE4072/4447336/F Y.ent/YE4072/4447834/R	*E. coli*	E.coliStxF E.coliStxR
Y. pseudotuberculosis	Y.pse/YPTB0154/178134/F Y.pse/YPTB0154/178436/R	SEOV	SEOV/S/472/F SEOV/S/971/R
Y. pestis	Y.pes/pPCP/8374/F Y.pes/pPCP/8902/R	PUUV	PUUV/S/29/F PUUV/S/475/R
Yersinia spp.	Y.gen/YOPH/62151/F Y.gen/YOPH/62664/R	Hantavirus	HAN-L-F1 HAN-L-R1
T. gondii	T.gon/B1/12/F T.gon/B1/510/R	HEV	HEV/ORF1/4109/F HEV/ORF1/4724/R
C. jejuni	Camp/flgS/776650/F Camp/flgS/777053/R	Bartonella	Bart/gltA/362/F Bart/gltA/740/R
Cowpox	Cow/HA/178301/F Cow/HA/178929/R	MRSA	MRSA/mecA/2343/F MRSA/mecA/2925/R
LCMV	LCMV/NP/779/F LCMV/NP/1448/R	*Rickettsia*	Rick/17kD/1047487/F Rick/17kD/1047921/R
Leptospira	Lept/sphH/3501394/F Lept/sphH/3501818/R	*S. monilliformis*	S.monil/asp/F S.monil/asp/R
Leptospira	Lept/hemo/3184769/F Lept/hemo/3185246/R	*T. spiralis*	FMSSU1 RMLSU1
S. Typhimurium	Salm/CDP/2167279/F Salm/CDP/2005357/R	*Mycobacterium*	TB 16S-F TB 16S-R
Salmonella spp.	Salm/flag/1366055/F Salm/flag/1366482/R	*Francsiella*	FT RD-F FT RD-R

Table 2.6. Multiplex PCR protocol

Step	Temperature(°C)	Time	
Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	40 cycles
Annealing	60°C	60 s	
Extension	72°C	60 s	
Final extension	72°C	10 min	
Final hold	4°C	Indefinitely	

Table 2.7. Pathogen mix for multiplex PCR

Pathogen	Volume (µl)	Concentration (ng/ µl)	Copy number
LCMV	9	1.66	$2.33x10^{9}$
T. gondii	9	10.78	$1.97x10^{10}$
C. jejuni	2	84.55	$1.91x10^{11}$
S. Typhimurium	6	1.89	$2.6x10^{9}$
S. Enteritidis	4	8.76	$1.87x10^{10}$
CPXV	2	35.17	$5.1x10^{10}$
E. coli	2	38.12	$5.35x10^{10}$
Y. pseudotuberculosis	2	78.94	$2.38x10^{11}$
Y. enterocolitica	2	112.5	$2.06x10^{11}$
Y. pestis	2	61.97	$1.07x10^{11}$
Leptospira	2	62.34	$1.19x10^{11}$

2.6 AGAROSE GEL ELECTROPHORESIS

The presence and size of the amplified products was assessed using electrophoresis on 1% (w/v) agarose gels stained with either ethidium bromide or Nancy-520 for a final concentration of 0.5 µg/mL. 5 µl of each reaction (including a non-template control) was mixed with 1 µl of 6x loading dye (New England Biolabs, Ipswich, MA) (2.5% Ficoll-400, 11 mM EDTA, 3.3 mM Tris-HCL, 0.017% SDS and 0.015% bromophenol blue) and 5 µl of each mixture was added into the wells. Samples were run at 80 V for 65 min alongside a 5 µl 1 kbp DNA ladder (New England Biolabs, Ipswich MA) to visualise amplicon sizes. Gel images were captured on an IQuant Capture 300 system.

2.7 LABELLING PCR

Direct biotin labelling of the product produced by non-biotinylated primers was performed by an additional PCR step. A 50 µl reaction was prepared as follows: 32.5 µl of nuclease-free water, 1.5 µl of 50mM $MgCl_2$, 5.0 µl 10x Mg-free buffer, 5.0 µl of 10x Biotin labelling mix (for a 100 µl total: 1µl dATP, 1 µl dGTP, 1 µl dCTP, 0.65 µl dTTP, 35 µl Biotin-dUTP, 61.35 µl water), 0.5 µl of 100µM Primer B, 0.5 µl of *Taq* DNA polymerase and 5.0 µl template/Round B product. Labelling was only required for the singleplex sequence-specific and sequence-independent amplification methods and not for the multiplex approach as this already used biotinylated probes. The protocol can be seen in Table 2.8.

Table 2.8. Protocol for labelling of sequence-specific and sequence-independent PCR products

Step	Temperature(°C)	Time
Initial denaturation	94°C	10 s
Denaturation	94°C	30 s
Annealing	50°C	30 s
Extension	72°C	2 min
Final extension	72°C	2 min
Final hold	4°C	Indefinitely

25 cycles

2.8 ARRAYTUBE HYBRIDISATION

The ArrayTube (AT) was first conditioned by washing with 500 µl of water for 20 min at 30°C followed by incubation with 500 µl of pre-hybridisation buffer (5x SSC (Life Technologies), 0.1% SDS (Sigma), 4x Denhardt's solution (Life Technoligies)) for 30 minat 50°C. Both steps were performed using a BioShake iQ (QUANTIFOIL Instruments GmbH) thermomixer at 550 rpm, which was used for all incubation steps where appropriate. A 10 µl aliquot of the labelled DNA sample was added to 90 µl of hybridisation buffer (5x SSC, 1% SDS, 4x Denhardt's solution). The mixture was then denatured at 95°C for three min and then kept on ice. The denatured sample (100 µl) was then pipetted into the AT and allowed to hybridise for 30 min at 50°C at 550 rpm. The sample solution was then removed by pipette and each tube was washed with 500 µl of Washing Buffer 1 (1x SSC, 0.2% SDS) for 20 min at 50°C at 550 rpm. Wash Buffer 1 was then removed and 500 µl of Wash Buffer 2 (0.1x SSC, 0.2% SDS) was added and the array washed for 20 min at 40°C at 550 rpm. Wash Buffer 2 was then removed and 500 µl of Wash Buffer 3 (0.1x SSC) was added and the array washed for 20 min at 30°C at 550 rpm. This buffer was then removed and a blocking solution (100 µl) of 2% biotin-free milk (Marvel) in PBS containing 1% BSA (New England BioLabs) and 0.1% Tween 20 was added to the AT and incubated for 60 min at 30°C at 300 rpm. The blocking solution was then removed and replaced with 100 µl conjugation solution (Streptavidin Poly-Horse radish peroxidase (HRP, Thermo Scientific) diluted 1:100 in the blocking solution) and incubated a further 15 min at 30°C at 300 rpm. The Post-conjugation washes were performed as described for the post-hybridisation washes. Wash Buffer 3 was then left in the AT and a snap shot of the array was taken on the Alere ArrayTube Reader (ATR 03). Following removal of Wash Buffer 3, 100 µl of the tetramethylbenzidine coupled with hydrogen peroxide (TMB/H_2O_2) staining solution (TrueBlue, Insight BioTechnology LTD, Middlesex, UK) was added and incubated for 10 min at 25°C without shaking. The HRP molecule bound to

the streptavidin was acted upon by the TMB/H_2O_2 solution and a precipitate was formed. The AT was then inserted once again into either the ArrayMate (Alere) or the ArrayTube Reader (Alere), and the array image was recorded. The recorded image was analysed using Alere's integrated IconoClust software and analysis script. Iconoclust processes the signals and automatically normalises the signal value after an algorithm processes the average intensity of the spot and the local background noise. The output range of the signals was between 0 and 1, with 0 being negative and 1 being the maximal possible signal value. The normalised intensity of the spots was automatically calculated by subtracting the local background noise from the average intensity of the automatically recognised spot.

2.9 ARRAYSTRIP HYBRIDISATION

The same protocol was followed for the ArrayStrips, however different volumes were used. The ArrayStrip (AS) was conditioned by washing with 150 µl of water followed by a wash with 150 µl of pre-hybridisation buffer. The denatured sample (100 µl) was pipetted into the AS and allowed to hybridise for 30 min at 55°C at 550 rpm. Each well was washed separately with 150 µl of Wash Buffer 1, 2 and 3 for 20 min at 60°C at 550 rpm. The blocking and conjugation steps were the same as for the AT. Post-conjugation washes were performed as described for the post-hybridisation washes. The AS was then inserted into the ArrayMate (Alere) and the array image was recorded.

2.10 SENSITIVITY TESTING

Quantitative real-time PCR (qPCR) was performed with the Applied Biosystems 7500 Fast Real-Time PCR System to test the sensitivity of the arrays. Samples of Infectious Bronchitis Virus (IBV) RNA (provided by A. Abu-Median, University of Nottingam) were initially reverse-transcribed into cDNA, and then qPCR was performed using the *Taq*Man

Universal PCR Master Mix kit (Life Technologies). The IBV sample was used as an assay control, as the RNA was not present in any of the other pathogens used. Initially, a primer optimisation step was carried out. The purpose of this procedure was to determine the minimum primer concentration which resulted in the maximum ΔRn (the difference between the Rn+ and the Rn- value, which reliably indicates the magnitude of the signal generated by the given set of PCR conditions). A PCR master mix was prepared to run four replicates of each of the nine conditions as shown in Table 2.9. These conditions tested different ratios of forward and reverse primers, to determine which one was optimal. The components of the master mix are listed in Table 210. The conditions required for the primer optimisation are listed in Table 2.11.

Table 2.9. Primer optimisation ratios of forward and reverse primer

	Forward primer (nM)		
Reverse primer (nM)	50	300	900
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Table 2.10. Volume of reagents required for qPCR master mix

Reaction component	**Volume per sample (µl)**	**Final concentration**
*Taq*Man Universal Master Mix (2x)	5.0	1x
Forward primer	1.0	50 to 900 nM
Reverse primer	1.0	50 to 900 nM
*Taq*Man probe (2.5 µM)	1.0	250 nm
DNA sample	1.0	10 to 100 ng
Water	1.0	-
Total	10.0	-

Table 2.11. Primer optimisation protocol

Step	**UNG incubation**	**Ampli*Taq* Gold Activation**	**PCR protocol**	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/Extend
Temperature	50°	95°C	95°C	60°C
Time	2 min	10 min	15 s	1 minute
Volume	50 µl			

Once primer optimisation was completed, probe optimisation for IBV was undertaken to determine the minimum probe concentrations that would result in the minimum C_T above the designated cut-off point. For single-probe assays, the optimal probe concentration was determined by running four replicates, at 50 nM increments from 50 to 250 nM. The PCR conditions were the same as those for the primer optimisation and are shown in Table 2.11.

Primer and probe optimisation was then carried out for *Y. pestis*. *Y. pestis* was selected for this testing, as this was the reference pathogen for which the most abundant nucleic acid was available, and therefore optimisation steps could be carried out without using up valuable reference material for the other pathogens. The sensitivity of the arrays was then conducted using diluted samples of *Y. pestis*. The DNA concentration of a *Y. pestis* reference sample was measured using a spectrophotometer, and serial dilutions (200-fold) were then prepared. The concentration of DNA in the first sample was 0.45 ng/µl (copy number $3.47x10^9$). This was then serially diluted to reach concentrations of 2.28^{x10-3} ng/µl (copy number $1.76x10^7$), $1.11x10^{-5}$ ng/µl (copy number $8.57x10^4$), 5.69^x10^{-8} ng/µl (copy number $4.39x10^2$), and 0.30^x10^{-10} ng/µl (copy number 1). The standard PCR primers used were Y.pes/pPCP/8374/F and Y.pes/pPCP8902/R, which amplified the *pla* region on the PCP plasmid, and the PCR products were then hybridised on the Yersinia_01 ArrayTube.

2.11 SHIPMENT OF SAMPLES

Fresh tissue was sliced (less than 0.5 cm in any one dimension) and submerged in approximately 5 volumes of RNA*later*, which preserved the nucleic acid and allowed the user to transport the sample at 2-8 °C. If RNA*later* was not used then fresh tissue was frozen immediately and then thawed at the testing laboratory, at which point homogenisation and extraction was carried out (see SOP in Appendix).

2.12 TISSUE HOMOGENISATION AND NUCLEIC ACID EXTRACTION

Over 700 samples of liver, kidney and lung from *R. rattus* and *R. norvegicus* were received from Canada (provided by C. Himsworth, British Colombia Ministry of Agriculture). However, in several cases the full sample set was not provided for each rodent. Therefore only 652 samples from Canada (33 *R. rattus*, 619 *R. norvegicus*), where all three of the tissues were available, were used. 84 samples of liver, kidney and lung from *R. norvegicus* were received from France (provided by F. Ayral, VetAgro Sup). 329 samples of liver and 174 samples of kidney from *R. norvegicus* (131), *M. musculus* (35) and *Apodemus sylvaticus* (163) were received from the UK (provided by K. Pounder, University of Liverpool). Automated nucleic acid extraction was performed on the samples at the University of Nottingham using the QIAcube (Qiagen) and appropriate kit (*cador* Pathogen Mini Kit (Qiagen)), following partial homogenisation of each tissue sample. Mechanical or enzymatic disruption of the tissue structure in each sample was necessary for liberation of cells and subsequent release of nucleic acids. This kit is intended for the extraction of pathogen nucleic acids (viral RNA and DNA and bacterial DNA) from a variety of organic samples including tissue.

Small sections of tissue (around 25 mg) were cut from the archived material and partially homogenised in 500 µl of PBS containing a 5-mm steel bead (Qiagen). The scissors used to cut the samples were sterilised in 100% ethanol for one minute

between each sample to prevent cross contamination. The partial homogenisation was performed using a Qiagen® TissueLyser II set at 30 Hz (1800 oscillations per minute) for 10 s. This time was found to be sufficient to partially disrupt the tissue without complete homogenisation. Partial homogenisation has proven sufficient to separate the pathogens from host cells.

The QIAcube performs fully automated purification of nucleic acids; Figure 2.2 shows the QIAcube workbench. The instrument is able to process 12 samples simultaneously. Integrated components include a centrifuge, a heated shaker, a pipetting system and a robotic gripper. The necessary plastic ware, samples and reagents were loaded onto the QIAcube worktable. The selected protocol provided the necessary commands for sample lysis and purification using Qiagen spin columns. A fully automated load check ensured correct loading of the worktable. The protocol 'Purification of Pathogen Nucleic Acids from Fluid Samples' (Qiagen) was used. This protocol is intended for the purification of viral RNA and DNA and the DNA of easy-to-lyse bacteria from fluid samples or pretreated tissue samples.



Figure 2.2. The QIAcube workbench with all parts labelled.

1. Centrifuge lid
2. Centrifuge
3. Shaker
4. Reagent bottle rack
5. Tip sensor
6. Microcentrifuge tube slots
7. Tip racks
8. Robotic arm
9. Display

200 µl of homogenised tissue was pipetted into the QIAcube sample rack. During extraction, 20 µl of proteinase K (Qiagen) was pipetted into the 2 ml safe-lock tube containing the sample. 100 µl Buffer VXL was then added and the contents mixed by vortexing in the shaker. Samples were lysed with proteinase K and buffer VXL (Qiagen) at room temperature, both of which have been designed to inactivate nucleases. The sample was then incubated at 20-25°C for 15 min, then 350 µl of Buffer ACB was added and the sample was mixed again by vortexing. Adding buffer ACB adjusts the binding conditions for the co-purification of DNA and RNA as it allows optimal binding of nucleic acid to the silica membrane in the spin column. The lysate was then transferred to the QIAamp Mini column. During centrifugation, nucleic acids were adsorbed onto the silica membrane in each column while contaminants passed through. The sample was then centrifuged at 6000 x*g* for one minute. Two wash steps removed the remaining contaminants and enzyme inhibitors, and 600 µl of Buffer AW1 was then added and the column centrifuged for one minute at 6000 x*g*. Buffer AW1 contains guanidine hydrochloride which was used to denature proteins in the sample. Then 600 µl of Buffer AW2 was added, and again the column was centrifuged for one minute at 6000 x*g*. Buffer AW2 is principally 70% ethanol, and is used to help purify the DNA present in the sample by further precipitating the DNA and removing salts. The sample was then centrifuged at full speed (13,000 x*g*) for two min to dry the membrane. The QIAamp Mini column was then placed in a clean 1.5 ml microcentrifuge tube and 100 µl of Buffer AVE was added, which eluted the nucleic acid by affecting the salt concentration. This was once again centrifuged at full speed for one minute. The resulting elute contained the extracted nucleic acids for use in PCR amplification. An aliquot of the eluted nucleic acid from each tissue of the same animal was pooled. This pooled approach, in theory, would allow us to detect as many pathogens as possible in just one sample. PCR amplification was then performed on the pooled material. Some of the samples from

the UK only had one tissue type available (liver), in these scenarios, the single tissue was used in the screening steps.

2.13 CONFIRMATORY TESTING

2.13.1 PCR/qPCR

Confirmatory work was performed on the samples which tested positive on the arrays using standard end-point PCR, qPCR and sequencing. All of the qPCR reactions were carried out in an Applied Biosystems (California, USA) 7500 Fast Real-Time PCR System. The PCR and qPCR primers for the tests came from published journals. The primers for standard PCR which were obtained from the literature are given in Table 2.12. These were tested with reference material (where available) prior to confirmatory PCR work. The protocol used for the sequence-specific amplification was also used for the confirmatory PCR, though during confirmatory PCR the primers were obtained from published journals. Further confirmatory tests using qPCR were also performed. Primers were obtained from the literature and the sequences of the primers and probes are given in Table 2.13. The protocol used for each primer set followed the one in the literature.

Table 2.12. Primers for standard PCR

Pathogen	Primer name	Sequence	Target	Reference
Leptospira	2For 504Rev	TGAGCCAAGAAGAAACAAGCTACA ATGGTTCCGCTTTCCGAAGA	*gyrB*	(Slack et al., 2006)
SEOV	SEOVF SEOVR	GTGGACTCTTCTTCTCATTATTG TGGGCAATCTGGGGGGTTGCA	M segment	(Plyusnina, 2004)
T. gondii	TOX4 TOX5	CGCTGCAGGGAGGAAGACGAAAGTTG CGCTGCAGACACAGTGCATCTGGATT	*B1*	(Durnez et al., 2011)
Bartonella	BARTON-1 BARTON-2	TAACCGATATTGGTTGTGTTGAAG TAAAGCTAGAAAGTCTGGCAACATAACG	*ribC*	(Buxton and Fraser, 1977)
Y. pestis	Yp1 Yp2	ATCTTACTTTCCGTGAGAAG CTTGGATGTTGAGCTTCCTA	*pla*	(Hinnebusch et al., 1998)
Y. pseudotuberculosis	invF invR	CGGTACGGCTCAAGTTAATCTG CCGTTCTCCAATGTACGTATCC	*inv*	(Samuel et al., 2001)
C. jejuni	HIP400F HIP1134R	GAAGAGGGTTTGGGTGGTG AGCTAGCTTCGCATAATAACTTG	*hip*	(Maneerat et al., 1996)
S. Typhimurium	Fli15 Typ04	CGGTGTTGCCCAGGTTGGTAAT ACTGGTAAAGATGGCT	*fliC*	(Carter, 1975)
E. coli	EAE157-F EAE157-R	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	*eaeA*	(Gannon et al., 1997)

Table 2.13. Primers for real-time PC

Pathogen	Primer and probe name	Sequence	Target	Reference
Leptospira	Lepto F	CCCGCGTCCGATTAG	*rrs* (16S)	(Smythe et al., 2002)
	Lepto R	TCCATTGTGGCCGRA/GACAC		
	Lepto P	5'FAM-CTCACCAAGGCGACGATCGGTAGC-3'TAMRA		
T. gondii	TOXO-F	TCCCCTCTGCTGGCGAAAAGT	*B1*	(Lin et al., 2000)
	TOXO-R	AGCGTTCGTGGTCAACTATCGATTG		
	TOXO-P	5'FAM-TCTGTGCAACTTTGGTGTATTCGCAG-3'TAMRA		
C. jejuni	VS15	GAATGAAATTTTAGAATGGGG	*VS*	(Kamani et al., 2013)
	VS16	GATATGTATGATTTTATCCTGC		
	VSgeneP	5'FAM-TTTAACTTGGCTAAAGGCTAAGGCT-3'TAMRA		
Y. pseudotuberculosis	Yps1	CGTCTGTTAATGTGTATGCCGAAG	*ail*	(Philips and Parker, 1938)
	Yps2	GAACCTATCACTCCCCAGTCATTATT		
	YpsP3	5'VIC-CGTGTCAAGGACGATGGGTACAAGTTGG-3'TAMRA		
Y. pestis	cafpri_f	CCAGCCCGCATCACT	*caf1*	(Leek et al., 2010)
	cafpri_r	ATCTGTAAAGTTAACAGATGTGCTAGT		
	Tqpro_caf	JOE-AGCGTACCAACAAGTAATTCTGTATCGATG-BHQ1		

2.13.2 SEQUENCING

The 'QIAquick PCR purification' protocol was used to purify some of the PCR products for sequencing, to verify the identity of the products. 125 µl of Buffer PB was mixed with 25 µl of the PCR sample. The DNA was bound by placing the sample in a QIAquick column and centrifuging the column at 13, 000 x *g* for 60 s. The flow-through was discarded and 0.75 ml of Buffer PE was added and the column centrifuged for an additional 60 s at 13, 000 x *g*. The QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube, and a volume of water (30-50 µl) was added to the column. To increase the final DNA concentration, 30 µl of water was added instead of 50 µl. This was centrifuged at 13, 000 x *g* for 60 s. The eluate (purified PCR product) was collected in a clean microcentrifuge tube and stored at -20°C.

The DNA sequencing was performed in a 96-well plate. For each sample to be sequenced, a working stock of 10 µM of sequencing primer (forward primer) was diluted 1:15 with water. Four µl of this diluted primer was dispensed onto the bottom of the well. A BigDye master mix (187.5 µl water, 187.5 µl 5 x buffer and 25 µl BigDye) (Life Technologies) was then prepared for the 96 wells. Four µl of this master mix was then dispensed into each well. Two µl of the purified PCR product was then added to the appropriate well. The plate was then spun at 500 *x g* for 60 s to ensure the reaction mix was at the bottom of each well. The sequencing protocol was 30 cycles of 96°C for 10 s, 50°C for 5s and 60°C for 2 min.

The plate was then removed from the thermal cycler and 15 µl of water was dispensed into each well. A mixture of 7 ml of absolute ethanol and 280 µl 3M NaOAc (Sodium acetate, pH 5.2) was prepared and 52 µl of this was added to each well. The plate was then incubated at room temperature for 45 min. The plate was then centrifuged for one hour at 2750 *xg* (4°C). Immediately after centrifugation the plate was inverted onto absorbent paper and spun again at 500 *xg* for 60 s (ambient

temperature). Each well was washed with 150 µl 70% ethanol, and the plate was then centrifuged at 2750 *xg* for 10 min (ambient temperature). The ethanol was removed as before by inverting the plate onto absorbent paper and centrifuging. The dry pellet at the bottom of each tube was then stored at -20°C until it was sent to be sequenced at the Institute of Zoology, Oxford.

2.14 PHYLOGENETIC TREE

The sequenced results were used to produce a phylogenetic tree with SeaView 4.5.1. The default settings were used to create a Bootstrap PhyML tree with 100 replicates. The invariable sites were set to none. The across site variation had a number of rate categories set to 4 and optimised. The tree searching option used was the default one; nearest-neighbour interchange (NNI). Finally, the starting tree option was set at BioNJ (neighbour-joining method), with the optimise tree topology option selected and 5 random starts.

CHAPTER THREE

MICROARRAY DESIGN

3.1 SUMMARY

Array design is often considered the most important process in any microarray experiment and can be the deciding factor in the success of a study. There is inherent signal variation produced on microarrays, and this needs to be reduced to a minimum. This can be done with good probe design, which can be achieved for example, by designing probes that have uniform characteristics. This chapter shows the steps required to design a microarray for pathogen detection. The array platforms used were the Alere ArrayTube and ArrayStrip; these were chosen as they allowed the testing of a large number of samples simultaneously for a number of pathogens. Probe design began with a literature review on the genomes of the pathogens for which screening was to be conducted. Once a potential target gene had been identified probes were designed using publicly available software (OligoWiz, Unique Probe Selector). The oligonucleotide length was set at 60 bp as this length offers the best trade-off between sensitivity and specificity. Primers were then designed so that the target sequences would be amplified in the samples. The ArrayTube was used as a pilot experiment and the ArrayStrip was then used to screen the field samples. Three ArrayTube types were produced: Hanta_HEV_01, Yersinia_01 and Toxoplasma_01. A pilot ArrayStrip (WT_Rodent_Chip_02) was also synthesised and used for optimisation procedures before the final screening chip was synthesised. The final screening chip (Rodent_Chip_03) contained probes for all the pathogens that were to be screened, and included the best probes from both the ArrayTubes and pilot ArrayStrip. Over 800 probes were designed during the study using various design software tools, and the probes that performed well were used on the final screening chip.

3.2 INTRODUCTION

The most commonly used application for microarrays has been to study gene expression. In recent years however, they have increasingly been used to detect and classify pathogens (Severgnini, 2011). Different microarray platforms are available for diagnostic purposes, and can be characterised by variables which include the length of the probe, the array surface, and the method used for target detection (Miller, 2009).

In situ-synthesised arrays are extremely high-density microarrays where oligonucleotide probes (20-25 bp) are synthesised directly on the surface of a solid substance (usually glass). Affymetrix designed the first in situ-synthesised arrays, and oligonucleotides were synthesised directly onto a silicon surface (Fodor, 1991). The technology used with Affymetrix arrays is expensive, making their application in microbial diagnostics a costly one. Other in situ-synthesised and commercially available DNA microarray products are available, and include arrays manufactured by Roche NimbleGen and Agilent Technologies. The arrays produced by both of these companies utilise a longer probe length (60-100 bp), than the Affymetrix arrays (Severgnini, 2011). A low to medium-density (which can range from hundreds to several thousand probes) is regarded as the most effective for diagnostic laboratories, as it offers reliable results that can be obtained with simple protocols (Mikhailovich, 2008). The main advantage of low to medium-density arrays is the ability to design custom arrays at more affordable costs.

Printed arrays have probes that are spotted onto the microarray surface; a glass microscope slide is most commonly used. Printed arrays can also be categorised based upon the properties of the probe: double-stranded DNA (150-200 bp), cDNA or oligonucleotides (25-80 bp). Microbial pathogens including *Bacillus anthracis*, Newcastle disease and influenza viruses have all been identified by microarrays in the

past 10 years (Burton et al., 2005, Wang et al., 2008). The Alere ArrayTube and ArrayStrip are types of printed oligonucleotide microarrays.

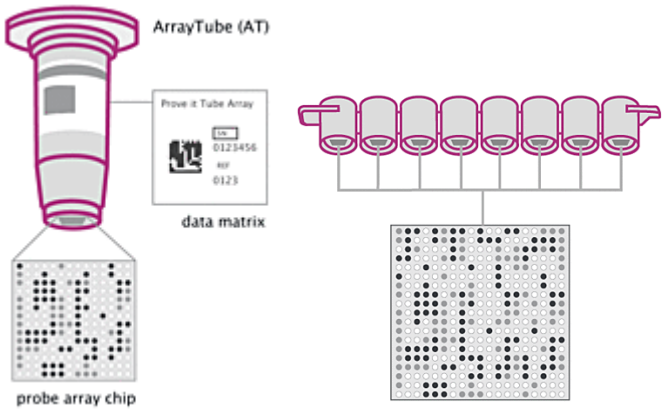
The primary objective of the project was to develop microarrays which could be used to test for the presence of a range of zoonotic pathogens in wild rodents, and to apply these arrays to screen wild rodent samples from the UK, France and Canada. This was accomplished using a variety of methods.

Data obtained from any microarray experiment is subject to variation which can be caused by a number of sources. A good microarray experiment should be designed and implemented so that sources of unwanted variation are reduced as much as possible. The probe set designed for all the pathogens should have uniform properties, i.e. they should all show the same thermodynamic behaviour under the experimental conditions used. At the same time the probes must be highly specific for their targets; therefore, they should only detect the sequence for which they were designed. Identifying a target set and designing a good probe set can be challenging since there are several conditions that must be addressed. These include sensitivity, specificity and noise which were discussed in Chapter 1. Through careful selection of probe sequences, specificity and sensitivity can be optimised.

3.2.1 Microarray platforms

The commercially available ArrayTube and ArrayStrip platforms (Alere Technologies GmbH) were used during this study because they allowed high-throughput testing of up to 96 samples simultaneously for multiple pathogens, unlike other available platforms. The ArrayTubes were employed for initial array evaluation, as the platform is less expensive than the ArrayStrip, and it was more cost-effective to use this platform during the array testing phase. The reader (ArrayMate) enables image acquisition of the resulting precipitation patterns and generation of data from up to six ArrayTubes or 12

ArrayStrips within min. This renders the system particularly user-friendly and economical. The 3 x 3mm chip in the ArrayTube is fitted at the bottom of a 1.5 ml micro-reaction tube and accommodates up to 225 spots (Figure 3.1A). Each single ArrayStrip is made up of eight individual wells, with a 4 x 4 mm chip at the bottom of each well with over 600 spots (Figure 3.1B). Up to 12 ArrayStrips can be assembled into a microplate frame enabling the parallel testing of up to 96 samples. Because the chips are placed at the bottom of the tube or well, all steps of the hybridisation reaction can be conducted in the ArrayTube or ArrayStrip. As visualisation of the hybridisation reaction is based on enzyme-catalysed precipitation rather than fluorescence, a transmission reader can be used to measure signal intensities which can reduce costs. A biotin-labelled target (PCR amplicon) binds with its complementary probes on the array. A conjugation reaction follows, when horseradish peroxidase (HRP) molecules conjugate to bound target molecules. Precipitation occurs when tetramethylbenzidine (TMB) is added and acted upon by HRP. This precipitation is then measured by the AT Reader or ArrayMate.



A B

Figure 3.1. Schematic representation of the ArrayTube (A) and ArrayStrip (B) platforms (Figure is adapted from Alere website http://alere-technologies.com/en/products/lab-solutions/platform-components.html).

3.2.2 Microarray design

A work flow for the project is shown (Figure 3.2). The first step was to select pathogens for which to screen. The pathogens selected for the array were those considered to be zoonotic and which had been previously detected in rodent species. These pathogens were reviewed in Chapter 1.

The next step was the probe design. After the target regions had been identified, probe design software was used to determine probe sequences (see Chapter 2). Following this, an *in silico* analysis was performed, using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), and the probes were then spotted onto the arrays by Alere. Any probe sequences that showed cross-hybridisation with any other sequences with identity >75% were discarded.

Several tools have been developed that aid large-scale probe design. Whilst they differ in the precise algorithms used to select probes, they all follow a similar format. They search for specific probes that are unique within the genome, that would not be self-binding and that, as a set, are thermodynamically similar. It is advantageous to try to design probes that have uniform behaviour at the hybridisation temperature selected. In practice, this is achieved by limiting the melting temperature of each probe to a narrow range, by varying the probe length between 57-60 bp for example. If the probes are limited to a single length, for example 60 bp, there will be a wider range in melting temperature due to a difference in GC content, which can reduce the number of possible targets.

After the design and fabrication of the arrays, an evaluation phase was required, which is described in detail in Chapter 4. The ArrayTubes and ArrayStrips were tested using reference material (where available) and the probes were checked for correct hybridisation with their targets, as well as for any non-specific hybridisation which might

occur. After stages 4, 6 and 8 had been reached (Figure 3.2), additional probes were designed to replace under-performing probes. In the final stage, evaluated probes were grouped in a single array for screening (application phase).

3.2.3 Aims

The aims of the array design stage were to:

- Design oligonucleotide probes specific for each pathogen on the array
- Perform an *in silico* analysis for each of these probes
- Design PCR primers to amplify the sequence of interest

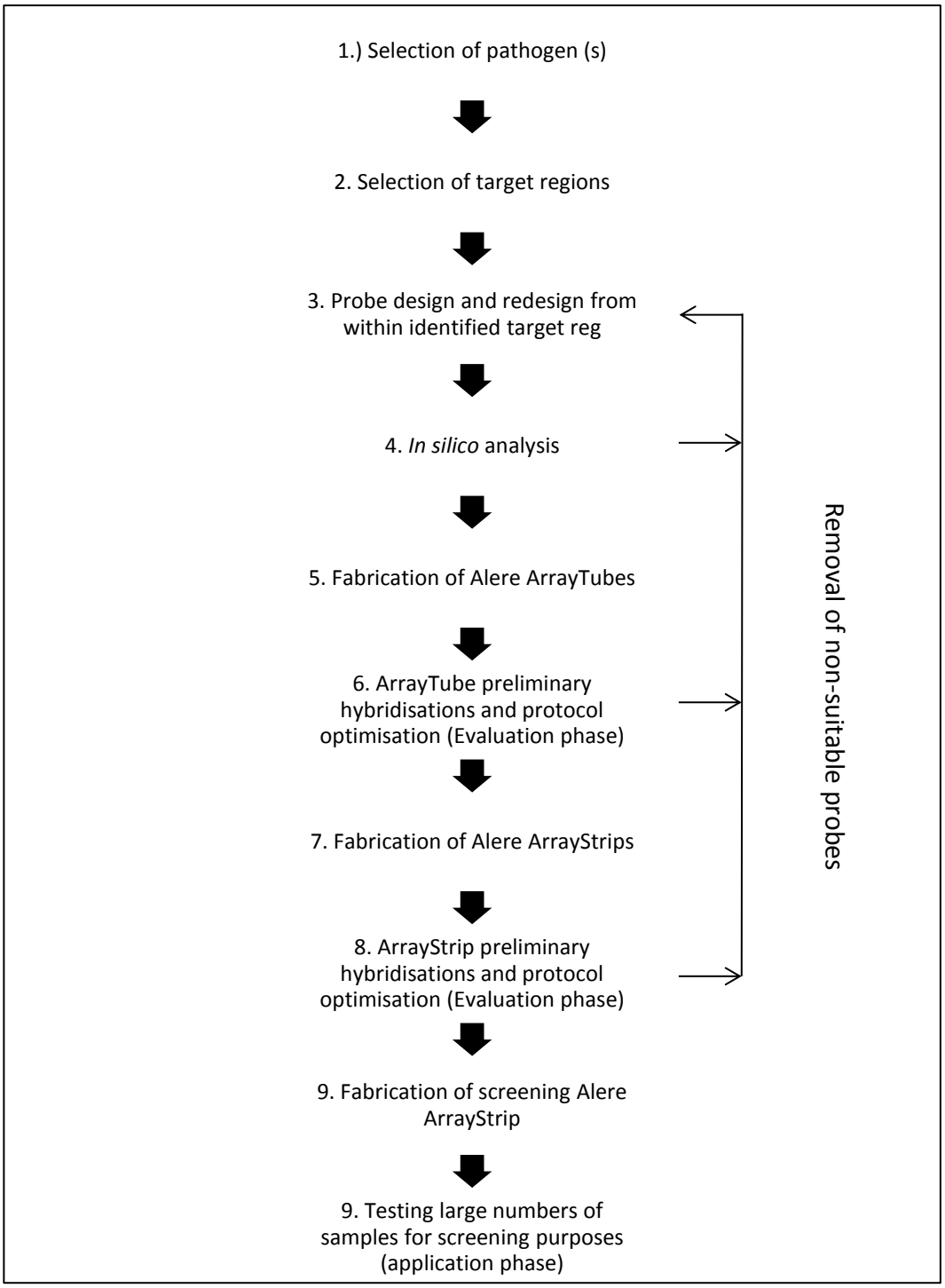


Figure 3.2. Design steps involved in the project.

3.3 RESULTS

3.3.1 Probes

Over 800 probes were designed using probe design software. Once a target gene was identified for each pathogen, an alignment was then made for that gene across different strains or subtypes of that pathogen. This helped in the design stages, as certain regions of the gene could be targeted for primer and probe design. The full list of probes used on each of the arrays and the genes which they target can be seen in the Appendix. Figure 3.3 shows an alignment of the gene sequence encoding the nucleocapsid protein of SEOV, with 11 strains used to generate the alignment. The selected target sequences were initially aligned using the programme ClustalX2. This alignment was then saved as a PostScript file (.ps) and opened using the programme GSView4.9. The region highlighted in the red box is the sequence used for one of the SEOV probes on the array (SEOVUPS_531). There were 3 positions that did not match across all 11 strains. However, with a similarity of 95%, it was expected that this probe sequence would be able to hybridise with all the strains shown in Figure 3.3. An *in silico* analysis with BLAST of this particular region of SEOV had over 100 matches. All of these matches were with other SEOV strains, and the lowest identity was 95%. This region, therefore, appeared to be highly conserved across all strains of SEOV. There was no cross-hybridisation with other hantaviruses, so it also appeared to be specific to SEOV. Probes for the other pathogens were designed in the same way.

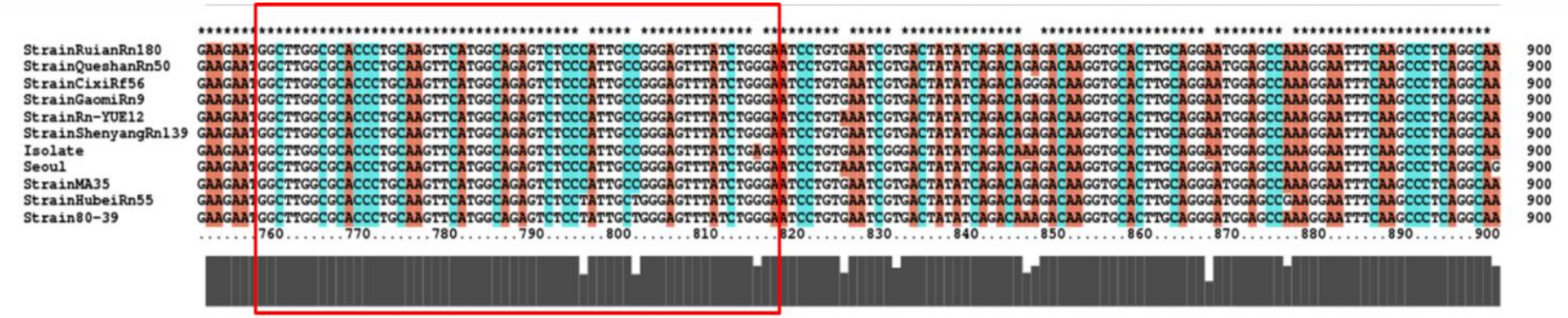


Figure 3.3. Multiple sequence alignment of 11 strains of SEOV.
The sequence highlighted in the red box shows the sequence used for probe design.

3.3.2 ARRAY LAYOUT

Three microarrays were produced in the ArrayTube format for a selection of viruses (HEV and hantaviruses), bacteria (*Yersinia* species) and parasites (*T. gondii*) (Figure 3.4). The blank, non-numbered probe spots were not used, as these were the locations occupied by the orientation markers. The location of the biotin marker (assay control) is also indicated as is the spotting buffer. The number of probes that had been designed and then spotted for each pathogen on the relevant microarray is shown in Table 3.2.

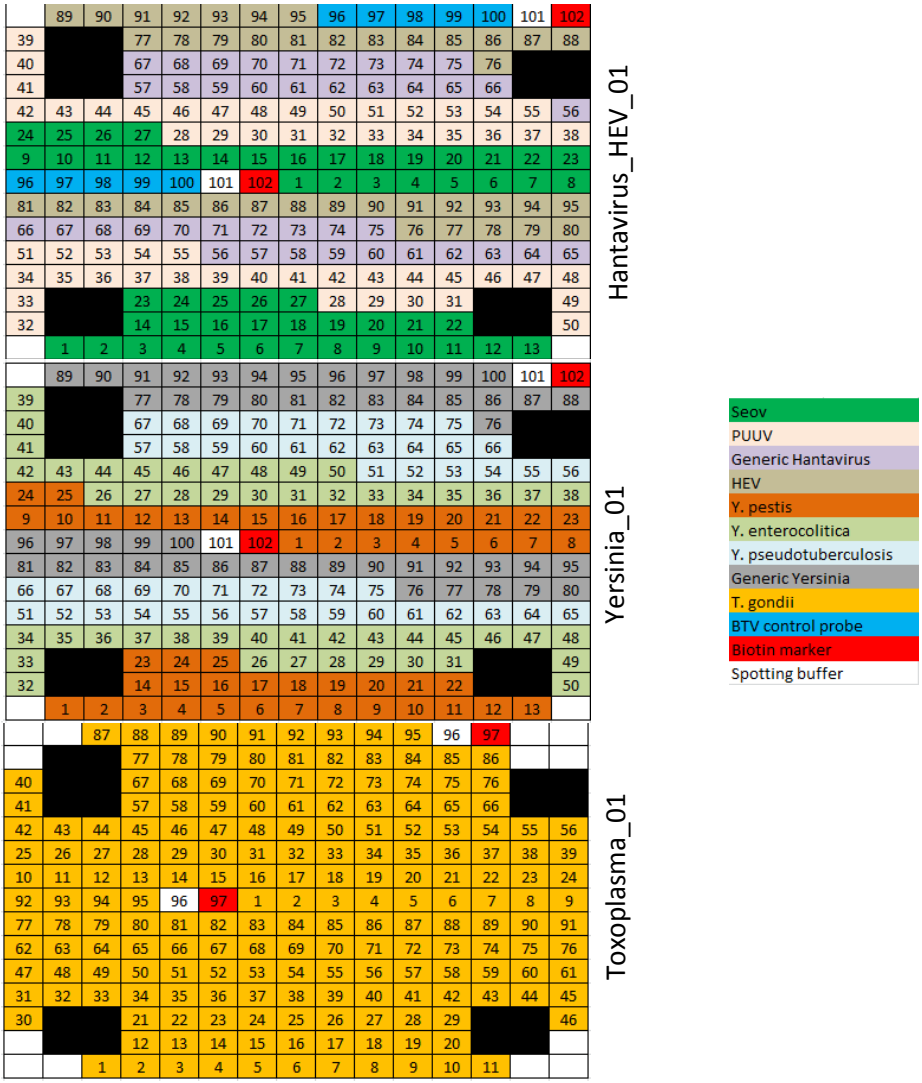


Figure 3.4. A colour-coded layout of the ArrayTubes Hanta_HEV_01, Yersinia_01 and Toxoplasma_01.

Table 3.1. Number of probes designed and probes that were spotted onto each ArrayTube.

Pathogen	Number of probes designed	Array	Number of probes on Array
Generic *Yersinia*	90	Yersinia_01	25
Y. pestis	58	Yersinia_01	25
Y. enterocolitica	57	Yersinia_01	25
Y. pseudotuberculosis	58	Yersinia_01	25
Generic hantavirus	20	Hanta_HEV_01	20
SEOV	27	Hanta_HEV_01	27
PUUV	28	Hanta_HEV_01	28
HEV	20	Hanta_HEV_01	20
T. gondii	95	Toxoplasma_01	95

The layout of the pilot ArrayStrip WT_Rodent_Chip_02 can be seen in Figure 3.5. Different arrays were ordered at different stages of the evaluation process. There were 15 spots printed in duplicate for each pathogen present on this array. The orange non-numbered spots were the locations occupied by the orientation markers. The biotin marker (assay control) was marked at position 301. The biotin markers on the arrays were used as an assay control to determine if the hybridisation reaction was successful.

The final ArrayStrip (WT_Rodent_Chip_03) included working probes from both the ArrayTubes and the pilot ArrayStrip. There were also probes for *Mycobacterium* and *Francisella*, which had beed designed and tested by a colleague at the SVMS using the same methodology. The layout of the screening ArrayStrip is shown in Figure 3.6 and the number of probes for each pathogen is shown in Table 3.3. The number of probes for each pathogen varied on the screening ArrayStrip. This was because for some of the pathogens (e.g. *S*. Typhimurium) the probes hybridised strongly during the evaluation stage and it was decided that as such, only a select few probes would be needed to confirm the presence of the pathogen. The black non-numbered spots were the locations occupied by the orientation markers. The biotin markers (in red) were spotted at position 328.



Figure 3.5. A colour-coded layout of the ArrayStrip WT_Rodent_Chip_02.

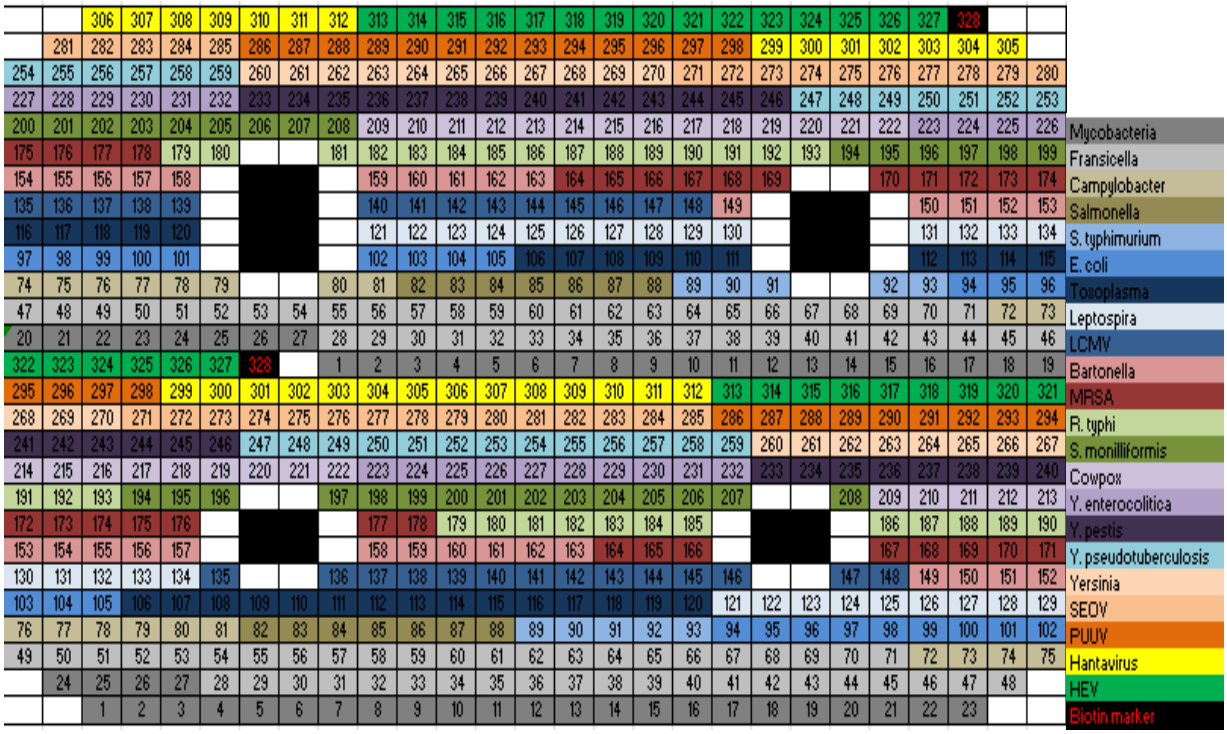


Figure 3.6. A colour-coded layout of the ArrayStripWT_Rodent_Chip_03.

Table 3.2. The number of probes for each pathogen that were spotted on the WT_Rodent_Chip_03 ArrayStrip.

Pathogen	Number of probes
Mycobacterium	27
Francisella	44
C. jejuni	10
Salmonella	7
S. Typhimurium	5
E. coli	12
T. gondii	15
Leptospira	14
LCMV	14
Bartonella	15
MRSA	15
Rickettsia	15
S. monilliformis	15
Cowpox	14
Y. enterocolitica	10
Y. pestis	14
Y. pseudotuberculosis	13
Generic *Yersinia*	11
SEOV	15
PUUV	13
Hantavirus	14
HEV	15

3.4 DISCUSSION

The nucleic acid arrays described in this chapter represent a potential novel screening tool for several different zoonotic pathogens. The information presented provides a suggested approach for setting up and managing a microarray experiment from start to finish, highlighting some of the most important factors to consider. These include probe design, primer design, microarray fabrication and evaluation. The design of oligonucleotide probes is a complex problem for a variety of reasons, including defining the target sequences to be screened and understanding the thermodynamics of probe-target interactions during hybridisation (Russell et al., 2009). The length of the oligonucleotides was set at 60 bp, as several studies have indicated that this offers the best compromise between specificity and sensitivity (Relogio et al., 2002, Kane et al., 2000, Wang et al., 2003a). Shorter oligonucleotide probes (15-25 mers) have a very high specificity, but they have been criticised for having a lack of sensitivity whereas longer oligonucleotides (40-90 mers) are thought to have a good sensitivity whilst maintaining a high specificity (Russell et al., 2009). It has been reported that 60-mer oligonucleotides have an eightfold higher sensitivity than 25-mers (Chou et al., 2004).

As well as probe design, primer design was also an important step to consider. As pathogenic nucleic acid is usually only present in low levels in host tissue samples, a PCR step was required to amplify the pathogen nucleic acid. In addition to standard PCR primers, a set of biotinylated primers was used. Biotinylation is the process by which a biotin molecule is covalently bound to a protein, nucleic acid or other molecule. The biotinylated primers negate the need for an additional biotin-labelling step of the PCR product. This can reduce the time and cost of the PCR steps, making it a more cost-effective approach. For some primers there was more than one primer set. This is because reference material was available for these pathogens and therefore, primer evaluation could take place.

However thorough the design stage is, variation within microarray experiments can still occur. The typical sources of variation can be broadly divided into three main categories: Variation within the biological sample, the performance of the technology itself, and finally variation in the spot signal measurements. The majority of variance in microarray experiments is generally biological rather than technical (Koskinen et al., 2009).

Arrays are generally made in batches and variation can occur between batches. These can include different probe concentrations, which can lead to incorrect conclusions being drawn from data (Leek, 2010). Variation at the array production stage can occur for a number of reasons, for example the particular printing pin used, the humidity, and temperature during printing. These variables can lead to slight differences in the amount of probe that is deposited on the slide surface, the amount that remains on the array surface after processing, and the level of deviation from the expected spot location. All of these factors can have an impact on the amount of labelled target that can bind to the probe, and on the efficiency of subsequent spot finding and data extraction steps (Russell et al., 2009). The level of deviation from the expected location can result in the array reader making inaccurate readings or the signals of neighbouring probes becoming merged. Batch variation was eliminated with the final ArrayStrips as these were all produced at the same time (in the same batch).

In order to reduce the variability that is inherent in all biological experiments, experimental replication is essential. One obvious form of technical replication is through array probe replication. It is advantageous to at least have duplicates (these were present on the arrays), or preferably multiples, of all probes spotted on the same array, however this may not be possible due to spotting density constraints. The precision of particular probe measurements will be more reliable if the spot intensities of the replicate spots are averaged for each sample (Tran et al., 2002).

The design stage can be the deciding factor in the success of any microarray experiment. The choice of array platforms or probe types can be challenging. However, it is now becoming increasingly clear that when a careful design is followed, the results obtained with different platforms are likely to be comparable (Russell et al., 2009, Jarvinen et al., 2004, Irizarry et al., 2005). The user can decide whether to invest time and resources in developing their own arrays, utilise one of the commercial providers who can assist with array design and fabrication, or use off-the-shelf commercial arrays. Oligonucleotide design is a key step when using custom made arrays. The oligonucleotides on the array surface need to hybridise well with the target sequence, and not with any non-target sequences. Cross-hybridisation, which is the non-specific binding of the probe to other sequences, is to be avoided.

In this study, there were 20 primer pairs designed using Primer3 and over 800 probes designed using OligoWiz and UPS (other primers were obtained from published journals). Primer3 was used because it has an easy-to-use interface, is publicly available and can generate primers from specific Fasta sequences. OligoWiz and UPS were used in this study to see if there was any difference in probe effectiveness depending on the design method used. Both OligoWiz and UPS are user-friendly, though OligoWiz does not currently have the capacity to design probes for viral pathogens. UPS was the easier of the two to use and was therefore the preferred probe design tool. For a researcher without previous bioinformatics experience, both OligoWiz and UPS are recommended tools.

To achieve an efficient hybridisation step, it is important to have probes with a narrow melting temperature distribution, because the hybridisation step takes place at the same temperature for all probes on the array (Haque et al., 2007). The algorithms used in both OligoWiz and UPS are able to make minor alterations to the length of each probe so that a narrow melting temperature range is achieved. Determination of

melting temperature thresholds is a difficult task as this determines the conditions under which probes will bind to the target sequence. Melting temperatures can cause a loss of signal if too high, and non-specific signal if too low (Blalock, 2003). As a single temperature is used during the hybridisation step, it is advisable that the narrowest melting temperature range be used to maximise signal detection (Lemoine et al., 2009).

Oligonucleotide design must be optimised for all the various factors that can influence the hybridisation between the probe and the sample. The probes may not perform as expected when they are evaluated using reference samples, so it may be necessary to design additional probes. Whilst an *in silico* analyss must be performed prior to probe synthesis, there is still the potential for cross-hybridisation, so it is necessary to also have an array evaluation stage.

Both the ArrayTube and ArrayStrip (which have hundreds of probes) have the advantage of being a more economical microarray than the glass-based microarray slides (which can millions of probes). Numerous studies have found that the ArrayTube and ArrayStrip platforms, as well as being user-friendly and economical, have a sensitivity equivalent to that of qPCR, rendering it suitable for use in the diagnostic laboratory (Sachse et al., 2005). The hybridisation process was also a simple one and can be accomplished in around 5 hours. The disadvantage to the ArrayTube and ArrayStrip however, is that they are a lower density array with at most up to 600 spots available. For a pathogen screening tool such as the one in this project, it is only necessary to have a few probes per pathogen so a lower density tool is acceptable. Five arrays were designed and manufactured for the current project. At the early stages of the study, The ArrayTubes were used as a pilot platform and the targeted pathogens initially only included *Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *T. gondii*, HEV, SEOV and PUUV. The additional pathogens were added later in the project, at the request of a collaborator, which is why there were no ArrayTubes with probes for these

additional pathogens. *Mycobacteria* and *Fransicella* probes were then added to the array. After array evaluation (detailed in the next chapter), the probes that performed well were used on the final screening array.

CHAPTER FOUR

ARRAY EVALUATION

4.1 SUMMARY

While array design is important for *in silico* analysis, the actual performance of the probes will not be known until they are tested in the laboratory. Several considerations need to be checked for each probe: Does it hybridise with appropriate target sequences, does it produce a detectable signal with the appropriate target sequence, and does it cross-hybridise with any inappropriate sequences. Sequence-independent PCR, sequence-specific PCR and multiplex sequence-specific PCR were also compared to discover the most effective one on the array. The arrays were also tested for their sensitivity. This was accomplished by serially diluting samples of rodent tissue spiked with a known DNA concentration of a selected pathogen, in this case *Y. pestis* and then performing PCR, qPCR and hybridisation on the array. Reference samples of pathogen nucleic acid, where available, were used to test the efficiency of the probes. After the probes were evaluated with reference samples, the most effective probes on the ArrayTube and pilot ArrayStrips were selected for inclusion on the final screening chip. The signal produced on the arrays indicated that the sequence-specific multiplex approach generated the best signal on the arrays. For time efficiency, this approach was further modified with the use of biotinylated primers. This negated the need for a biotin labelling step and thus decreased the overall time taken to process the samples. The use of biotin labelled primers used in a multiplex format in conjunction with microarrays has not previously been reported. Because the multiplex approach produced signal comparable to the signal obtained using standard PCR, the multiplex approach was selected for use with the field samples due to the superior time and cost effectiveness. The sensitivity of the arrays, as indicated by the test results for *Y. pestis*, suggested that it was comparable with standard PCR, though less sensitive than qPCR.

4.2. INTRODUCTION

Before the field samples were tested on the arrays, each probe was evaluated for performance under experimental conditions. This included specific and non-specific hybridisation and quality of the signal produced. Array evaluation allows optimisation of the conditions required for hybridisation. The hybridisation temperature is one of the most important factors to consider in the hybridisation process. If the hybridisation temperature is too low then non-specific probe-target binding will occur, and specificity would decrease. In contrast, if the temperature is too high, hybridisation of the specific targets will be reduced, with a resultant loss of signal, so the sensitivity would decrease (Russell et al., 2009).

To fully evaluate any new screening tool, nucleic acids derived from specific samples are needed. These include cultured pathogens, samples from an experimentally infected host and clinical samples with known and unknown infection states. Testing with clinical samples is an essential step in the evaluation of array performance. In clinical samples, it is possible that there are low levels of pathogen, so the array must be sensitive enough to detect these. In contrast, the amount of pathogen in a cultured sample may be much higher, and therefore easier to detect.

Although PCR-based assays have any advantages (detailed in chapter 1), they also have a set of drawbacks. As successful identification relies heavily on effective primer sets, all PCR-based testing requires prior knowledge of the sequence of the infecting pathogen (Vora et al., 2004). Three PCR methods were used as part of the array evaluation: Sequence-independent amplification, individual sequence-specific amplification and multiplex sequence-specific amplification. Sequence-independent or random amplification has been used in the past to identify novel virus species (Wang et al., 2002a, Wang et al., 2003b). A large array consisting of approximately 10,000 oligonucleotides from approximately 1,000 viruses was developed by Wang and

colleagues. Their objective was to design a microarray that could detect the widest possible range of both known and unknown viruses. With a sequence-independent approach, all the nucleic acid in the sample was amplified and the resulting hybridisation led to the discovery of new viruses (Severe Acute Respiratory Syndrome). Sequence-specific primers, by contrast, offer a greater specificity, but they do not have the flexibility of random primers, which amplify all nucleic acid in a sample. However, for sequence-specific primers the sequence of the target must be known. The region amplified by the primers may also be different in various strains or subtypes of some pathogens, rendering them unable to amplify this target region despite the pathogen being present in the sample. In an attempt to improve sequence-specific amplification, researchers have developed multiplex PCR assays that amplify several unique regions within a single experimental reaction (Singh et al., 2002, Volokhov et al., 2002). However, multiplex assays are limited in the number of pathogens they can detect, though this has been reported as being up to 19 (Kong et al., 2002, Caliendo, 2011, Anderson et al., 2013, Puppe et al., 2013). Another limitation is the overall throughput that is now necessary for pathogen identification (Vora et al., 2004).

4.2.1 Aims

The aim of the work in this chapter was to evaluate, using reference pathogen material, the ArrayTubes and ArrayStrips that had been designed and produced. This consisted of testing how well the probes on the array hybridised with the target pathogen, and whether any non-specific cross-hybridisation occurred. The signal intensity observed after amplification with sequence-independent PCR, singleplex sequence-specific PCR and multiplex sequence-specific PCR was analysed for any statistical differences with a student's T test. The sensitivity of the array was tested through comparison with standard end-point PCR and qPCR.

4.3. RESULTS

4.3.1 ArrayTubes

Individual ArrayTubes were prepared which contained probes for the Yersinia species (*Y. pestis, Y. enterocolitica, Y. pseudotuberculosis*), *T. gondii*, the hantaviruses and HEV. The additional pathogens which were studied were added later on in the project, so they did not undergo initial evaluation. Three ArrayTube types were ordered, one which contained all the Yersinia probes (WT_Yersinia_01), one which contained all the *T. gondii* probes (WT_Toxoplasma_01) and one which had a combination of hantavirus and HEV probes (WT_Hanta_HEV_01).

4.3.1.1. Y. pestis

The WT_Yersinia_01 ArrayTubes were tested using reference samples of *Y. pestis* DNA from pure culture. They were first tested using Y.Pes/PcP/8532/F and Y.pes/PcP/9011/R primers (*pla* gene), which were expected to hybridise with four probes on the array. However, only two probes (Y.pestis_Owiz_117 and Y.pestis_Owiz_127) showed a mean hybridisation signal >0.4 after repeat experiments. These results are illustrated in Table 4.1. The next test was with sequence-independent amplification, and this resulted in 10 of the 25 generic probes hybridising with a mean signal intensity >0.4. After sequence-independent amplification 18 of the 25 *Y. pestis* probes produced a mean signal > 0.4. However, there was a degree of variation observed in the probe signal after sequence-independent variation. This was more commonly observed for the probes specific to *Y. pestis*. There was very little cross-hybridisation observed for *Y. enterocolitia* and *Y. pseudotuberculosis* probes after sequence-independent amplification.

Table 4.1. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of *Y. pestis* with the WT_Yersinia_01 ArrayTube.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Y.pestis_Owiz_97	Sequence-independent	pPCP	0.567 (4)	0.214
Y.pestis_Owiz_98	Sequence-independent	pPCP	0.548 (4)	0.250
Y.pestis_Owiz_103	Sequence-independent	pPCP	0.621 (4)	0.225
Y.pestis_Owiz_109	Sequence-independent	pPCP	0.481 (4)	0.180
Y.pestis_Owiz_110	Sequence-independent	pPCP	0.546 (4)	0.209
Y.pestis_Owiz_111	Sequence-independent	pPCP	0.561 (4)	0.227
Y.pestis_Owiz_112	Sequence-independent	pPCP	0.507 (4)	0.192
Y.pestis_Owiz_113	Sequence-independent	pPCP	0.518 (4)	0.198
Y.pestis_Owiz_114	Sequence-independent	pPCP	0.542 (4)	0.202
Y.pestis_Owiz_115	Sequence-independent	pPCP	0.575 (4)	0.203
Y.pestis_Owiz_116	Sequence-independent	pPCP	0.501 (4)	0.210
Y.pestis_Owiz_117	Sequence-independent	pPCP	0.469 (4)	0.179
Y.pestis_Owiz_117	Y.Pes/PcP/8532/F and Y.pes/PcP/9011/R	pPCP	0.697 (10)	0.150
Y.pestis_Owiz_118	Sequence-independent	pPCP	0.537 (4)	0.240
Y.pestis_Owiz_122	Sequence-independent	pPCP	0.568 (4)	0.227
Y.pestis_Owiz_124	Sequence-independent	pPCP	0.504 (4)	0.171
Y.pestis_Owiz_126	Sequence-independent	pPCP	0.513 (4)	0.204
Y.pestis_Owiz_127	Sequence-independent	pPCP	0.590 (4)	0.254
Y.pestis_Owiz_127	Y.Pes/PcP/8532/F and Y.pes/PcP/9011/R	pPCP	0.831 (8)	0.055
Y.pestis_Owiz_128	Sequence-independent	pPCP	0.587 (4)	0.220
Yersinia_Owiz_271	Sequence-independent	YOPB	0.584 (4)	0.057
Yersinia_Owiz_272	Sequence-independent	YOPB	0.546 (4)	0.025
Yersinia_Owiz_273	Sequence-independent	YOPB	0.616 (4)	0.022
Yersinia_Owiz_274	Sequence-independent	YOPB	0.734 (4)	0.040
Yersinia_Owiz_292	Sequence-independent	YOPH	0.590 (4)	0.061

Yersinia_Owiz_293	Sequence-independent	YOPH	0.522 (4)	0.046
Yersinia_Owiz_316	Sequence-independent	YOPT	0.586 (4)	0.018
Yersinia_Owiz_318	Sequence-independent	YOPT	0.405 (4)	0.044
Yersinia_Owiz_331	Sequence-independent	YOPP	0.400 (4)	0.023
Yersinia_Owiz_332	Sequence-independent	YOPP	0.408 (4)	0.037

Probe signal intensity 0-1

4.3.1.2. Y. enterocolitica

The WT_Yersinia_01 ArrayTubes was also tested with a reference sample of *Y. enterocolitica*, again from pure culture. This was tested using both sequence-specific and sequence-independent ampliifcation. Results of this testing can be seen in Table 4.2. After sequence-independent amplification, 3/25 *Y. enterocolitica* specific probes hybridised with a mean hybridisation signal >0.4 on the array and seven of the generic probes showed a mean hybridisation signal >0.4. Table 4.2 also shows results for sequence-dependent amplification; primers Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R (bcsC gne) were used to amplify a 499 bp region of *Y. enterocolitica*. There were five probes that were expected to hybridise using this primer set. However, as shown in Table 4.2, there was one additional probe that also showed a positive signal. This additional probe (Y. entero_Owiz_178) was designed from the same region as the other hybridising probes; however, it was not expected to hybridise, as it did not fall within amplicon generated using the primers Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R.

Table 4.2. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of *Y. enterocolitica* with the WT_Yersinia_01 ArrayTube.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Y.enteroco_Owiz_155	Sequence-independent	YE3228	0.508 (6)	0.062
Y.enteroco_Owiz_166	Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R	YE4072	0.631 (4)	0.100
Y.enteroco_Owiz_171	Sequence-independent	YE4072	0.484 (5)	0.123
Y.enteroco_Owiz_171	Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R	YE4072	0.852 (4)	0.052
Y.enteroco_Owiz_172	Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R	YE4072	0.798 (4)	0.115
Y.enteroco_Owiz_173	Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R	YE4072	0.692 (4)	0.143
Y.enteroco_Owiz_176	Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R	YE4072	0.742 (4)	0.163
Y.enteroco_Owiz_177	Sequence-independent	YE4072	0.493 (5)	0.079
Y.enteroco_Owiz_178	Y.ent/YE4072/4447336/F and Y.ent/YE4072/4447834/R	YE4072	0.895 (4)	0.060
Yersinia_Owiz_271	Sequence-independent	YOPB	0.637 (4)	0.038
Yersinia_Owiz_273	Sequence-independent	YOPB	0.715 (6)	0.048
Yersinia_Owiz_274	Sequence-independent	YOPB	0.704 (5)	0.038
Yersinia_Owiz_292	Sequence-independent	YOPH	0.626 (5)	0.040
Yersinia_Owiz_293	Sequence-independent	YOPH	0.413 (4)	0.051
Yersinia_Owiz_318	Sequence-independent	YOPT	0.581 (6)	0.050
Yersinia_Owiz_332	Sequence-independent	YOPP	0.416 (5)	0.034

Probe signal intensity 0-1

4.3.1.3. Y. pseudotuberculosis

Y. pseudotuberculosis probes on the WT_Yersinia_01 were tested using reference samples of *Y. pseudotuberculosis* from pure culture. They were first tested using primers Y.pse/YPTB0154/178134/F and Y.pse/YPTB0154/178436/R (YPTB0154 gene) to amplify a 303 bp region, which were expected to hybridise with three probes on the array. These results are illustrated in Table 4.3. The mean signal intensity could not be calculated for these probes as the repeat experiments failed to show any hybridisation which was likely a result of defective reagents. Instead, shown in Table 4.3 is the range in signal observed between the two probes after successful hybridisation. The next test, with sequence-independent amplification, resulted in 8 of the 25 generic probes hybridising with a mean signal intensity >0.4 and there was very little cross-hybridisation seen with probes specific to *Y. pestis* or *Y. enterocolitica*. Table 4.3 also shows two generic *Yersinia* probes (Yersinia_Owiz_342 and Yersinia_Owiz_344) that displayed hybridisation after sequence-specific amplification, which were not expected to hybridise with the primer set used.

Table 4.3. Probes that showed hybridisation after sequence-independent and sequence-specific amplification of *Y. pseudotuberculosis* with the WT_Yersinia_01 ArrayTube. Where only two probes showed a signal >0.4, the signal recorded for both probes is shown.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Y.pseudo_Owiz_216	Y.pse/YPTB0154/178134/F and Y.pse/YPTB0154/178436/R	YPTB0154	0.874-0.877	NA
Y.pseudo_Owiz_218	Y.pse/YPTB0154/178134/F and Y.pse/YPTB0154/178436/R	YPTB0154	0.846-0.847	NA
Y.pseudo_Owiz_219	Y.pse/YPTB0154/178134/F and Y.pse/YPTB0154/178436/R	YPTB0154	0.852-0.852	NA
Y.pseudo_Owiz_224	Sequence-independent	YPTB0244	0.499 (5)	0.179
Y.pseudo_Owiz_234	Sequence-independent	YPTB0245	0.481 (4)	0.147
Yersinia_Owiz_271	Sequence-independent	YOPB	0.600 (4)	0.135
Yersinia_Owiz_272	Sequence-independent	YOPB	0.422 (4)	0.070
Yersinia_Owiz_273	Sequence-independent	YOPB	0.683 (4)	0.156
Yersinia_Owiz_274	Sequence-independent	YOPB	0.701 (5)	0.143
Yersinia_Owiz_292	Sequence-independent	YOPH	0.458 (4)	0.115
Yersinia_Owiz_293	Sequence-independent	YOPH	0.513 (4)	0.147
Yersinia_Owiz_342	Y.pse/YPTB0154/178134/F and Y.pse/YPTB0154/178436/R	YPTB0154	0.860-0.864	NA
Yersinia_Owiz_344	Y.pse/YPTB0154/178134/F and Y.pse/YPTB0154/178436/R	YPTB0154	0.804-0.858	NA

Probe signal intensity 0-1

4.3.1.4. T. gondii

T. gondii probes on the WT_Toxoplasma_01 ArrayTube were tested using reference samples of *T. gondii* from pure culture. A variety of primer sets were used to see which ones would work best. They were first tested using primers T.gon/B1/12/F and T.gon/B1/510/R (B1 gene), which were expected to hybridise with seven probes on the array. Additionally, there were seven probes designed from the region amplified by the primers T.gon/ROP16/440/F and T.gon/ROP16/915/R (ROP16 gene). Seven probes hybridised with a mean signal intensity >0.4 with the primer set T.gon/B1/12/F and T.gon/B1/510/R but only one probe hybridised with a mean signal intensity >0.4 with the primer set T.gon/ROP16/440/F. These results are illustrated in Table 4.4. The next test, with sequence-independent amplification resulted in 5 of the 95 generic probes hybridising with a mean signal intensity >0.4. The remaining 90 probes (not shown) had a weaker signal.

Table 4.4. Probes that showed hybridisation after sequence-independent and sequence-specific amplification of *T. gondii* with the WT_Toxoplasma_01 ArrayTube.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
ToxoplasmaUPS_359	Sequence-independent	B1	0.422 (4)	0.086
ToxoplasmaUPS_359	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.535 (6)	0.062
ToxoplasmaUPS_360	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.512 (6)	0.092
ToxoplasmaUPS_361	Sequence-independent	B1	0.494 (6)	0.064
ToxoplasmaUPS_361	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.611 (6)	0.059
ToxoplasmaUPS_362	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.726 (6)	0.073
ToxoplasmaUPS_363	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.777 (6)	0.046
ToxoplasmaUPS_364	Sequence-independent	B1	0.415 (6)	0.119
ToxoplasmaUPS_364	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.802 (6)	0.031
ToxoplasmaUPS_385	Sequence-independent	ROP1	0.906 (4)	0.030
Toxoplasma_433	Sequence-independent	GRA6	0.828 (4)	0.011
Toxoplasma_435	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.679 (4)	0.039
Toxoplasma_439	T.gon/ROP16/440/F and T.gon/ROP16/915/R	ROP16	0.643 (3)	0.288

Probe signal intensity 0-1

4.3.1.5 Hantavirus and HEV

Reference samples of Dobrava-Belgrade Virus (DOBV), Puumala Virus (PUUV) Hantaan Virus (HTNV) and Hepatitis E Virus (HEV) were obtained from a variety of sources (See Chapter 3). Despite repeated attempts with both sequence-independent and sequence-specific amplification, the pathogen was not detectable in these samples. Alternative samples were not available in the time available and therefore, reference material was not used on the arrays.

4.3.1.6 Cross-hybridisation assessment

A series of cross-hybridisation tests were performed using the ArrayTubes. The DNA was amplified using a sequence-independent approach. Only *Yersinia* and *T. gondii* reference samples were available to test for cross-hybridisation using the ArrayTubes, as at this point no other reference material was available. The probes that displayed cross-hybridisation are listed in Table 4.5. On the WT_Toxoplasma_01 array, there were seven probes in total that showed cross-hybridisation with *Y.pestis* and *Y. pseudotuberculosis*. One HEV probe was positive for all three *Yersinia* species, and one of the control probes (Ctrl_100) on the WT_Hanta_HEV_01 array yielded a positive signal with *Y. enterocolitica* and *Y. pseudotuberculosis* samples. The Ctrl_100 probe had a sequence based on Bluetongue virus, so in theory it should not have hybridised with any of the reference samples used. Two additional ArrayTubes had been designed by a colleague working on a parallel project. These arrays were for a variety of *Mycobacterium* species (WT_Mycobacteria_01) and *Francisella* species (WT_Francisella_01). There were several probes which showed cross-hybridisation on the WT_Mycobacteria_01 array. This was seen with all three *Yersinia* species and *T. gondii*. There was only one probe on the WT_Francisella_01 array that showed cross-hybridisation with *T. gondii*; there was no cross-hybridisation with any of the *Yersinia*

species. All of these probes that displayed cross-hybridisation were not used on any further microarray chips.

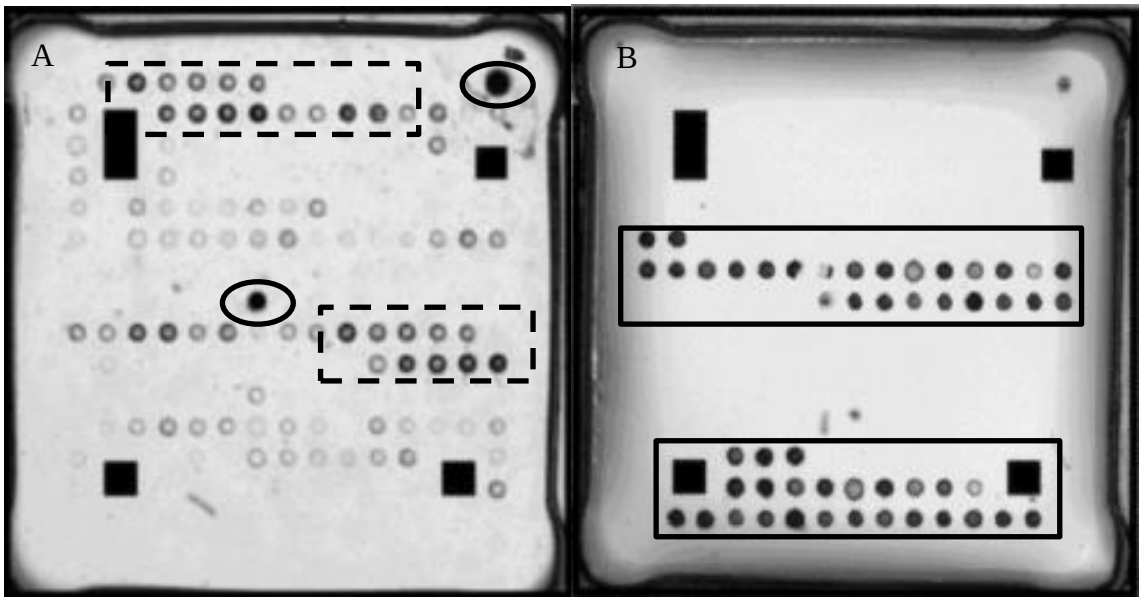
Table 4.5
Samples of *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis* and *T. gondii* were hybridised with other pathogen-specific ArrayTubes to test for any cross-hybridisation with probes specific for other pathogens. These were tested on the WT_Yersinia_01, WT_Toxoplasma_01, WT_Hanta_HEV_01, WT_Mycobacteria_01 and WT_Francisella_01 ArrayTubes. The probes on different ArrayTubes shown below are those that displayed cross-hybridisation with the pathogens specified.

Probe ID.	Sample	Array	Gene target
Toxoplasma_430	*Y. pestis*	WT_Toxoplasma_01	GRA7
Toxoplasma_440	*Y. pestis*	WT_Toxoplasma_01	ROP16
ToxoplasmaUPS_385	*Y. pestis*	WT_Toxoplasma_01	ROP1
HEV_93	*Y. pestis*	WT_Hanta_HEV_01	ORF2
M. bovis_11	*Y. pestis*	WT_Mycobacteria_01	
M. microti_05	*Y. pestis*	WT_Mycobacteria_01	gi|6013442|gb|AF189829.1|MTDRLN2 Mycobacterium microti strain 16240 direct repeat locus and insertion sequence IS6110, 3'-sequence_982-1037
M. tuberc+bovis_28	*Y. pestis*	WT_Mycobacteria_01	gi|755917060|gb|CP007299.1|:836139-836198 Mycobacterium tuberculosis 18b
Mycobacterium_01	*Y. pestis*	WT_Mycobacteria_01	gi|765685521|gb|KP116941.1|:630-688 Mycobacterium sp. GLR14 16S ribosomal RNA gene, partial sequence
Toxoplasma_425	*Y. enterocolitica*	WT_Toxoplasma_01	ROP1
Toxoplasma_433	*Y. enterocolitica*	WT_Toxoplasma_01	GRA6
ToxoplasmaUPS_373	*Y. enterocolitica*	WT_Toxoplasma_01	GRA7
ToxoplasmaUPS_385	*Y. enterocolitica*	WT_Toxoplasma_01	ROP1
Ctrl_100	*Y. enterocolitica*	WT_Hanta_HEV_01	
HEV_93	*Y. enterocolitica*	WT_Hanta_HEV_01	ORF2
M. avium_16	*Y. enterocolitica*	WT_Mycobacteria_01	gi|756081930|gb|CP010114.1|:4051480-4051539 Mycobacterium avium subsp. paratuberculosis strain E93, complete genome
M. bovis_11	*Y. enterocolitica*	WT_Mycobacteria_01	gi|31619300|emb|BX248343.1|:194113-194170 Mycobacterium bovis subsp. bovis AF2122/97 complete genome; segment 10/14
M. marinum_05	*Y. enterocolitica*	WT_Mycobacteria_01	gi|183173361|gb|CP000854.1|:5603-5658 Mycobacterium marinum M, complete genome
M. tuberc+bovis_28	*Y. enterocolitica*	WT_Mycobacteria_01	gi|755917060|gb|CP007299.1|:836139-836198 Mycobacterium tuberculosis 18b
M. ulcerans_04	*Y. enterocolitica*	WT_Mycobacteria_01	gi|118568029|gb|CP000325.1|:5600-5658 Mycobacterium ulcerans Agy99, complete genome
Mycobacterium_01	*Y. enterocolitica*	WT_Mycobacteria_01	gi|765685521|gb|KP116941.1|:630-688 Mycobacterium sp. GLR14 16S ribosomal RNA gene, partial sequence

Toxoplasma_425	*Y. pseudotuberculosis*	WT_Toxoplasma_01	ROP1
Toxoplasma_433	*Y. pseudotuberculosis*	WT_Toxoplasma_01	GRA6
ToxoplasmaUPS_385	*Y. pseudotuberculosis*	WT_Toxoplasma_01	ROP1
Ctrl_100	*Y. pseudotuberculosis*	WT_Hanta_HEV_01	Bluetongue virus 2 isolate sheep/08/Ind/ABT/Hisar segment 8, complete sequence
HEV_93	*Y. pseudotuberculosis*	WT_Hanta_HEV_01	ORF2
M. bovis_11	*Y. pseudotuberculosis*	WT_Mycobacteria_01	gi|31619300|emb|BX248343.1|:194113-194170 Mycobacterium bovis subsp. bovis AF2122/97 complete genome; segment 10/14
M. marinum_05	*Y. pseudotuberculosis*	WT_Mycobacteria_01	gi|183173361|gb|CP000854.1|:5603-5658 Mycobacterium marinum M, complete genome
M. tuberc+bovis_28	*Y. pseudotuberculosis*	WT_Mycobacteria_01	gi|755917060|gb|CP007299.1|:836139-836198 Mycobacterium tuberculosis 18b
M. ulcerans_04	*Y. pseudotuberculosis*	WT_Mycobacteria_01	gi|118568029|gb|CP000325.1|:5600-5658 Mycobacterium ulcerans Agy99, complete genome
Mycobacterium_01	*Y. pseudotuberculosis*	WT_Mycobacteria_01	gi|765685521|gb|KP116941.1|:630-688 Mycobacterium sp. GLR14 16S ribosomal RNA gene, partial sequence
Ctrl_100	*T. gondii*	WT_Hanta_HEV_01	Bluetongue virus 2 isolate sheep/08/Ind/ABT/Hisar segment 8, complete sequence
FTH_FSC155_03	*T. gondii*	WT_Francisella_01	gi|754282044|gb|CP010290.1|:1062076-1062133 Francisella tularensis subsp. tularensis SCHU S4, complete genome
M. bovis_11	*T. gondii*	WT_Mycobacteria_01	gi|31619300|emb|BX248343.1|:194113-194170 Mycobacterium bovis subsp. bovis AF2122/97 complete genome; segment 10/14
M. marinum_05	*T. gondii*	WT_Mycobacteria_01	gi|183173361|gb|CP000854.1|:5603-5658 Mycobacterium marinum M, complete genome
M. ulcerans_04	*T. gondii*	WT_Mycobacteria_01	gi|118568029|gb|CP000325.1|:5600-5658 Mycobacterium ulcerans Agy99, complete genome
Mycobact. sp_02	*T. gondii*	WT_Mycobacteria_01	gi|126232413|gb|CP000580.1|:6880-6939 Mycobacterium sp. JLS, complete genome

4.3.1.7 ArrayTube anomalies

There was an anomalous result obtained on one of the arrays. A *Y. pestis* reference sample was amplified using sequence-independent amplification, and then hybridised on the WT_Yersinia_01 ArrayTube in two separate trials. Figure 4.1 illustrates the results of the two trials. Although both images used the same sample of *Y. pestis*, there were clear differences in the spots that hybridised. Results from the first trial, seen in Figure 4.1A, indicated that generic *Yersinia* probes had hybridised to the sample as expected (shown in the dashed boxes), although the *Y. pestis* specific spots failed to hybridise with the sample. By contrast, results from the second trial can be seen in Figure 4.1B. In this trial, the specific *Y. pestis* probes hybridised (shown in a solid rectangle), but no generic Yersinia probes hybridised. The very weak signal of the biotin marker shown in Figure 4.1B was also noted. The biotin marker, which is circled in Figure 4.1A, should be present on all array images to act as an assay control as described in the Materials and Methods section. This apparent error did not occur again with the ArrayTubes or ArrayStrips, and was likely because of an error in the spotting of the probes on this particular ArrayTube.



Figures 4.1A and 4.1B. Anomalous Yersinia_01 hybridisation images.
4.2A Generic Yersinia probes show a positive signal after hybridisation with a *Y. pestis* sample (shown in dashed box)
4.2B *Y. pestis* specific probes show a positive signal after hybridisation with the same *Y. pestis* sample on another day.
Both sets of probes would be expected to hybridise with a *Y. pestis* sample. A different hybridisation pattern was observed on separate days despite using the same sample on the same ArrayTube.

4.3.2 ArrayStrip

At the request of a collaborating partner on the project, a number of additional pathogens were added to the arrays. These included: *Rickettsia typhi, Bartonella, Streptobacillus monilliformis, C. jejuni, E. coli, Salmonella*, MRSA, Cowpox virus, and LCMV. Probes for these pathogens were designed using the same methods described for the other pathogens; as these pathogens were added later in the project there was not enough time to test them on an ArrayTube before spotting them onto an ArrayStrip.

After the probes on the ArrayTubes were evaluated for their specificity, the successful probes for the different pathogens of interest were selected for use on a higher density array, the ArrayStrip (WT_Rodents_2). Probes from the ArrayTubes which did not perform as expected were not included on the ArrayStrips, and new probes were designed for inclusion on this array. These new probes for *T. gondii* and

the *Yersinia* species were then tested on the ArrayStrips. As with the ArrayTubes, reference material for each pathogen was amplified and hybridised onto the ArrayStrip to identify the working probes, and eliminate those which failed to hybridise.

4.3.2.1 C. jejuni

Reference material from *C. jejuni* was amplified using both sequence-specific and sequence-independent amplification, and then hybridised onto the array (see Figure 4.2). Results from hybridisation of the product following sequence-independent amplification can be seen (Figure 4.3A), as well as results from hybridisation of the products following amplification using three different sets of *C. jejuni* specific primers (Figures 4.3B, C and D). The signal observed on each probe can be seen in Table 4.6. The array layout is shown to the right of each of the recorded array images, with each of the *C. jejuni* probes highlighted (yellow probes hybridised as expected, green probes failed to hybridise). As can be seen from the images, there was a much stronger signal from the products of sequence-specific amplification (4.3B, C and D) compared to sequence-independent amplification (4.3A); however, the product of non-specific amplification hybridised with a larger number of the *C. jejuni* probes. There were 15 probes on the array designed to hybridise with *C. jejuni*, and these were all expected to hybridise with products of sequence-independent amplification. However, as can be seen from the recorded array image, only four probes had a mean signal intensity >0.4 (Figure 4.2A). There was no cross-hybridisation of the reference sample of *C. jejuni* with any of the other pathogen specific probes. Figure 4.2B shows the image recorded after hybridisation of *C. jeuni* DNA following amplification with primers Camp/hipO/1662/F and Camp/hipO/1984/R (hipO gene). The image shows two expected probes that hybridised with strong signal intensity. However, there were four additional probes that were expected to hybridise with this primer set, as shown in the array layout but they

did not. Figures 4.3C and 4.3D show the array image recorded after hybridisation of *C. jejuni* DNA following amplification using primers Camp/flgS/776650/F, Camp/flgS/777053/R (flgS gene) and Camp/neuA3/1316412/F, Camp/neuA3/1316858/R (neuA3 gene), respectively. For each of these primer sets, the two expected probes showed a strong positive signal with a mean signal intensity >0.4.

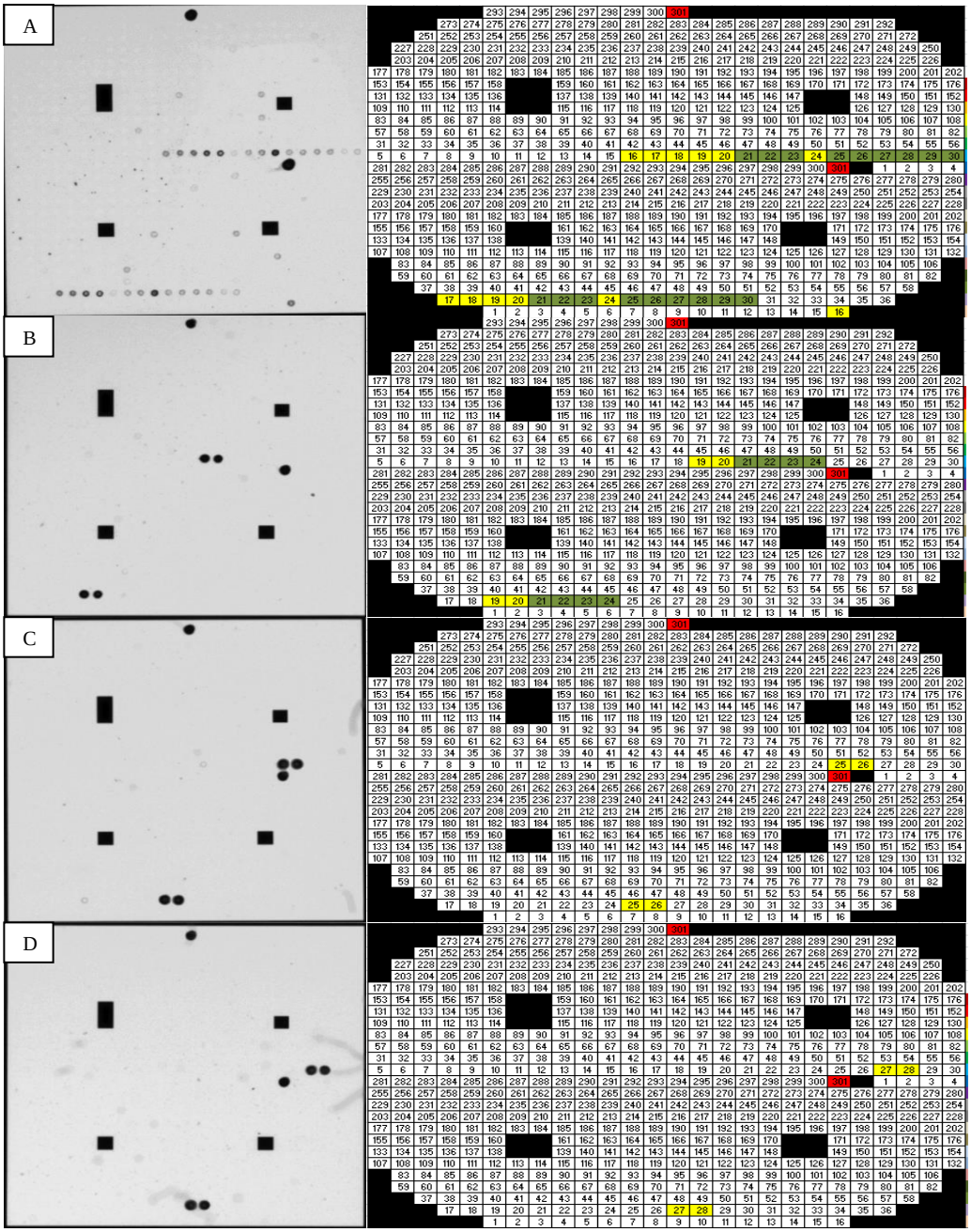


Figure 4.2. Hybridisation of amplified *C. jejuni* reference material on the WT_Rodent_Chip_02 array.

Left image: Array hybridisation image. Right image: Numerical layout of probes spotted on the arrays. Probe 301 (in red) was the spot occupied by the biotin markers. Probes in yellow were those which hybridised as expected. Green probes are those that were expected, but failed to hybridise.

A. *C. jejuni* sequence-independent amplification
B. *C. jejuni* amplification using primers Camp/hipO/1662/F and Camp/hipO/1984/R
C. *C. jejuni* amplification using primers Camp/flgS/776650/F and Camp/flgS/777053/R
D. *C. jejuni* amplification using primers Camp/neuA3/1316412/F and Camp/neuA3/1316858/R

Table 4.6. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of *C. jejuni* with the WT_Rodent_Chip_02 array ArrayStrip.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Campylo_Owiz_472	Sequence-independent	23srRNA	0.470 (8)	0.053
Campylo_Owiz_473	Sequence-independent	23srRNA	0.493 (11)	0.063
Campylo_Owiz_474	Sequence-independent	23srRNA	0.510 (12)	0.093
Campylo_Owiz_475	Camp/hipO/1662/F and Camp/hipO/1984/R	hipO	0.879 (4)	0.019
Campylo_Owiz_476	Camp/hipO/1662/F and Camp/hipO/1984/R	hipO	0.883 (4)	0.009
Campylo_Owiz_480	Sequence-independent	hipO	0.563 (8)	0.128
Campylo_Owiz_481	Camp/flgS/776650/F and Camp/flgS/777053/R	flgS	0.881 (6)	0.022
Campylo_Owiz_482	Camp/flgS/776650/F and Camp/flgS/777053/R	flgS	0.860 (6)	0.026
Campylo_Owiz_483	Camp/neuA3/1316412/F and Camp/neuA3/1316858/R	neuA3	0.826 (6)	0.048
Campylo_Owiz_484	Camp/neuA3/1316412/F and Camp/neuA3/1316858/R	neuA3	0.670 (6)	0.193

Probe signal intensity 0-1

4.3.2.2 T. gondii

Reference material was amplified using sequence-independent amplification, and one primer set for sequence-specific amplification. Each product was then hybridised on the arrays. There were 15 probes specific for *T. gondii* on the ArrayStrip. The product of sequence-independent amplification only hybridised with two probes with a mean signal intensity >0.4. Some of the other probes did produce a signal, but the signal was judged to be too weak to be of use in later stages. Sequence-specific amplification was done with the primers T.gon/B1/12/F and T.gon/B1/510/R (B1 gene) and showed a mean signal intensity >0.4 with five probes. Table 4.7 also shows some of the probes that showed cross-hybridisation with *T. gondii* DNA. These five probes were removed from the final screening array.

Table 4.7. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of *T. gondii* with the WT_Rodent_Chip_02 array. Probes marked with a ** indicates those that displayed cross-hybridisation.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
T.gondiiUPS_364	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.830 (6)	0.038
T.gondiiUPS_361	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.687 (4)	0.155
T.gondii_422	Sequence-independent	SAG3	0.525 (4)	0.032
T.gondii_435	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.837 (4)	0.049
T.gondiiUPS_755	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.500 (5)	0.120
T.gondiiUPS_756	T.gon/B1/12/F and T.gon/B1/510/R	B1	0.471 (4)	0.031
T.gondiiUPS_768	Sequence-independent	GRA6	0.734 (4)	0.029
BartonellaUPS_461	Sequence-independent	gltA	0.504 (2)	NA
HANTAUPS_570	Sequence-independent	RNA-	0.700	0.137

		dependent RNA polymerase	(4)	
LeptospiraUPS_625	Sequence-independent	LA1027	0.587 (4)	0.262
SEOVUPS_535	Sequence-independent	N protein	0.483 (4)	0.189
Y.pseudo_Owiz_224	Sequence-independent	YPTB2044	0.390 (2)	NA

Probe signal intensity 0-1

4.3.2.3 Y. pestis

Reference material of *Y. pestis* was amplified using both sequence-independent and sequence-specific amplification and then hybridised onto the array. After sequence-independent amplification, hybridisation resulted in a strong signal (>0.4) from the *Y. pestis* specific probes, and 13/15 probes hybridised (see Table 4.8). However, only one of the generic *Yersinia* probes showed a mean signal intensity >0.4 (Yersinia_Owiz_292), and the remaining 14 probes had low signal intensity. In addition, as can be seen in Table 4.8, two non-*Yersinia* probes showed cross-hybridisation with *Y. pestis* after sequence-independent amplification. After sequence-specific amplification 5/6 of the probes which were expected to hybridise showed a strong positive signal (>0.7) on the array.

Table 4.8. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of *Y. pestis* with the WT_Rodent_Chip_02 array ArrayStrip. Probes marked with a ** indicates those that displayed cross-hybridisation.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Y.pestis_Owiz_120	Sequence-independent	pPCP	0.865 (8)	0.020
Y.pestis_Owiz_121	Sequence-independent	pPCP	0.839 (8)	0.026
Y.pestis_Owiz_129	Sequence-independent	pPCP	0.875 (8)	0.012
Y.pestis_Owiz_147	Sequence-independent	pPCP	0.852 (8)	0.025
Yersinia_Owiz_292	Sequence-independent	YOPH	0.569 (4)	0.153
Y.pestisUPS_785	Sequence-independent	pPCP	0.819 (8)	0.040
Y.pestisUPS_785	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	pPCP	0.814 (8)	0.015
Y.pestisUPS_786	Sequence-independent	pPCP	0.814 (8)	0.035
Y.pestisUPS_786	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	pPCP	0.816 (16)	0.078
Y.pestisUPS_787	Sequence-independent and	pPCP	0.815 (8)	0.018
Y.pestisUPS_787	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	pPCP	0.722 (14)	0.164
Y.pestisUPS_788	Sequence-independent	pPCP	0.823 (6)	0.025

Y.pestisUPS_789	Sequence-independent	pPCP	0.846 (8)	0.022
Y.pestisUPS_789	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	pPCP	0.746 (12)	0.156
Y.pestisUPS_790	Sequence-independent	pPCP	0.793 (8)	0.051
Y.pestisUPS_791	Sequence-independent	pPCP	0.847 (8)	0.030
Y.pestisUPS_791	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	pPCP	0.784 (10)	0.185
Y.pestisUPS_792	Sequence-independent	pPCP	0.822 (8)	0.092
Yersinia_Owiz_292	Sequence-independent	YOPH	0.569 (4)	0.153
LeptospiraUPS_625	Sequence-independent	LA1027	0.597 (5)	0.090
SEOVUPS_535	Sequence-independent	N protein	0.511 (6)	0.068

Probe signal intensity 0-1

4.3.2.4 Y. enterocolitica

Only sequence-independent amplification of *Y. enterocolitica* was performed, as there was insufficient reference material and ArrayStrips remaining to test individual sequence-specific amplification. There were 15 specific *Y. enterocolitica* probes on the WT_Rodents_2_1.0 array, though only two showed a mean signal intensity >0.4. Of the generic probes, 3/15 produced a mean signal intensity >0.4. As can also be seen in Table 4.9, three probes for other pathogens cross-hybridised with *Y. enterocolitica* DNA.

Table 4.9. Probes that showed hybridisation after sequence-independent amplification of *Y. enterocolitica* with the WT_Rodent_Chip_02 array ArrayStrip. Probes marked with a ** indicates those that displayed cross-hybridisation.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Y.enteroco_Owiz_156	Sequence-independent	YE3228	0.517 (4)	0.062
Y.enteroco_Owiz_157	Sequence-independent	YE3228	0.555 (4)	0.013
Yersinia_Owiz_292	Sequence-independent	YOPH	0.470 (4)	0.050
YersiniaUPS_796	Sequence-independent	pCD1	0.586 (4)	0.083
YersiniaUPS_797	Sequence-independent	pCD1	0.780 (4)	0.026
LeptospiraUPS_625	Sequence-independent	LA1027	0.630 (4)	0.022
SEOVUPS_535	Sequence-independent	N protein	0.434 (4)	0.028
Y.pseudo_Owiz_224	Sequence-independent	YPTB2044	0.568 (4)	0.011

Probe signal intensity 0-1

4.3.2.5 Y. pseudotuberculosis

Sequence-independent amplification of the pure culture reference material for *Y. pseudotuberculosis* was performed, and the product hybridised on the ArrayTubes and ArrayStrips. Unfortunately, there was insufficient reference material and strips remaining to also carry out sequence-specific amplification of *Y. pseudotuberculosis* for comparison. However, assessment of the probes following sequence-independent amplification suggested that the probes performed well on both the ArrayTubes and the ArrayStrips. ArrayStrip test results can be seen in Table 4.10. Of the three *Yersinia* species, *Y. pseudotuberculosis* performed best on the WT_Rodents_2_1.0 array, and a positive signal was seen for most of the generic and *Y. pseudotuberculosis*-specific probes on the array. Eleven of the 15 generic Yersinia probes had a mean signal intensity >0.4. Similarly, 12/15 specific *Y. pseudotuberculosis* probes had a mean signal intensity >0.4. There were two probes that cross-hybridised with *Y. pseudotuberculosis* DNA and these are also shown in Table 4.10.

Table 4.10. Probes that showed hybridisation after sequence-independent amplification of *Y. pseudotuberculosis* with the WT_Rodent_Chip_02 array ArrayStrip. Probes marked with a ** indicates those that displayed cross-hybridisation.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
Y.pseudo_Owiz_224	Sequence-independent	YPTB0244	0.714 (4)	0.015
Y.pseudo_Owiz_226	Sequence-independent	YPTB0244	0.453 (4)	0.022
Y.pseudo_Owiz_227	Sequence-independent	YPTB0244	0.445 (4)	0.040
Y.pseudo_Owiz_229	Sequence-independent	YPTB0244	0.508 (4)	0.018
Y.pseudo_Owiz_234	Sequence-independent	YPTB0245	0.821 (4)	0.013
Y.pseudo_Owiz_236	Sequence-independent	YPTB0245	0.602 (4)	0.030
Y.pseudo_Owiz_237	Sequence-independent	YPTB0245	0.597 (4)	0.058
Y.pseudo_Owiz_238	Sequence-independent	YPTB0245	0.636	0.067

			(4)	
Y.pseudo_Owiz_243	Sequence-independent	YPTB0245	0.721 (4)	0.057
Y.pseudo_Owiz_244	Sequence-independent	YPTB0245	0.541 (4)	0.024
Y.pseudo_Owiz_245	Sequence-independent	YPTB0245	0.795 (4)	0.020
Y.pseudo_Owiz_246	Sequence-independent	YPTB0245	0.433 (4)	0.036
Yersinia_Owiz_271	Sequence-independent	YOPB	0.534 (4)	0.051
Yersinia_Owiz_272	Sequence-independent	YOPB	0.460 (4)	0.014
Yersinia_Owiz_273	Sequence-independent	YOPB	0.657 (4)	0.034
Yersinia_Owiz_274	Sequence-independent	YOPB	0.819 (4)	0.076
Yersinia_Owiz_292	Sequence-independent	YOPH	0.534 (4)	0.010
Yersinia_Owiz_293	Sequence-independent	YOPH	0.682 (4)	0.025
YersiniaUPS_796	Sequence-independent	pCD1	0.643 (4)	0.038
YersiniaUPS_797	Sequence-independent	pCD1	0.854 (4)	0.041
YersiniaUPS_798	Sequence-independent	pCD1	0.538 (4)	0.107
YersiniaUPS_800	Sequence-independent	pCD1	0.856 (4)	0.035
YersiniaUPS_801	Sequence-independent	pCD1	0.505 (4)	0.040
LeptospiraUPS_625	Sequence-independent	LA1027	0.757 (4)	0.095
SEOVUPS_535	Sequence-independent	N protein	0.658 (4)	0.218

Probe signal intensity 0-1

4.3.2.6 Salmonella

Reference material from pure culture *Salmonella enterica* serovars (*S*. Typhimurium and *S*. Enteritidis) was amplified using sequence-independent amplification, and then hybridised onto the array. Only three generic *Salmonella* probes hybridised with signal intensity >0.4 for *S*. Typhimurium and *S*. Enteritidis (see Table 4.11). When *S*. Typhimurium was hybridised after sequence-independent amplification, the five expected probes hybridised with a mean signal intensity >0.4. Several probes also showed cross-hybridisation.

Sequence-dependent amplification was performed on DNA from pure cultures of *S.* Typhimurium and *S.* Enteritidis, and the products hybridised on the arrays (see Table 4.11). Three generic probes were expected to hybridise with products resulting from amplification using primers Salm/CDP/2167279/F and Salm/CDP/2005357/R. For both Salmonella types, all three probes hybridised strongly (>0.7) with no background noise recorded. Five probes were designed to hybridise specifically with products resulting from amplification using the *S*. Typhimurium specific primers Salm/flag/1366055/F and Salm/flag/1366482/R. All five of these probes hybridised with a mean signal intensity >0.8.

Table 4.11. Probes that showed hybridisation after sequence-independent and sequence-specific amplification of *Salmonella* spp with the WT_Rodent_Chip_02 array ArrayStrip. Probes marked with a ** indicates those that displayed cross-hybridisation.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
SalmonellaUPS_709	Sequence-independent	Endonuclease	0.508 (12)	0.121
SalmonellaUPS_709	Salm/CDP/2167279/F and Salm/CDP/2005357/R	Endonuclease	0.880 (10)	0.023
SalmonellaUPS_710	Sequence-independent	Endonuclease	0.527 (10)	0.170
SalmonellaUPS_710	Salm/CDP/2167279/F and Salm/CDP/2005357/R	Endonuclease	0.863 (10)	0.034
SalmonellaUPS_711	Sequence-independent	Endonuclease	0.404 (8)	0.071
SalmonellaUPS_711	Salm/CDP/2167279/F and Salm/CDP/2005357/R	Endonuclease	0.875 (10)	0.035
SalmonellaUPS_733	Sequence-independent	CDP-abeguose synthase	0.802 (4)	0.015
SalmonellaUPS_733	Salm/flag/1366055/F and Salm/flag/1366482/R	CDP-abeguose synthase	0.887 (4)	0.010
SalmonellaUPS_734	Sequence-independent	CDP-abeguose synthase	0.813 (4)	0.007
SalmonellaUPS_734	Salm/flag/1366055/F and Salm/flag/1366482/R	CDP-abeguose synthase	0.878 (4)	0.021
SalmonellaUPS_735	Sequence-independent	CDP-abeguose synthase	0.654 (4)	0.018
SalmonellaUPS_735	Salm/flag/1366055/F and Salm/flag/1366482/R	CDP-abeguose synthase	0.878 (4)	0.024
SalmonellaUPS_736	Sequence-independent	CDP-abeguose synthase	0.650 (4)	0.014
SalmonellaUPS_736	Salm/flag/1366055/F and Salm/flag/1366482/R	CDP-abeguose synthase	0.898 (4)	0.002
SalmonellaUPS_737	Sequence-independent	CDP-abeguose synthase	0.616 (4)	0.013
SalmonellaUPS_737	Salm/flag/1366055/F and Salm/flag/1366482/R	CDP-abeguose synthase	0.887 (4)	0.015
LeptospiraUPS_625	Sequence-independent	LA1027	0.576 (4)	0.001
T.gondiiUPS_768	Sequence-independent	GRA6	0.437 (4)	0.012

Probe signal intensity 0-1

4.3.2.7 Lymphocytic Choriomeningitis Virus (LCMV)

Reference material of LCMV was amplified using both sequence-independent and sequence-specific amplification, and then hybridised onto the array; results are listed in Table 4.12. After sequence-independent amplification, hybridisation resulted in a strong signal from the LCMV specific probes, and 12/15 probes hybridised with a mean signal intensity >0.4. There were two primer sets designed for LCMV: LCMV/S/1742/F and LCMV/S/2391/R (S segment), and LCMV/NP/779/F and LCMV/NP/1448/R (nucleoprotein). These were designed to hybridise with three and five probes, however as seen from Table 4.12, the first primer set only showed hybridisation with one of the probes. The second primer set performed as expected and all five probes had a positive hybridisation signal (>0.7). There were several probes that showed cross-hybridisation with LCMV DNA after sequence-independent amplification, and these are also listed in Table 4.12.

Table 4.12. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of LCMV with the WT_Rodent_Chip_02 array ArrayStrip. Probes marked with a ** indicates those that displayed cross-hybridisation.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
LCMVUPS_595	Sequence-independent	Glycoprotein C	0.865 (4)	0.034
LCMVUPS_597	Sequence-independent	Glycoprotein C	0.885 (4)	0.016
LCMVUPS_601	Sequence-independent	S segment	0.857 (4)	0.031
LCMVUPS_603	Sequence-independent	S segment	0.487 (4)	0.058
LCMVUPS_606	Sequence-independent	Glycoprotein C	0.876 (4)	0.021
LCMVUPS_606	LCMV/S/1742/F and LCMV/S/2391/R	S segment	0.640 (4)	0.195
LCMVUPS_611	Sequence-independent	Nucleoprotein	0.852 (4)	0.014
LCMVUPS_612	Sequence-independent	Nucleoprotein	0.831 (4)	0.030
LCMVUPS_612	LCMV/NP/779/F and LCMV/NP/1448/R	Nucleoprotein	0.895 (6)	0.004
LCMVUPS_613	Sequence-independent	Nucleoprotein	0.784 (4)	0.039
LCMVUPS_613	LCMV/NP/779/F and LCMV/NP/1448/R	Nucleoprotein	0.867 (6)	0.033
LCMVUPS_614	Sequence-independent	Nucleoprotein	0.819 (4)	0.038
LCMVUPS_614	LCMV/NP/779/F and LCMV/NP/1448/R	Nucleoprotein	0.892 (6)	0.009
LCMVUPS_615	Sequence-independent	Nucleoprotein	0.823 (4)	0.017
LCMVUPS_616	Sequence-independent	Nucleoprotein	0.794 (4)	0.018
LCMVUPS_616	LCMV/NP/779/F and LCMV/NP/1448/R	Nucleoprotein	0.892 (6)	0.002
LCMVUPS_617	Sequence-independent	Nucleoprotein	0.822 (4)	0.027
LCMVUPS_617	LCMV/NP/779/F and LCMV/NP/1448/R	Nucleoprotein	0.895 (6)	0.005
HANTAUPS_570	Sequence-independent	RNA-dependent RNA polymerase	(0.556) 4	0.104
LeptospiraUPS_625	Sequence-independent	LA1027	0.592 (4)	0.033
SEOVUPS_535	Sequence-independent	Nucleoprotein	0.714 (4)	0.061
T.gondiiUPS_768	Sequence-independent	GRA6	0.576 (4)	0.023

Probe signal intensity 0-1

4.3.2.8 Cowpox Virus (CPXV)

Reference material from CPXV was amplified using both sequence-specific and sequence-independent amplification, and then hybridised onto the array (see Figure 4.3). Results from hybridisation of the product following sequence-independent amplification (Figure 4.3A) can be seen, as well as results from hybridisation of the product following amplification with CPXV specific primers (Figure 4.3B). The array layout is shown to the right of each of the recorded array images, with each of the CPXV probes highlighted (yellow probes hybridised as expected, green probes failed to hybridise). As can be seen from Figure 4.3A, there was no hybridisation with the products of sequence-independent amplification. There were 15 probes on the array designed to hybridise with CPXV, and these were all expected to hybridise with products of sequence-independent amplification. However, as can be seen from the recorded array image, none of the probes displayed a positive signal. This was repeated on three separate occasions, all with the same result. Figure 4.3B shows the image recorded after hybridisation of CPXV DNA following amplification with primers Cow/HA/178301/F and Cow/HA/178929/R (HA gene). The image shows three expected probes that hybridised with strong signal intensity (>0.8). However, there were two additional probes that were expected to hybridise with this primer set, as shown in the array layout that did not produce a positive hybridisation signal. Table 4.13 shows the identity of the hybridising probes.

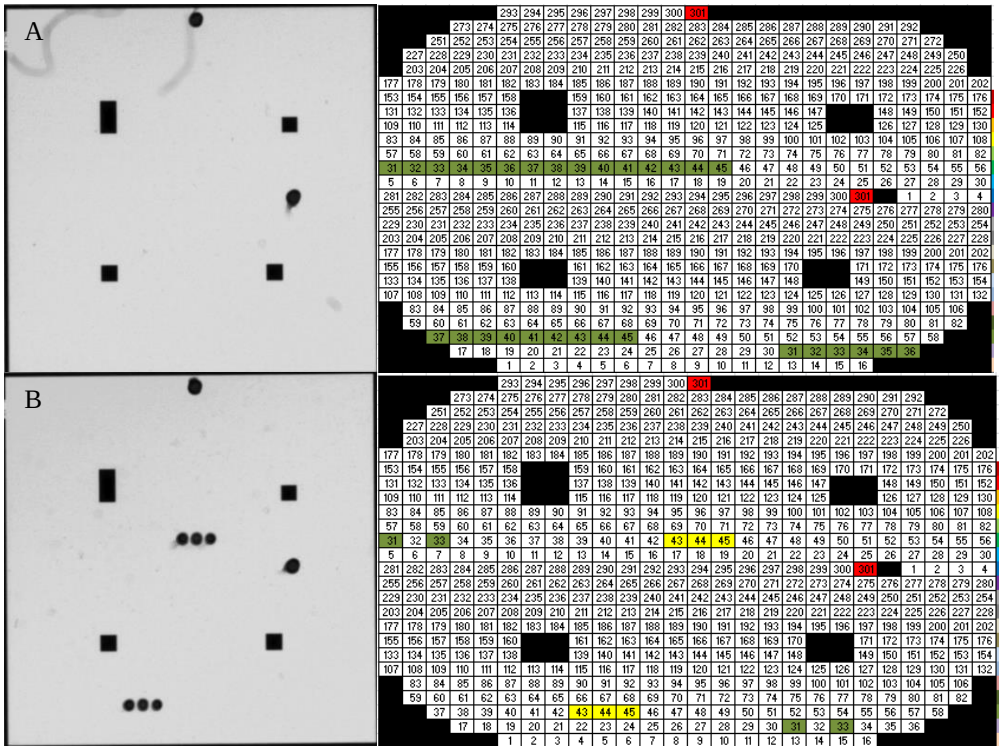


Figure 4.3. Hybridisation of CPXV reference material on the WT_Rodent_Chip_02 array.

Left column: Array hybridisation image. Right column: Numerical layouy of probes spotted on the arrays. Probe 301 (in red) was the spot occupied by the biotin markers. Probes in yellow were those which hybridised as expected. Green probes were expected, but failed to hybridise. Areas that have been blacked out in the four corners are unused spots.

A. CPXV sequence-independent amplification
B. CPXV amplification using primers Cow/HA/178301/F and Cow/HA/178929/R

Table 4.13. Probes that showed hybridisation after sequence-independent and sequence-dependent amplified CPXV with the WT_Rodent_Chip_02 array ArrayStrip.

Probe ID.	Amplification method		Gene target	Probe mean signal (number of probes)	Standard deviation
CowpoxUPS_821	Cow/HA/178301/F Cow/HA/178929/R	and	HA gene	0.872 (10)	0.017
CowpoxUPS_822	Cow/HA/178301/F Cow/HA/178929/R	and	HA gene	0.816 (10)	0.018
CowpoxUPS_823	Cow/HA/178301/F Cow/HA/178929/R	and	HA gene	0.867 (10)	0.867

Probe signal intensity 0-1

4.3.2.9 *Leptospira*

Reference material of *Leptospira* was amplified using both sequence-independent and sequence-specific amplification, and then hybridised onto the array; results are listed in Table 4.14. After sequence-independent amplification, hybridisation resulted in a mean hybridisation signal >0.4 from 4/15 of the *Leptospira* specific probes (see Table 4.14). There was no cross-hybridisation observed after sequence-independent amplification. Two primer sets were designed for *Leptospira*: Lept/sphH/3501394/F and Lept/sphH/3501818/R (*sphH* gene), and Lept/hemo/3184769/F and Lept/hemo/3185246/R (haemolysin). Three probes were expected to hybridise with the amplicons produced by these primers, however only two probes showed a positive signal.

Table 4.14. Probes that showed hybridisation after sequence-independent and sequence-dependent amplification of *Leptospira* with the WT_Rodent_Chip_02 array ArrayStrip.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
LeptospiraUPS_623	Sequence-independent	LA1027	0.465 (6)	0.036
LeptospiraUPS_625	Sequence-independent	LA1027	0.563 (8)	0.076
LeptospiraUPS_629	Sequence-independent	LA1029	0.488 (7)	0.067
LeptospiraUPS_630	Lept/hemo/3184769/F and Lept/hemo/3185246/R	LA1029	0.672 (4)	0.033
LeptospiraUPS_636	Sequence-independent	SphH	0.514 (14)	0.059
LeptospiraUPS_636	Lept/sphH/3501394/F and Lept/sphH/3501818/R	SphH	0.873 (4)	0.028

Probe signal intensity 0-1

4.3.2.10 E. coli

Reference material of both Stx-positive and Stx-negative *E. coli* was amplified using sequence-independent and sequence-specific amplification, and then hybridised onto the array; results are listed in Table 4.15. After sequence-independent amplification, hybridisation resulted in no signal produced from the *E. coli* specific probes for either sample. There was one primer set for Stx-positive *E. coli* types: E.coliStxF and E.coliStxR (*stx* gene), and two probes were expected to hybridise after using these specific primers. Both of the probes designed for this region hybridised as expected with a strong positive signal (>0.8) and no cross-hybridisation was observed with any other probes. There was no amplification observed after sequence-specific amplification with these primers for the Stx-negative sample so hybridisation was not performed with this sample.

Table 4.15. Probes that showed hybridisation after sequence-dependent amplification of *E. coli* with the WT_Rodent_Chip_02 array ArrayStrip.

Probe ID.	Amplification method	Gene target	Probe mean signal (number of probes)	Standard deviation
E.coliUPS_519	E.coliStxF and E.coliStxR	Stx1	0.885 (10)	0.025
E.coliUPS_520	E.coliStxF and E.coliStxR	Stx1	0.860 (10)	0.038

Probe signal intensity 0-1

4.3.2.11. Multiplex testing

After sequence-independent and singleplex sequence-specific amplification had been performed with the arrays, multiplex sequence-specific amplification was then tested. The primers shown in Table 2.4 from Chapter 2 were chosen to be used in the multiplex reaction as a result of their superior performance in singleplex PCR reactions. Initial tests which used the recommended primer concentrations proved unsuccessful for some pathogens (CPXV and LCMV) when used in a multiplex format. To address this, a higher primer concentration was used for primer sets that did not appear to work as expected in the initial tests. Of the samples available the primer sets that did not work as expected were Cow/HA/178301/F-Cow/HA/178929/R and LCMV/S/1742/F-LCMV/S/2391/R. For these, instead of using 10 µl per primer (2 µM) in the primer mix, 15 µl of each of these primers was used for a concentration in the primer mix of 3 µM.

Figure 4.4 shows the gel electrophoresis image of the sequence-specific PCR amplification of individual pathogens. As it was unlikely that a sample would contain all these pathogens it was important to test the effectiveness of the primer mix when only one pathogen was present. As seen here, there were strong bands in the majority of lanes. The amplicons produced by the specific and generic primers for *Y. pestis* and *Y. enterocolitica* in Lanes I and K were very similar in size and would occupy the same space on the gel. Lane J shows the *Y. pseudotuberculosis* sample with only one band observed; from the size, this was expected to be the amplicons produced by the generic *Yersinia* primers (514 bp). The *Y. pseudotuberculosis* specific primers did not appear to produce an amplicon, as no amplicon was visible at the expected size (303 bp). At this point the reference material for *Y. pseudotuberculosis* had been exhausted, and therefore it was not possible to repeat this experiment. The amplicon in Lane D also appears to be of the incorrect size as the LCMV amplicon was expected to be 670 bp, whereas the band on the gel image looks like it is smaller than that of Cowpox (629 bp)

in Lane C. The two bands in Lane F were assumed to represent the specific *S.* Typhiumurium and the generic Salmonella amplicons. This was expected as the multi-pathogen primer mix contained primers specific for *S.* Typhimurium and primers to amplify a region which is common to many *Salmonella* serovars. Whilst the band seen in Lane L for *T. gondii* is very dim, analysis of the recorded image showed that all of the pathogens in the sample, including *T. gondii*, hybridised with the expected specific probes on the array. Figure 4.5 shows the image recorded after hybridisation with the pathogen mix after amplification with the multiplex primer mix.

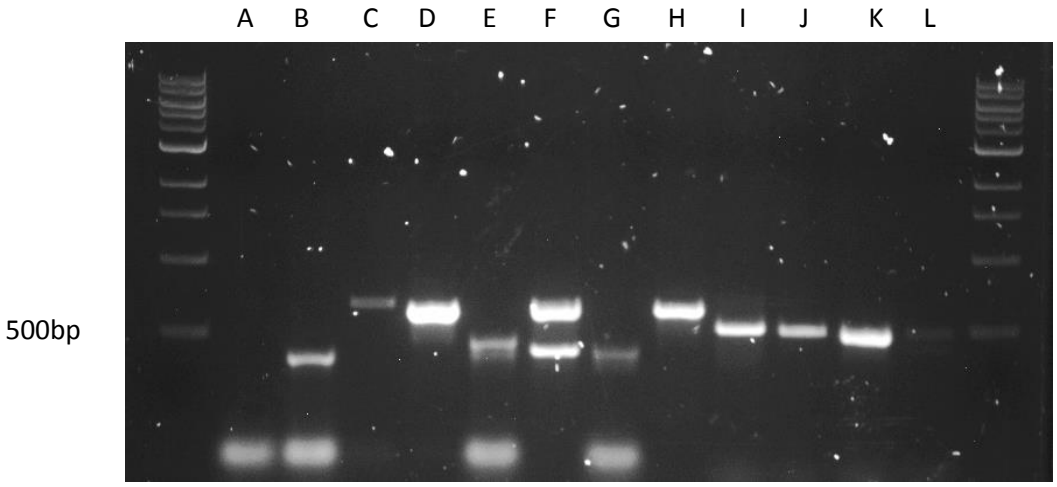


Figure 4.4. Gel electrophoresis image after multiplex amplification. Lanes A-K show the individual amplicons produced after amplification with the multiplex primer mix and the expected product size.

A.	Water sample	None expected
B.	*C. jejuni*	404 bp
C.	CPXV	629 bp
D.	LCMV	670 bp
E.	*Leptospira*	477 bp
F.	*S.*Typhimurium	633 bp
G.	*S.*Enteritidis	428 bp
H.	*E.coli*	620 bp
I.	*Y. pestis*	529 bp
J.	*Y. pseudotuberculosis*	303 bp
K.	*Y. enterocolitica*	499 bp
L.	*T. gondii*	499 bp

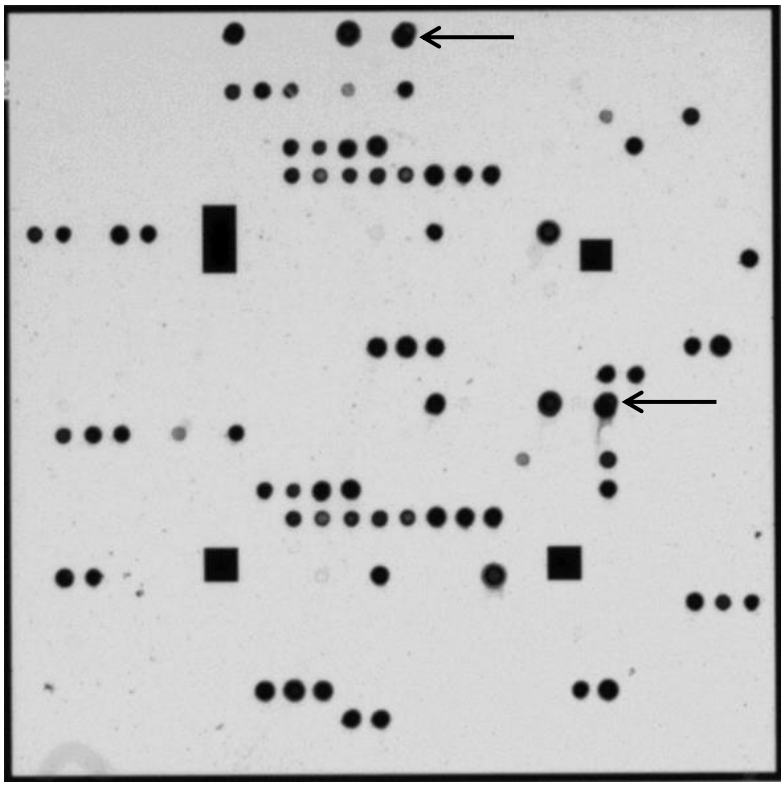


Figure 4.5. Array hybridisation image after multiplex amplification of multiple pathogens. The biotin markers are labelled by arrows. Probes have hybridised with amplified DNA of *C. jejuni*; CPXV; *E. coli*; LCMV; *Leptospira*; *Salmonella*; *T. gondii*; *Y. enterocolitica*; *Y. pestis*, and Generic *Yersinia*.

4.3.2.12 Mycobacterium and Francisella

As there were probes for *Mycobacterium* and *Fransicella* species on the final array, cross-hybridisation tests were conducted, using DNA from these pathogens on the pilot ArrayStrip. The products from sequence-independent and sequence-specific amplifications of the pathogen mix used in the multiplex testing were also hybridised onto the ScreenChip_01, which had been developed in a separate project. This array had probes for *Mycobacterium, Fransicella, T. gondii, Y. enterocolitica, Y. pestis, Y. pseudotuberculosis* and generic *Yersinia* probes. Following sequence-independent amplification of the pathogen mix used in the multiplex experiment, no cross-hybridisation was observed on any of the probes specific for *Mycobacterium* or *Francisella*. The products of sequence-specific amplification of *Mycobacterium* and *Fransicella* were hybridised on the WT_Rodents_2 array to see if there was any cross-hybridisation with any of the probes specific to the other pathogens. Table 4.16 shows the probes that produced a signal after hybridisation with *Mycobacteria* and *Fransicella*. No probes exhibited cross-hybridisation with any of the *Fransicella* species used (*F. tularensis, F. novicida, F. holarctica, F. mediastatica*). There were two probes that cross-hybridised with the *Mycobacterium* species used.

Table 4.16. Probes that showed cross-hybridisation after *Mycobacterium* species were hybridised with the WT_Rodents_2 array after sequence-dependent amplification.

Pathogen	**Probe ID.**		**Amplification method**
Mycobacterium avium subspecies paratuberculosis	S.monilUPS_745	S.monil_750	TB_16S-F and TB_16S-R
Mycobacterium bovis	S.monilUPS_745	S.monil_750	TB_16S-F and TB_16S-R

4.3.2.13 Comparison of hybridisation signals after sequence-independent and sequence-specific amplification

In several cases, the signal observed after sequence-independent amplification had a lower intensity than that for sequence-specific amplification on the same probe. An analysis was performed to determine if this was significant. Table 4.17 shows the mean signal produced after both sequence-independent and sequence-specific amplification of probes where the mean hybridisation signal after both amplification methods was >0.4. As no probes produced a signal after hybridisation with *E. coli* or CPXV after sequence-independent amplification, these were not included. The p value after a student's T test is also included. The null hypothesis was that no difference would be observed in signal intensity.

For the majority of probes, there was a significant difference (p-value <0.05) between the signal for sequence-independent and sequence-specific amplification. In general, a higher signal intensity was observed after hybridisation with sequence-specific amplicons (16/26 probes). There were however, a few exceptions to this and 4/26 probes showed a significantly higher hybridisation signal after sequence-independent amplification. There were six probes for which there was not a significant difference between the two amplification methods and for these probes the signal intensity was comparable.

Table 4.17. Results of a t.test comparison of the signal measured between sequence-independent and sequence-specific amplification for the same probe (** indicates a significant result).

Pathogen	Probe ID.	Sequence-independent mean signal (number of probes)	Standard deviation	Sequence-specific mean signal (number of probes)	Standard deviation	p-value
LCMV	LCMVUPS_606	0.876 (4)	0.021	0.640 (4)	0.195	0.037**
	LCMVUPS_612	0.831 (4)	0.030	0.895 (6)	0.004	0.013**
	LCMVUPS_613	0.784 (4)	0.039	0.867 (6)	0.033	0.004**
	LCMVUPS_614	0.819 (4)	0.038	0.892 (6)	0.009	0.011**
	LCMVUPS_616	0.794 (4)	0.018	0.892 (6)	0.002	0.001**
	LCMVUPS_617	0.822 (4)	0.027	0.895 (6)	0.005	0.006**
Leptospira	LeptospiraUPS_636	0.514 (14)	0.059	0.873 (4)	0.028	<0.001**
Salmonella	SalmonellaUPS_709	0.508 (12)	0.121	0.880 (10)	0.023	<0.001**
	SalmonellaUPS_710	0.613 (14)	0.200	0.887 (6)	0.009	0.005**
	SalmonellaUPS_711	0.404 (8)	0.071	0.875 (10)	0.035	<0.001**
	SalmonellaUPS_733	0.802 (4)	0.015	0.887 (4)	0.010	<0.001**
	SalmonellaUPS_734	0.813 (4)	0.007	0.878 (4)	0.021	0.006**
	SalmonellaUPS_735	0.654 (4)	0.018	0.878 (4)	0.024	<0.001**
	SalmonellaUPS_736	0.650 (4)	0.014	0.898 (4)	0.002	<0.001**

	SalmonellaUPS_737	0.616 (4)	0.013	0.887 (4)	0.015	<0.001**
T. gondii	ToxoplasmaUPS_359	0.422 (4)	0.086	0.535 (6)	0.062	0.063
	ToxoplasmaUPS_361	0.494 (4)	0.064	0.611 (6)	0.059	0.071
	ToxoplasmaUPS_364	0.802 (6)	0.031	0.415 (4)	0.119	0.003**
Y. enterocolitica	Y.enteroco_Owiz_171	0.484 (5)	0.123	0.852 (4)	0.052	0.001**
Y. pestis	Y.pestis_Owiz_117	0.469 (4)	0.179	0.697 (10)	0.150	0.003**
	Y.pestis_Owiz_127	0.590 (4)	0.254	0.831 (8)	0.055	0.048
	Y.pestisUPS_785	0.819 (8)	0.040	0.814 (8)	0.015	0.401
	Y.pestisUPS_786	0.814 (8)	0.035	0.816 (16)	0.078	0.163
	Y.pestisUPS_787	0.815 (8)	0.018	0.722 (14)	0.164	0.007**
	Y.pestisUPS_789	0.846 (8)	0.022	0.746 (12)	0.156	0.019**
	Y.pestisUPS_791	0.847 (8)	0.030	0.784 (10)	0.185	0.160

Probe signal intensity 0-1

4.3.2.14 Comparison of hybridisation signals after singleplex and multiplex sequence-specific amplification

A comparison between the signal recorded between singleplex and multiplex amplification was then made to see if there was any statistical difference between results from these two methods. Table 4.18 shows the probes that hybridised after singleplex and multiplex PCR. The mean signal intensity for each probe is shown together with the p-value for the difference between the two intensities. The table shows that there were not many significant differences between the two (11/28 probes). Even when there was a significant difference, the actual signal intensity was still relatively high. Occasionally the signal intensity was stronger after singleplex amplification; however the opposite was true for other probes, neither method appeared to produce consistently higher signals than the other.

Table 4.18. Results of a t.test comparison of the signal measured between multiplex and singleplex PCR amplification for the same probe (** indicates a significant result).

Pathogen	Probe ID.	Multiplex amplification (number of probes)	Standard deviation	Singleplex amplification (number of probes)	Standard deviation	p-value
C. jejuni	Campylo_Owiz_481	0.893 (6)	0.009	0.881 (6)	0.022	0.149
	Campylo_Owiz_482	0.863 (6)	0.027	0.860 (6)	0.026	0.419
CPXV	CowpoxUPS_821	0.647 (4)	0.016	0.872 (10)	0.017	<0.001**
	CowpoxUPS_822	0.565 (4)	0.023	0.816 (10)	0.018	<0.001**
	CowpoxUPS_823	0.562 (4)	0.032	0.867 (10)	0.026	<0.001**
E. coli	E.coliUPS_519	0.843 (6)	0.041	0.860 (10)	0.038	0.422
	E.coliUPS_520	0.878 (6)	0.031	0.885 (10)	0.025	0.476
LCMV	LCMVUPS_612	0.895 (6)	0.002	0.895 (6)	0.004	0.430
	LCMVUPS_613	0.842 (6)	0.023	0.867 (6)	0.033	0.133
	LCMVUPS_614	0.884 (6)	0.004	0.892 (6)	0.009	0.080
	LCMVUPS_616	0.886 (6)	0.015	0.892 (6)	0.002	0.211
	LCMVUPS_617	0.891 (6)	0.006	0.895 (6)	0.005	0.246
Salmonella	SalmonellaUPS_709	0.895 (6)	0.004	0.880 (10)	0.023	0.011**
	SalmonellaUPS_710	0.893 (6)	0.006	0.887 (6)	0.009	0.123
	SalmonellaUPS_711	0.888 (6)	0.006	0.875 (6)	0.035	0.076

T. gondii	T.gondiiUPS_361	0.729 (6)	0.137	0.611 (12)	0.056	0.061
	T.gondiiUPS_364	0.860 (6)	0.054	0.772 (7)	0.084	0.049**
	T.gondii_435	0.860 (6)	0.050	0.774 (10)	0.092	0.297
	T.gondiiUPS_755	0.655 (5)	0.153	0.500 (5)	0.120	0.174
	T.gondiiUPS_756	0.663 (4)	0.073	0.471 (4)	0.031	0.002**
Y. enterocolitica	Y.enteroco_Owiz_178	0.843 (6)	0.027	0.895 (4)	0.060	0.054
Y. pestis	Y.pestisUPS_785	0.757 (6)	0.033	0.814 (8)	0.015	0.002**
	Y.pestisUPS_786	0.882 (6)	0.004	0.816 (16)	0.078	0.022**
	Y.pestisUPS_787	0.880 (6)	0.006	0.722 (14)	0.164	0.014**
	Y.pestisUPS_789	0.797 (6)	0.017	0.746 (12)	0.156	0.339
	Y.pestisUPS_791	0.864 (6)	0.014	0.784 (10)	0.185	0.105
Yersinia	Yersinia_Owiz_292	0.896 (6)	0.003	0.744 (8)	0.143	0.009**
	Yersinia_Owiz_293	0.866 (6)	0.015	0.526 (4)	0.126	0.005**

Probe signal intensity 0-1

4.3.2.15 Analysis of probe performance after sequence-independent amplification

Using the predicted probe melting temperature, an analysis was performed to see whether this had any effect on probe hybridisation. This was only performed with probes that hybridised after sequence-independent amplification. The probes on the ArrayTube WT_Yersinia_01 were used for this analysis. A total of 33 probes hybridised with a signal intensity >0.4 on the WT_Yersinia_01 array, with a mean Tm of 81.52°C. These included probes for *Y. pestis, Y. enterocolitica*, *Y. pseudotuberculosis* and the generic *Yersinia* probes. This temperature was lower than that for the non-hybridising probes (52 in total) with a mean Tm of 82.02°C. A Wilcoxon rank sum test was used as the data were found to be not normally distributed. The result of the Wilcoxon rank sum test indicated that there was not a significant difference between the two with a p value of 0.82.

Table 4.19. Analysis of probe performance based on predicted melting temperature (Tm).

	Mean Tm (°C)	Standard deviation	p-value
Hybridising probes (33)	81.52	2.55	0.82
Non-hybridising probes (52)	82.02	3.69	

4.3.3 Sensitivity testing

The sensitivity of the array was measured by using serial dilutions of *Y. pestis* DNA. Figure 4.6 shows the image recorded after gel electrophoresis of serially diluted spiked samples of rodent tissue with *Y. pestis* DNA. No band was visible when the tissue sample was spiked with water. Lanes B (copy number 3.47×10^9), C (copy number 1.76×10^7) and D (copy number 8.57×10^4) all had visible bands in them, although the band visible in lane D was not as intense as the band in lanes B and C. There was no band visible in lanes E (copy number 4.39×10^2) and F (copy number 1).

The array images shown in Figure 4.7 were produced after hybridisation on the Yersinia_01 ArrayTube with the PCR products shown in Figure 4.6. The biotin markers on each array are labelled with an arrow. On the Yersinia_01 array only two probes were expected to hybridise with the primer set used. As can be seen from the images produced after hybridisation, the two expected probes hybridised with samples B, C and D. For samples E and F there was no apparent hybridisation. Table 4.19 shows the signal intensity for the two probes on each array. There was a negligible signal, as expected, on the non-spiked tissue sample. For samples B and C there was a similar signal intensity, both of which were easily detectable. The signal intensity for sample D was variable between the two probes. The signal for probe Y.pestis_Owiz_117 was 0.235, and was therefore likely to be too low for detection. The signal for probe Y.pestis_Owiz_127 on the other hand was 0.443, which was above the threshold limit for detection. The signal intensity produced by sample E was negligible for probe Y.pestis_Owiz_117. However, the signal was higher for probe Y.pestis_Owiz_127 (0.194) and was visible, although it was extremely faint. This would, however be considered too low for positive identification. The signal for sample F was negligible, which indicated that no hybridisation took place.

Figure 4.9 shows the results of qPCR testing on the samples. A control sample of IBV was included as an assay control and is shown in image G. As expected for the negative control sample, there was no amplification. Samples B, C, D and E were detectable by qPCR. For each image the curve had shifted progressively further to the right, as a greater number of PCR cycles were required to amplify the product above the threshold value. The DNA in sample B was detectable after 18 cycles. For samples C, D, and E the cycle number was 24, 32 and 36 respectively. The DNA in sample F appeared to have been too low for qPCR to detect and no amplification was observed.

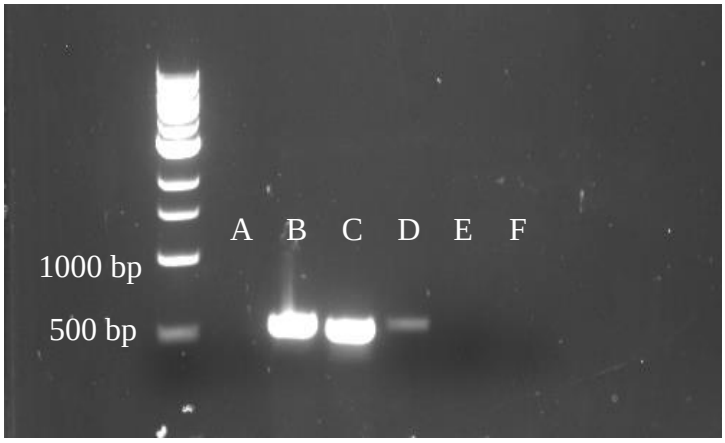


Figure 4.6. Gel electrophoresis image after a 200-fold serial dilution of *Y. pestis* DNA. The expected amplicon size was 529 bp.

A. Water sample
B. Copy number 3.47×10^{9}
C. Copy number 1.76×10^{7}
D. Copy number 8.57×10^{4}
E. Copy number 4.39×10^{2}
F. Copy number 1

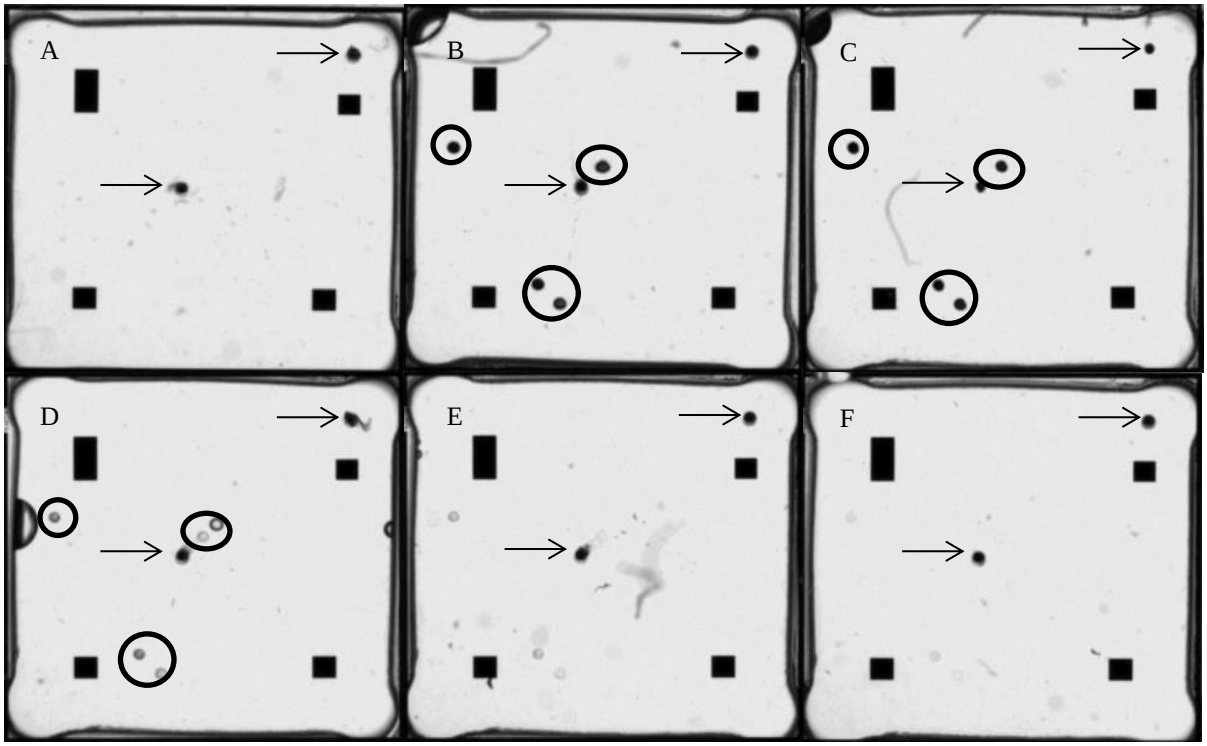
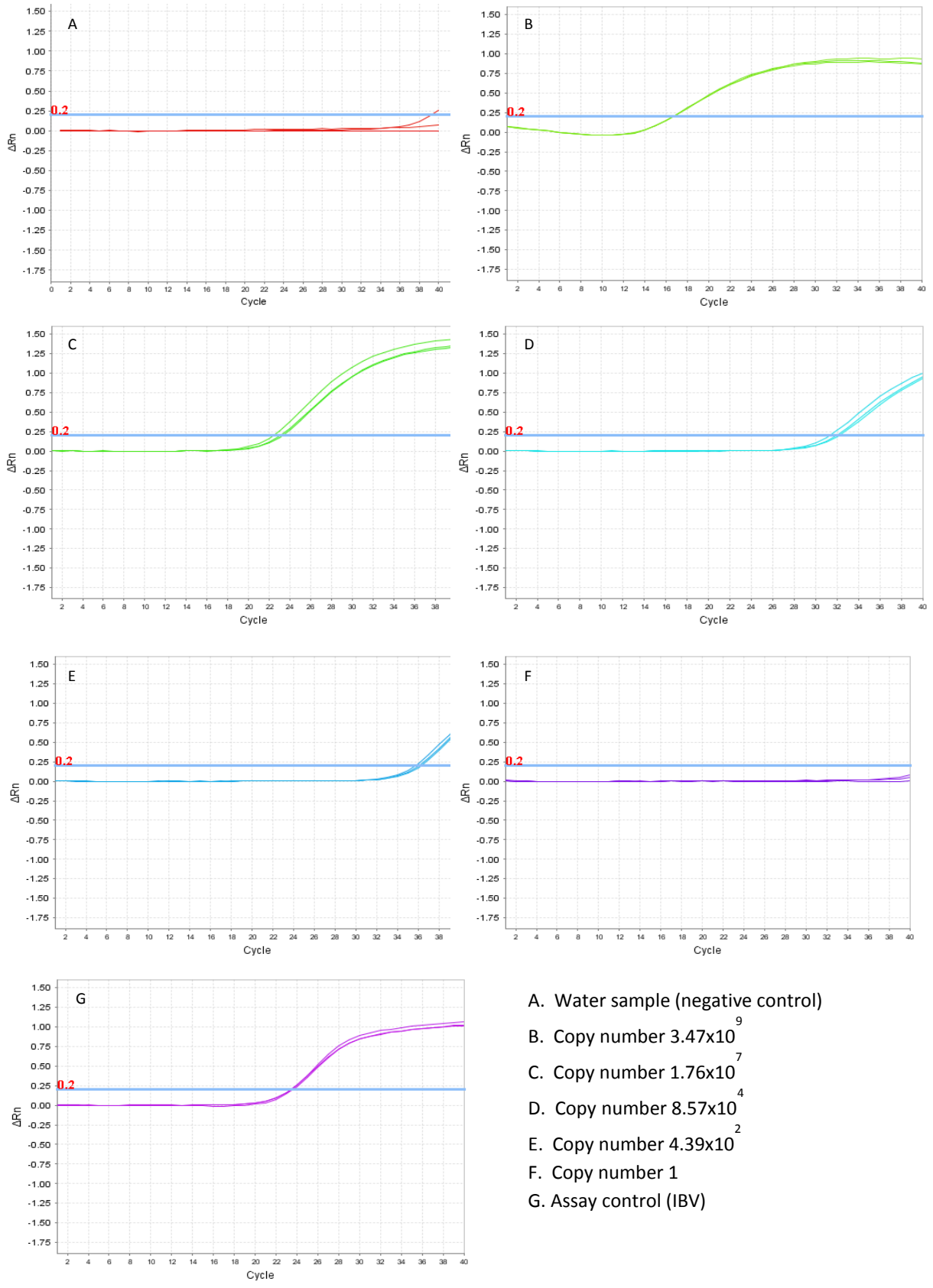


Figure 4.7. Images A-F shows the hybridisation of the PCR products from Lanes A-F in Figure 7.
The probes displaying hybridisation are circled. The biotin markers on each array are labelled with an arrow.

Table 4.20. Probe signal intensity for serially diluted samples of *Y. pestis*

Array Image	Probe ID	Signal intensity
A (water sample)	Y.pestis_Owiz_117	0.009
	Y.pestis_Owiz_127	0.003
B (copy number $3.47x10^9$)	Y.pestis_Owiz_117	0.797
	Y.pestis_Owiz_127	0.890
C (copy number $1.76x10^7$)	Y.pestis_Owiz_117	0.827
	Y.pestis_Owiz_127	0.897
D (copy number $8.57x10^4$)	Y.pestis_Owiz_117	0.235
	Y.pestis_Owiz_127	0.443
E (copy number $4.39x10^2$)	Y.pestis_Owiz_117	0.063
	Y.pestis_Owiz_127	0.194
F (copy number 1)	Y.pestis_Owiz_117	0.019
	Y.pestis_Owiz_127	0.076

Probe signal intensity 0-1



A. Water sample (negative control)
B. Copy number 3.47×10^{9}
C. Copy number 1.76×10^{7}
D. Copy number 8.57×10^{4}
E. Copy number 4.39×10^{2}
F. Copy number 1
G. Assay control (IBV)

Figure 4.8. qPCR curves recorded after amplification of serially diluted samples of *Y. pestis* shown in Figure 4.6.

4.4. DISCUSSION

The information presented in this chapter represents the stages of array evaluation which should be performed prior to screening of field samples. From PCR protocol optimisation (there are generally low levels of pathogen nucleic acid in samples, which is the reason that PCR was carried out prior to hybridisation) to array hybridisation optimisation and sensitivity testing, many steps are required before work can begin on field samples. If there are too few cycles, less cDNA is produced during PCR. The annealing temperature must be optimised to ensure maximum binding of the primer and target sequence.

The array design step is instrumental in any microarray experiment. However, until the probes are actually used in a laboratory setting, their performance will be unknown (Lemoine et al., 2009). The final array (WT_Rodent_Chip_03) had probes that had been through multiple rounds of evaluation, and were therefore judged ready to use for screening purposes. There were two stages to the array evaluation: one stage for the ArrayTubes and one for the ArrayStrip. When probes produced a very weak signal it was necessary to design several new probes and evaluate them. However probes for the pathogens *R. typhi, Bartonella, S. monoliformis*, MRSA, HEV, SEOV and PUUV could not be fully evaluated. This was because reference material was either unavailable or a PCR product was not obtained from the reference material, even when using previously published primers. As a consequence, it is possible that the probes for these pathogens on the array may not have worked as expected. Ideally additional samples for each pathogen would have been obtained and tested with the array to fully evaluate these probes, but this was not possible. It should be noted at this point that the WT_Rodent_Chip_03 did not have any probes that could have been used as internal positive controls. The biotin markers, which acted as an assay control were however, deemed sufficient. A set of probes could have been designed to detect a pathogen

known not to be present in rodents, and a control sample could have been spiked with that pathogen to confirm that the array was working properly. The arrays were also not tested with known pathogen-free rodent tissue, as it was not available. Ideally this would have been done to test for any probe cross-reactivity with host DNA, and should be done in any future work.

The evaluated probes were able to detect pathogens which would produce specific hybridisation patterns, cross-hybridisation was not expected to occur after evaluation. This was because any probes that did show cross-hyrbridisation were removed. The probes that were used on the WT_Rodent_Chip_03 array for each pathogen were selected based on how they performed during the evaluation stages. A mixture of probes that performed well in both sequence-independent and sequence-specific amplification were spotted on the array. This was done so that the array could still be used with a sequence-independent amplification step in any future work. However, some of the probes were not fully evaluated because reference material was not available; therefore, cross-hybridisation could have occurred with these probes. To check for this, confirmatory work was carried out on all positive probe results (detailed in Chapters 5 and 6).

The ArrayTubes were ordered first and array evaluation began with these. There were arrays for the *Yersinia* species, *T. gondii*, combined SEOV, PUUV and HEV. The probes for all three *Yersinia* species appeared to work for both sequence-independent and sequence-specific amplification. Functioning probes were noted and used on the other arrays produced in the project; non-functioning probes were not used in further array evaluation. The *T. gondii* ArrayTube did not perform well after sequence-independent amplification of *T. gondii* DNA. As all of the probes in that ArrayTube were designed to detect *T. gondii,* it was expected that many probes would hybridise. However, only 5/95 probes produced a detectable signal after sequence-independent

amplification. The signal measured after using the products of sequence-specific amplification was better. This could be because, after sequence-independent amplification, the whole genome of *T. gondii* was amplified and the quantity of target sequence was too low to produce a strong signal. Sequence-specific amplification on the other hand, is more specific, and all of the reagents in the reaction were used to amplify a small region of the *T. gondii* genome. This may explain why probes that did not produce a signal after sequence-independent amplification produced a strong signal after sequence-specific amplification. As the hantavirus and HEV RNA received did not appear to amplify, the probes on the Hanta_HEV_01 array were not fully evaluated. Efforts were made at acquiring additional samples, but none could be secured. However, the Hanta_HEV_01 ArrayTube, was used for cross-hybridisation experiments along with the other ArrayTubes.

Amplicons of all three Yersinia species, *T. gondii*, *Mycobacterium* and *Francisella* were hybridised on the ArrayTubes. Any probes that cross-hybridised were identified and removed from further analysis. Despite the *in silico* analyses indicating that none of these probes would cross-hybridise, some cross-hybridisation did occur. Reasons for this could include errors during the printing process, with a higher concentration of probe being deposited making it more likely to hybridise with non-specific target sequence or simply due to the nature of the probes being "sticky" (Tran et al., 2002). Two probes, for example M.bovis_11 and M.marinum_05, produced a positive signal when all three *Yersinia* species and *T. gondii* were hybridised on the WT_Mycobacteria_01 array. There were other errors seen during hybridisation with the arrays. Figures 4.2A and 4.2B show the images recorded after hybridisation of the Yersinia_01 ArrayTube with *Y. pestis* DNA. Both of these were hybridised after sequence-independent amplification, and yet the image produced is very different. In one image, the generic *Yersinia* probes hybridised, but not the specific *Y. pestis* ones,

and in the other the specific *Y. pestis* probes hybridised but not the generic ones. It is interesting to note that in Figure 4.1B the biotin marker did not appear to work yet the probes themselves did. As stated previously, no two arrays are identical (Russell et al., 2009), and errors during the printing process may explain this result. This is something that is very difficult to control for, and it is why technical replication is an essential part of microarray work.

Once the ArrayTube evaluation had been completed the pilot ArrayStrip, which contained probes for all pathogens apart from *Mycobacterium* and *Francisella*, was designed and printed. These final two pathogens were added to the WT_Rodent_Chip_03 after evaluation of the pilot ArrayStrip. To accommodate the greater number of pathogens there was a smaller number of probes (15) per pathogen. Similar to the ArrayTube evaluation, all of the pathogens for which reference material was available was amplified by sequence-independent and sequence-specific amplification and then hybridised onto the ArrayStrips. There were some variable results seen with the different pathogens. For some (*Y. pestis, Y. pseudotuberculosis*, LCMV) both types of amplification appeared to work well and many probes exhibited a positive signal. However, for other pathogens this was not the case. Sequence-independent amplification completely failed in several cases, for example with CPXV and *E. coli* and was poor with other pathogens (*T. gondii* and *Leptospira*). Sequence-specific amplification on the other hand, produced a detectable signal with several probes. This could be because, for this array and for these pathogens, sequence-independent amplification was not sensitive enough. As mentioned above, the higher specificity seen when using specific primers means that the sequence of interest is amplified in preference to all others and as a result, will be in greater quantities in the sample when hybridisation is performed. However, with greater specificity in sequence-specific amplification than sequence-independent, the likelihood of detecting novel

pathogens was decreased. Having a mixture of probes that can hybridise at different levels can be useful in detecting novel species. For example, a probe set could include a sequence that can hybridise at a genus level. This could be complemented by probes that hybridise at the species level. If a new pathogen is screened on the array then it could hybridise with the generic probes, that are conserved throughout the genus, but would not hybridise with any of the species-specific probes. This was the method used to detect SARS (Wang et al., 2003b).

As singleplex PCR of all the pathogens would be a time-consuming and expensive option, multiplex PCR was explored as an alternative. The sensitivity and specificity of multiplex has been shown to be comparable to that of singleplex PCR (Haque et al., 2007). Initial primer concentrations were determined using the Qiagen Multiplex Plus PCR protocol. After initial experiments however, when the expected probes did not hybridise fully, these conditions were altered. With the use of variable primer concentrations in the mixture, as primer concentration has been found to be critical to the success of multiplex PCR (Sint et al., 2012), all of the probes which were expected to hybridise on the ArrayStrip, produced detectable signals. This was the first time that this kit had been used with biotinylated primers, and the method was altered so that there was no need to perform an additional PCR labelling round.

The signal intensity was compared between sequence-independent amplification and singleplex sequence-specific amplification and also between singleplex sequence-specific amplification and multiplex sequence-specific amplification. A t-test was deemed appropriate to compare the two groups, as it is often used to determine if two sets of data are significantly different from one antother. Overall there was lower signal intensity observed after sequence-independent amplification than with sequence-specific amplification, with a few exceptions. As the samples tested were all from pure culture and were not from host tissue, it was likely that the signal observed after

extraction from tissue would be even lower as the pathogen would be present in lower levels in host tissue. The comparison between probe signal after singleplex PCR and multiplex PCR showed that there was a statistically significant difference in several cases, however the actual signal recorded did not vary much. Due to the superior performance of the sequence-specific primers compared to sequence-independent amplification, and the fact that the signal intensity seen after singleplex and multiplex was comparable, the multiplex approach using biotinylated primers was chosen for the screening stage of the project.

The arrays were also tested for sensitivity. This was done by serially diluting spiked samples of rodent tissue spiked with a known DNA concentration of *Y. pestis,* and then performing PCR and hybridisation on the array. A real-time PCR was also run on the same samples to determine the limit of detection of the arrays; qPCR was used as it is widely regarded as being more sensitive than standard end-point PCR (Azzari et al., 2010, Ioos et al., 2009), although this has been disputed by some (Bastien et al., 2008, Garland and Wood, 2011). The point at which the signal intensity on the array was below a threshold level set for detection was deemed the point at which the array could no longer detect a positive sample. The experiments carried out suggested that the array was not as sensitive as qPCR. The array reached its limit of detection before qCR. It would seem, therefore, that the array is as sensitive as standard end-point PCR but not quite as sensitive as qPCR. This is similar to what has been reported elsewhere (Stremmel 2002, Skacel 2002), and as PCR is still widely used as a diagnostic tool this suggests the array would certainly be sufficient for screening purposes.

CHAPTER FIVE

RODENT SCREENING

5.1 SUMMARY

Rodent tissue samples from Liverpool, Lyon and Vancouver were screened on the array once it was finalised. As there were over 3,000 individual tissues, an aliquot of the extracted nucleic acid from each tissue from each animal was pooled (to form a single pooled sample per animal) and PCR was then performed on the pooled sample. A reverse-transcription step was included, as some of the pathogens on the array were RNA viruses. The samples were then amplified with a biotinylated multiplex primer mix and hybridised with the array. The full set of tissues was unavailable for some of the sampled animals. As a result, pathogens which were most likely to have been present in either the lung or kidney were likely to have been missed when screening was performed with samples that did not include the lung or kidney. However, from the samples screened, there were positive signals on the array for *Leptospira* (72) and SEOV (2). Confirmatory testing, conducted at both the University of Nottingham and at the source laboratory, was used to determine the true infection status of the animal. The confirmatory testing included use of previously validated PCR, and sequencing on PCR positive samples. For both *Leptospira* and SEOV an alignment of the sequences from the array-positive samples and those taken from the NCBI database, showed a high degree of identity. Only array-positive samples that were also positive on a confirmatory test were included in the subsequent analysis. There were only two samples positive for SEOV, so detailed statistical analysis could not be performed. For the *Leptospira* positive samples, statistical analyses were performed to look at the possible importance of a number of variables which included species, size, weight, sex, month caught, pregnancy and lactation. Of these variables, age, size and the season when the animals were caught were significant.

5.2 INTRODUCTION

Once array design and evaluation had taken place, the final ArrayStrip (Rodent_Chip_03) was used to screen field samples from the UK, France and Canada for multiple pathogens. The rodent species sampled for the screening tests were *R. rattus, R. norvegicus, M. musculus* (UK only) and *A. sylvaticus* (UK only). Samples of liver, kidney and lung (where available) were used. To validate the positive results obtained on the array, confirmatory tests were performed which used previously published methods. Results from extensive confirmatory testing suggested that only the *Leptospira* and SEOV results were true positives. The results of the confirmatory work for the other array-positive pathogens are covered in Chapter 6. This chapter focuses on the true positive array results.

There are several factors which can affect the detection of the nucleic acid of a pathogen in a sample. These can include the stage of infection, how long after death the sample was taken, the method used to collect the sample, how long the sample was in transit, and how the sample was stored. The pathogen may have been successfully eliminated by the immune system of the animal in question, so no nucleic acid may be detectable; nucleic acid assays are only useful in detecting active infection. Furthermore, as pathogen RNA and DNA can be unstable, the time between sample collection after the death of the host, and appropriate storage of the sample can profoundly affect the integrity of the nucleic acid; degradation of RNA/DNA can invalidate many studies (Fleige and Pfaffi, 2006). Ideally all tissue samples should be collected immediately after death of the animal and immediately stored appropriately, i.e. placed in RNAlater or frozen at -20°C. Because of the importance of sample handling, a standard operating procedure (SOP) was developed (See Appendix) and sent to WildTech collaborators so that samples would be processed accordingly.

The pathogen of interest may only be present in certain tissues, and this can vary depending on the host species. Therefore, it was essential that the correct tissue be chosen to avoid missing the target pathogen. For each of the pathogens included in this project, information was compiled about the tissues most commonly infected (See Table 5.1.). This information was then used to determine the tissues which would be screened as part of this project.

5.2.1 AIMS

The aims of the rodent screening section the project were to:

- Screen tissue samples (liver, kidney, and lung) from rodent species for pathogens of zoonotic importance on newly designed microarrays
- Confirm any positive results by PCR, and sequence positive samples for further confirmation.

Table 5.1. The pathogens studied in this project and their most likely tissue distribution in rodent hosts.

Agent	Location in tissue
Mycobacterium	**Liver**, spleen, **lung**, lymph nodes and any external lesions (Durnez, 2011).
Salmonella	Rodents dying of acute infection: **liver**, spleen, heart blood, intestines; rodents with chronic infection: faeces, intestinal contents (Buxton and Fraser, 1977).
Francisella	**Liver**, spleen, lymph nodes, **lungs** (Williams and Barker, 2001)
C. jejuni	Intestine and faecal material (Rosef, 1983).
Leptospira	Acute infection: **liver**, spleen; in more chronic infection, **kidney** (for isolation of leptospires). In the living animal in acute infection, blood. Also from urine (Williams and Barker, 2001).
T. gondii	Multiple variable organs (Samuel, 2001).
HEV	**Liver**, spleen, lymph nodes, intestine and faeces (Maneerat et al., 1996).
Hantavirus	Particularly the **lungs**. Virus in the urine of infected mice (**kidneys**) (Mills et al., 1999).
CPXV	**Kidney** and **lung** tissue likely to be best for detecting CPXV (Marennikova, 1979).
LCMV	From rodents, blood, oral swabs or homogenates of most organs (Williams and Barker, 2001).
E. coli	**Liver**, spleen, **kidney**, large intestine contents, faeces (Le Moine et al., 1987).
Yersinia	Experimental infection and other small rodents suggest that spleen, **liver** and **lung** tissue is best for detection on *Yersinia* species (Riehm et al., 2011, Carter, 1975).
MRSA	Experimental infection saw bacteria accumulate in the **lungs** (Martinez-Olondris et al., 2010).
Bartonella	Blood and **liver** (Ellis et al., 1999, Kamani et al., 2013).
S. moniliformis	Upper respiratory tract, pharynx, lymph nodes (Boot et al., 2002, Elliott, 2007).
Rickettsia spp	Urine and **kidneys** in experimentally infected *M. musculus* (Philips and Parker, 1938). Direct amplification usually occurs from blood and rarely from other samples (Maurin, 2012).

5.3 RESULTS

5.3.1 *Leptospira*

Prior to analysis, the data were tested for normality. This was done to determine which statistical tests could be used on the data. The values for signal intensity for each probe that hybridised (Leptospira_826, and Leptospira_827) were examined and the normality determined. A Shapiro-Wilk test was performed to obtain a p-value which would indicate the normality of the data, which was needed for statistical purposes. Table 5.2 contains the p-values which tested the null hypothesis that the data were normally distributed. If the p-value was below 0.05, the null hypothesis could be rejected and it could be assumed that the data were not normally distributed. Because these tests indicated that some of the data were not normally distributed, statistical tests (Kruskal-Wallis chi-squared test and Fisher's exact test) were chosen that did not require a normal distribution.

Table 5.2. Shapiro-Wilk test for normality on the signal intensity values recorded from probes Leptospira_826 and Leptospira_827.

Probe ID	Canada p-value	France p-value	UK p-value	All three countries p-value
Leptospira_826	0.025	0.386	0.210	0.029
Leptospira_827	0.003	<0.05	0.013	<0.05

The mean signal intensity and standard deviations for both probes are given in Table 5.3, and boxplots of this data are shown in Figure 5.1. Signal intensity varied from 0.0 to 1.0. A cut-off point of 0.4 was used for the analysis. Overall, there was often quite a large range of values in the signal intensities for individual probes. Signal instensity is important as it can be used to determine cut-off values at which a result can be labelled as positive or negative. For example, the values for the probe Leptospira_827 ranged from 0.405 up to 0.823. Figure 5.1 shows the boxplots produced with the signal data

from probes Leptospira_826 and Leptospira_827. From the graphs it was apparent that the mean signal for Leptospira_826 was similar for France (0.508) and the UK (0.550), whereas in Canada it was higher (0.629). A Kruskal-Wallis analysis indicated that with a p-value <0.05 this was a significant difference. The mean signal for Leptospira_827 on the other hand, just from looking at the boxplots, shows that it was much closer between the three countries (Table 5.3). A Kruskal-Wallis analysis indicated that there was not a significant difference, with a p-value >0.05.

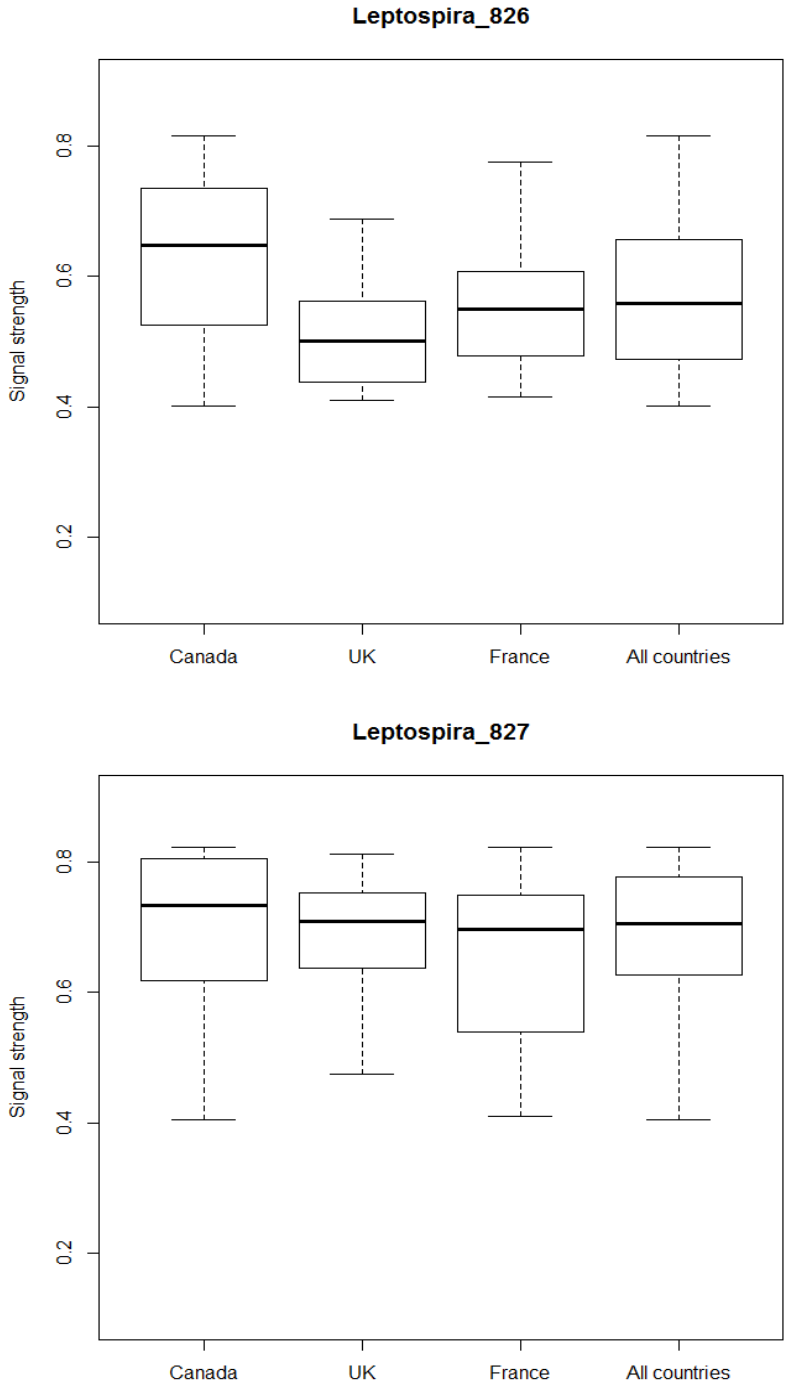


Figure 5.1. Boxplots of the signal intensity for each hybridising probe Leptospira_826 and Leptospira_827 by country.

Table 5.3. Descriptive statistics for the probes that displayed hybridisation to *Leprospira*.
Each value shown is the mean signal measured on the array for that probe; the scale was from 0.0-1.0.

Probe ID	**Mean signal (number of probes)**			**Standard Deviation**			**CI (95%)**			**Maximum**			**Minimum**		
	Canada	UK	France	Canada	UK	France	Canada	UK	France	Canada	UK	France	Canada	UK	France
*Leptospira*_826	0.629 (48)	0.550 (21)	0.508 (30)	0.124	0.101	0.079	0.035	0.043	0.028	0.815	0.775	0.687	0.401	0.416	0.410
*Leptospira*_827	0.688 (41)	0.653 (20)	0.692 (38)	0.126	0.132	0.081	0.039	0.058	0.026	0.823	0.822	0.812	0.405	0.410	0.474

A sample was regarded as positive if at least one of the probes had a signal intensity >0.4. The array detected 33 positive animals from Canada from a total of 652 animals. The positive samples were 12 mature female samples, two immature male samples and 19 mature male samples. All of these were members of the *R. norvegicus* species. An analysis of the signal intensity seen for each sample shows that there was not a uniform hybridisation signal intensity across the samples. There were 22 samples that were positive on the array which were also PCR positive at the source laboratory. Not all of the samples which were positive on the array were tested at the source laboratory. There were five samples that were positive on the array but were negative by PCR at the source laboratory. Four of these were confirmed as positive using standard end-point PCR (at the University of Nottingham); and one of these samples was used for sequence analysis to further confirm the presence of *Leptospira*. The one remaining negative sample was tested with qPCR and the result was inconclusive (cT value of 38). There were 40 animals that tested PCR positive at the source laboratory which were not positive on the arrays. To further investigate these conflicting findings, qPCR testing of the samples from these 40 animals was conducted at the University of Nottingham. Five of these samples were positive by qPCR, and the results were inconclusive. The remaining 32 results were all negative. However, it is important to note that different primers were used at the source laboratory which targeted a different gene (LipL32) to the primers used at the University of Nottingham.

The array detected 21 positive animals from France, from a total of 84 animals. Fifteen of the 21 array-positive animals were confirmed positive by other tests conducted at both the University of Nottingham and at the source laboratory (one of the samples was not tested by standard PCR). The remaining five animals that were positive on the array were confirmed positive using a standard end-point PCR at the University of Nottingham, but they did not test positive at the laboratory in France.

Sequencing of the PCR products for three of these array-and PCR-positive samples confirmed the presence of *Leptospira* presence in the samples. There were 17 animals identified as positive at the source laboratory that did not test positive on the array. Consequently, confirmatory qPCR was performed on these samples, and 5 were positive for *Leptospira*. There were two results that were inconclusive, and the remaining 9 samples were negative (one sample could not be tested as insufficient nucleic acid remained and there was not sufficient time to extract more nucleic acid).

In total, the array identified 18 *Leptospira* positive animals from the UK from a total of 329 animals. All of the animals tested negative at the source laboratory. Seventeen of the positive samples were confirmed by standard PCR or qPCR and sequencing of the PCR product confirmed the identity of *Leptospira* in five of the samples (one of the samples was not tested as the extracted material had been exhausted). 12 of these 17 animals were female and five were male. Three of the samples were negative with end-point PCR but tested positive with qPCR. The remaining two samples were negative by end-point PCR but were positive on the array and qPCR.

5.3.1.1 Prevalence

The confirmed positive results as identified by the arrays were used to determine the prevalence of *Leptospira* infection, based on the total number of samples tested from each country. The prevalence varied across the three countries. In Canada, the prevalence was 6.1%, in France it was 25.0% and in the UK it was 14.6%.

5.3.1.2 Species

The only species that had positive results for *Leptospira* was *R. norvegicus*. None of the *R. rattus, M. musculus*, or *A. sylvaticus* samples tested positive for *Leptospira*.

5.3.1.3 Sex of Animal

A chi-squared test was used to determine if the sex of the animal was a significant factor in contracting a *Leptospira* infection. Age data was available for a total of 744 animals. Overall there were 30 female (8.82% prevalence) and 42 male (10.40% prevalence) rodents which were positive for *Leptospira*. There were 310 female and 362 male rodents which were negative for *Leptospira*. The resulting chi-square test had a p-value >0.05; this suggests that sex of the animals was not a significant factor in *Leptospira* infection in rats when considering all three countries combined.

From France there were six female (22.2% prevalence) and 15 male (26.3% prevalence) rodents which were positive for *Leptospira*. There were 21 females and 42 males that were negative for *Leptospira*. The resulting chi-square test had a p-value >0.05, which suggests that sex of the animal was not a significant factor in *Leptospira* in rats in France.

From the UK there were 12 female (18.2% prevalence) and 6 male (10.5% prevalence) rodents which were positive for *Leptospira*. There were 54 females and 51 males that were negative for *Leptospira*. The resulting chi-square test had a p-value

>0.05, which suggests that sex of the animal was not a significant factor in *Leptospira* infection in rats in the UK.

From Canada there were 12 female (4.9% prevalence) and 21 male (7.2% prevalence) rodents which were positive for *Leptospira*. There were 235 females and 269 males that were negative for *Leptospira*. The resulting chi-square test had a p-value >0.05, which suggests that sex of the animal was not a significant factor in *Leptospira* infection in rats in Canada.

5.3.1.4 Age

A chi-squared test was performed to determine if age was a significant factor in *Leptospira* infection; results are shown in Table 5.4 and were presented as bar plots in Figure 5.2. The age of the rodents was not recorded in the UK, so only the data from Canada and France could be considered in this analysis. Weight has been considered as a good proxy for age (Davis, 1953), and the age of the animal was determined at the source laboratories using weight as a guide.

If results for Canada and France are considered together, there were 9 immature animals (4.1% prevalence) and 45 mature animals (11.2% prevalence) that were positive for *Leptospira*. There were 211 immature and 356 mature animals that were negative for *Leptospira*. The resulting chi-square test had a p-value of <0.05, which suggests that age was a significant factor in the infection status of the animal, and that older animals are more likely to be carrying *Leptospira*.

In samples from France, there were seven immature animals (25.9% prevalence) and 14 mature animals (24.6% prevalence) that were positive for *Leptospira*. There were 20 immature and 43 mature animals that were negative for *Leptospira*. The resulting chi-square test had a p-value of >0.05, which suggests that in the sampled location in France, age was not a significant factor in *Leptospira* infection status.

The age was only recorded in 621 animals from Canada, so only these animals were included in the age-based analysis of the data. In samples from Canada, there were two immature animals (1.0% prevalence) and 31 mature animals (9.0% prevalence) that were positive for *Leptospira*. There were 191 immature and 313 mature animals that were negative for *Leptospira*. The resulting chi-square test had a p-value of <0.05, which suggests that in Canada, increasing age was a significant factor in *Leptospira* infection status.

Table 5.4. Results of a chi-squared test of the association between age and *Leptospir*a infection status for animals from Canada, from France, or from both countries combined.
Age data was not available for UK animals sampled.

Country	Chi-squared value	Df	p-value
Combined	8.223	1	0.004
France	0.018	1	0.893
Canada	12.287	1	<0.001

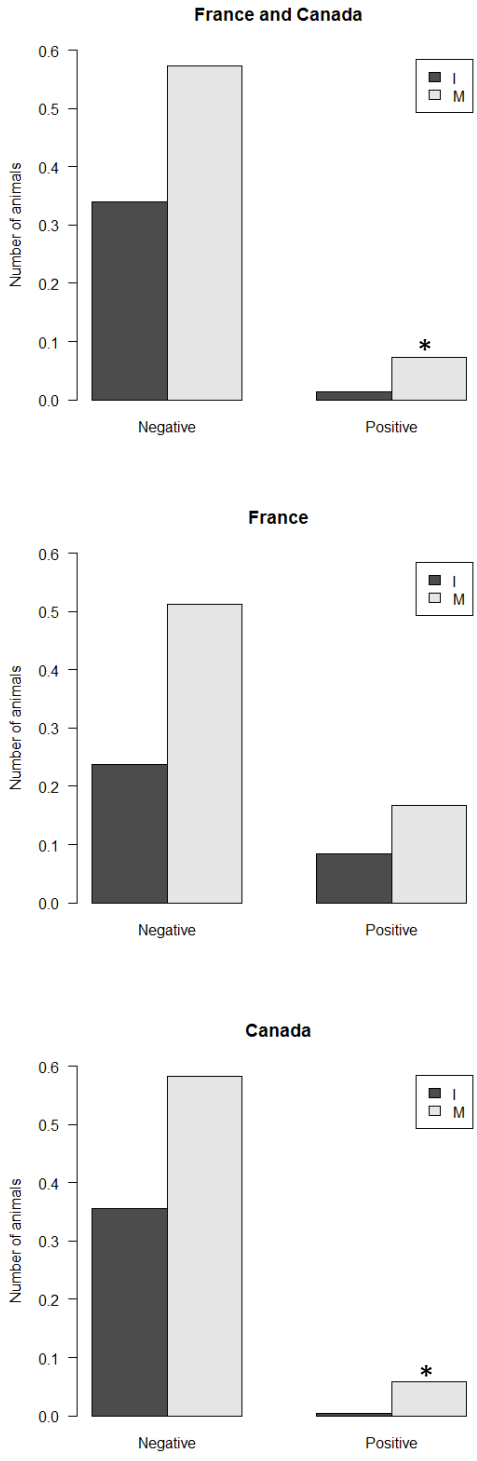


Figure 5.2. Bar plots showing the infection status of the animals from Canada and France. These have been separated according to the age of the animal: Immature (I) and Mature (M). * indicates a significant difference between I and M animals. For *Leptospira* positive animals there were significant differences for the combined group (p-value 0.004) and for the Canadian animals alone (p-value <0.001).

5.3.1.5 Size and weight

The size and weight of the rodents was recorded in France and Canada, but not in the UK. Therefore, logistic regression of the size and weight of the animal, and *Leptospira* infection status were only calculated for France and Canada. The z value for weight was not significant (z value = -1.114, p value = 0.253); however, there was a significant z value for size (z value = 3.525, p value = <0.001). These results indicate that as animal size increased, there was an increasing chance that the animal had a *Leptospira* infection.

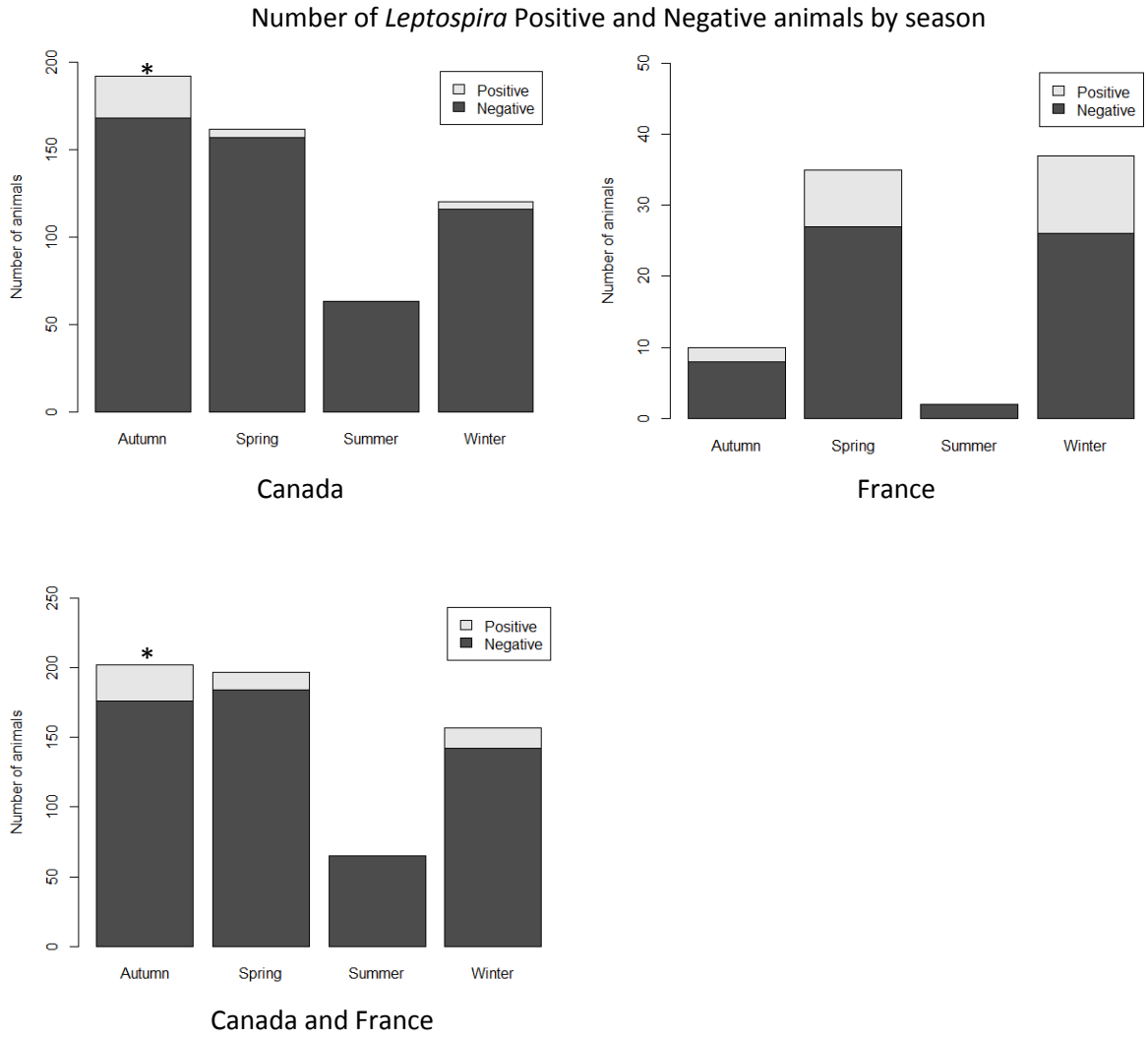
5.3.1.6 Season and month

The season in which the animals were trapped was only recorded in Canada and France. Therefore, data from the UK were excluded from the assessment to determine the association between season and infection status. Bar plots for the number of animals sampled, and the infection status in animals from France, from Canada, and a combined total can be seen in Figure 5.3. Overall there were 26/202 (12.9%) positive animals caught in the autumn; this represents 48.1% of all the positive animals caught. There were 15/157 (9.5%) positive animals caught in the winter, which represents 27.8% of all the positive animals caught. In the spring there were 13/197 (6.6%) positive animals caught, this represents 24.1% of all the positive animals caught. There were no positive animals caught in the summer; however, 65 negative animals were caught.

A pairwise comparison was performed using a Fisher's Exact Test to look at differences in infection status between seasons (Table 5.5). The odds ratio for the pairwise comparisons involving summer yielded infinite values, as there were no positive results identified from the animals caught in the summer. All of the pairwise comparisons were significant (p value = <0.05), with the exception of autumn/winter

and spring/winter. It was twice as likely for an animal caught in the autumn to test positive than if it was caught in the spring.

A chi-square test performed to determine if there were any significant differences in the prevalence of *Leptospira* infections between the different seasons for each country. In France there were 2/10 (20.0%) positive animals caught in the autumn. In the spring there were 8/35 (22.9%) positive animals. Only two animals were caught in the summer and both of these were negative. In the winter there were 11/37 (29.7%) positive animals. There was no significant association between season in the French data (p-value >0.05). From Canada there were 24/192 (12.5%) positive animals caught in the autumn. In the spring there were 5/162 (3.1%) positive animals. A total of 63 animals were caught in the summer and all of these were negative. In the winter there were 4/120 (3.3%) positive animals. There was a significant association (p-value <0.05) seen between season and infection status seen in the Canadian data. Positive animals were more likely to be found in the autumn.



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Figure 5.3. Number of animals caught in each season in Canada, in France, and both countries combined.
Positive cases are in light grey, negative cases are dark grey, * indicates a significant difference between the proportion of positive animals caught by season. The only season in which there was a significant difference was autumn (p-value <0.001), and only for the Canadian samples.

Table 5.5. Results of a pairwise comparison test of the different seasons. As no animals sampled in the summer tested positive, all odds ratios involving positive results from the summer yielded infinite values (** indicates a significant result with a p-value <0.05).

Pairwise comparison	p-value	Odds Ratio
Autumn/Spring	0.043**	2.087
Autumn/Summer	<0.001**	Infinite
Autumn/Winter	0.431	1.397
Spring/Summer	0.042**	Infinite
Spring/Winter	0.327	0.669
Winter/Summer	0.007**	Infinite

In Canada and France, the month in which the animals were trapped was recorded; a breakdown of positive results by month is given in Table 5.6. Looking at the combined totals for both countries, the largest percentage of positive animals was seen in November (22.8%), December (17.4%), March (14.9%) and February (10.4%). All of the positive animals in the month of November were caught in Canada (21 animals); this represents nearly two thirds of the total number of positive animals caught for the entire study. Although a large number of animals were caught during other months, very few of them were positives. In May, for example, there were 99 animals caught, but only one of these was positive. Likewise, in October, 100 animals were caught and only 5 were positive. A chi-square test performed on the combined monthly data indicated that there was a significant difference between months (p=<0.05). In France the highest number of positive animals was found in February and March, with eight positive animals identified. However, this was also the month in which the highest number of animals was captured. By contrast, the highest percentage of positive animals caught in Canada was in November, and the largest number of rodents trapped there was in May and October. A chi-square test performed on the data from France indicated that there was not a significant association between *Leptospira* infection and

month caught. A chi-square test performed on the Canadian data however, showed there was a significant association between infection status and month caught and November seemed to be the month where positive animals were most likely to be identified (p=<0.05).

Table 5.6. The number of animals caught from France, Canada and a combined total broken down into those that tested positive and negative.

	Canada		**France**		**Combined**	
Month	Positive	Negative	Positive	Negative	Positive	Negative
January	1	37	0	0	1	37
February	2	69	8	17	10	86
March	2	31	8	26	10	57
April	2	28	0	1	2	29
May	1	98	0	0	1	98
June	0	9	0	0	0	9
July	0	54	0	2	0	56
September	0	9	0	1	0	10
October	3	91	2	4	5	95
November	21	68	0	3	21	71
December	1	10	3	9	4	19

5.3.1.7 Pregnancy

Pregnancy information was only collected on the rodents from Canada. The mature female data was used to assess the association between pregnancy and *Leptospira* infection in Canadian rats. There were 109 non-pregnant rats caught; 8 of these were positive for *Leptospira*. Of the 25 pregnant animals caught, 4 were positive for *Leptospira*. A Fisher's Exact test and resulting Odds Ratio (OR) was used to look at the association between pregnancy and *Leptospira* infection. There was no significant association between pregnancy and *Leptospira* infection (p=>0.05). Although the OR

suggests that there was a higher chance of Leptospira being present if the animal is pregnant, this was not stastically significant.

5.3.1.8 Lactation

Information on lactation status was only available from Canada. Only the mature female data were used to assess the association between lactation and *Leptospira* infection. A Fisher's Exact test was used to look at the association between lactation and *Leptospira* infection, but none was found (p=>0.05). Although the OR suggests that there was a higher chance of Leptospira being present if the animal is lactating, this was not statistically significant.

5.3.1.9 Sequencing

Sequencing was performed on 15 of the *Leptospira* positive samples, which were selected as a representative subset of positives from all three of the sampled countries. The *gyrB* gene was partially sequenced and an alignment was made from all of these sequences. The alignment produced (Figure 5.4A), showed that the *Leptospira* region sequenced was similar in all positive samples. An asterisk above a nucleotide position in the alignment indicates that the same nucleotide was present in all samples at that location. The sequences obtained from positive samples in the UK, France and Canada were aligned with the sequences of the same gene from the NCBI database. The resulting alignment was almost identical, with very few differences in the individual base pairs (see Figure 5.4B). The tree produced from phylogenetic analysis with SeaView 4.5.1 shows the relationship between the different samples of *Leptospira* that were sequenced (Figure 5.5). This included sequences of different *Leptospira* serovars obtained from the NCBI database. Again, the tree produced shows that the *Leptospira* in all of the positive samples identified by the array were closely related to other

pathogenic *Leptospira* sequences on the NCBI database. There is a clear difference in the phylogenetic tree between the pathogenic *Leptospira* and the non-pathogenic *L. biflexa* sequences. The tree was replicated 100 times and there was a 100% bootstrap value which grouped the pathogenic *Leptospira* sequences together, with the non-pathogenic strains on a separate branch.

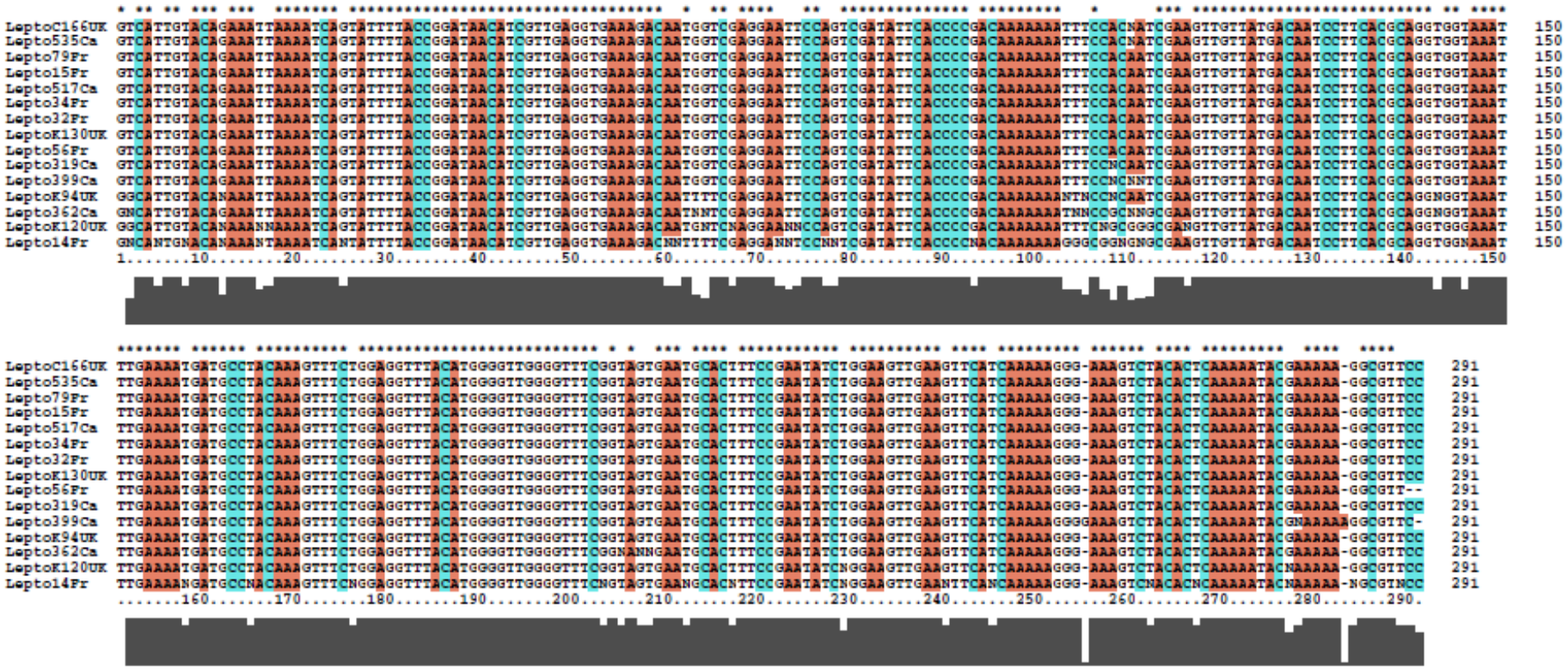


Figure 5.4A. The alignment produced from 15 *Leptospira gyrB* gene partial sequences obtained from positive samples on the array.
The name of each sample is shown on the left and each different base is represented by a symbol G, A, C, and T and separate colour. Samples were from all three countries; note they show a high degree of similarity for large regions of the alignment. A * above a nucleotide position in the alignment, represents an identical base for all samples.

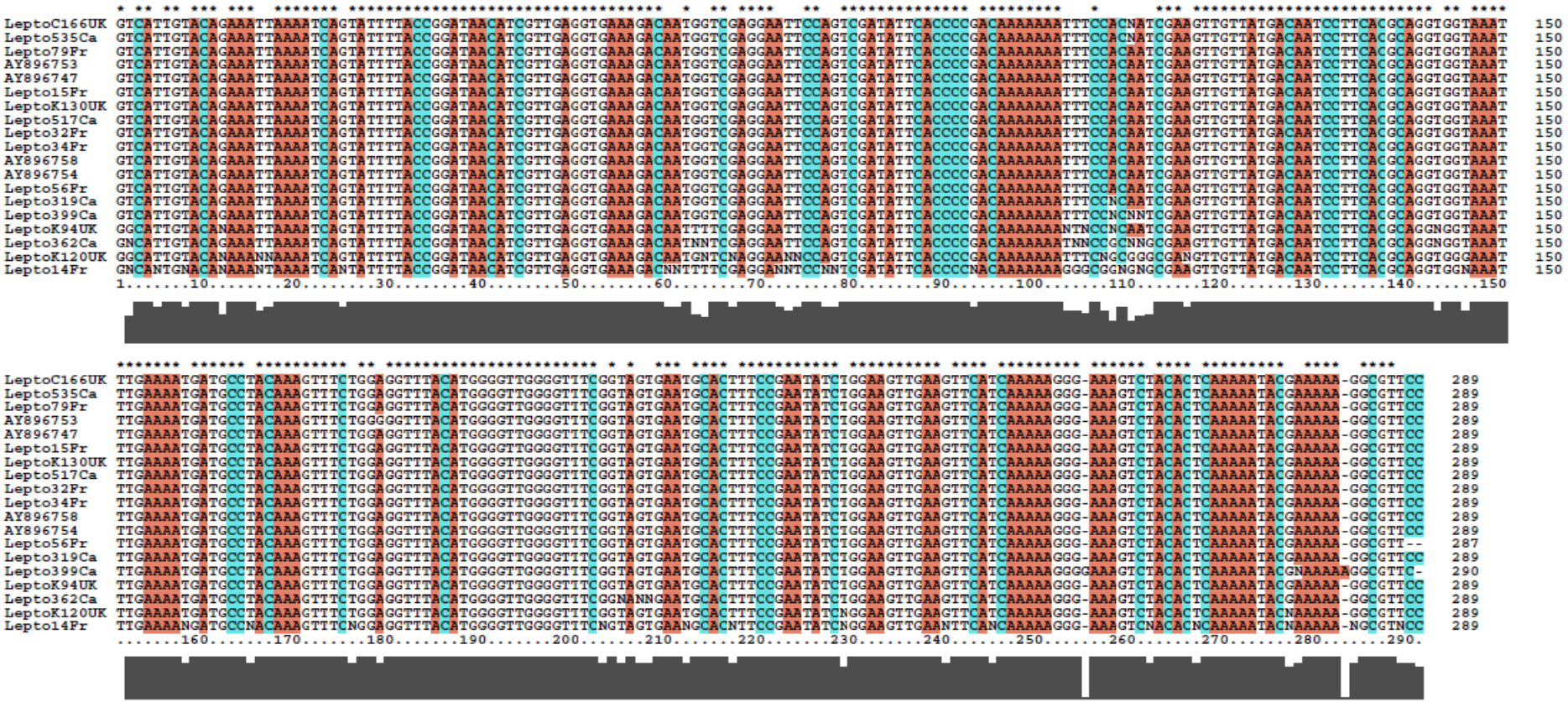


Figure 5.4B. The alignment produced from 15 *Leptospira gyrB* gene partial sequences identified by the array aligned with four sequences obtained from the NCBI database (AY896753, AY896747, AY896758, and AY986754). A * above a nucleotide position in the alignment, represents an identical base for all samples.



Figure 5.5. Phylogenetic tree produced using the partial *gyrB* gene sequence obtained from the array positive *Leptospira* samples.
The squared PhyMl tree includes other sequenced *gyrB* genes from two non-pathogenic strains *Leptospira* strains (*L. biflexa*). The branch scale is shown in the top right corner.

5.3.2. Seoul Hantavirus

There were 14 Seoul hantavirus positive rodents identified at the source laboratory. Only two of these were positive on the array, both of which were subsequently confirmed by end-point PCR with the glycoprotein gene as the target. The other samples that were detected as SEOV positive at the source laboratory (by qPCR) were also tested by PCR and qPCR at the University of Nottingham; however all of these were negative. The two array positive samples were from France, samples LYO0852 (adult female) and LYO0906 (adult male). Sample LYO0852 was positive for four probes, wheras LYO0906 had a single probe with a signal >0.4. The remaining three probes were between 0.2 and 0.4. Table 5.6 shows the mean signal for each probe.

The two array positive SEOV samples were also sequenced. An alignment of the two sequences was performed using Clustal X2 and GhostView. The alignment of the sequenced region of the glycoprotein gene is given in Figure 5.6, which includes SEOV strains obtained in other experiments. The alignment shows a high degree of identity for all the sequences with very few mismatches. A BLAST analysis was performed for both of these sequences. For sample LYO0852 the query coverage ranged from 90-98% with a maximum identity ranging from 95-99%. For sample LYO0906 the query coverage ranged from 89-94% with a maximum identity ranging from 86-89%. All of the matches were strains of SEOV.

The phylogenetic tree (Figure 5.7) demonstrates how closely related the SEOV isolates identified by microarray analysis were to other SEOV sequences obtained from NCBI in the alignment. This tree was obtained after 100 replicates. The sample Lyon/RN/FRA/2013/LYO852 and SEOV28G7 were both from the same animal. In addition to other SEOV strains, four additional Hantavirus sequences were aligned and used to make a phylogenetic tree. These were Dobrava, Tula, Puumala and Sin Nombre

virus. As shown in Figure 5.7, there is a clear clustering of the SEOV sequences and the other Hantavirus sequences as expected.

Table 5.7. Hybridising SEOV probes and the mean signal produced.

	Mean signal (number of probes)	**Standard deviation**	**CI (95%)**
SEOV_S27	0.630 (4)	0.213	0.208
SEOV_UPS_S15	0.505 (2)	NA	NA
SEOVUPS_531	0.490 (2)	NA	NA
SEOVUPS_532	0.304 (2)	NA	NA

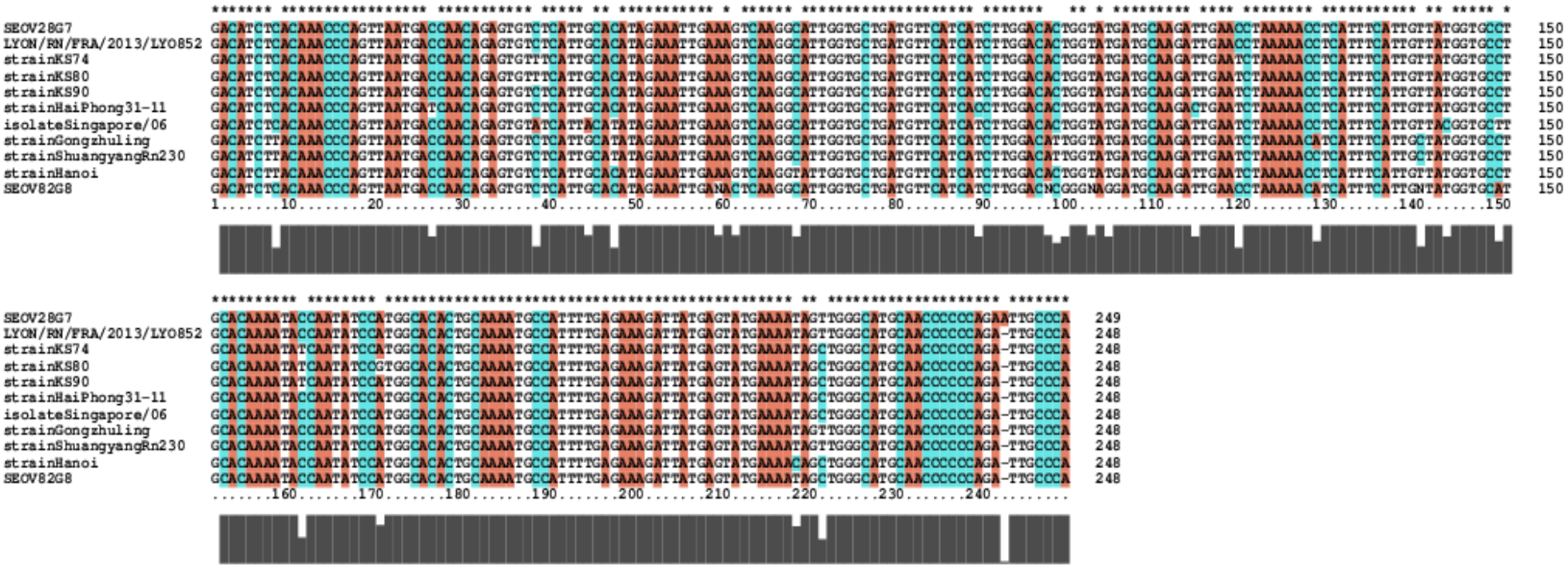


Figure 5.6. The alignment produced from a partial sequence of the glycoprotein gene obtained from positive samples on the array and from the NCBI database.

The name of each sample is shown on the left and each different base is represented by a symbol G, A, C, and T. The samples are from all three countries and yet show a high degree of similarity for large regions of the alignment. A * above a nucleotide position in the alignment, represents an identical base for all samples.

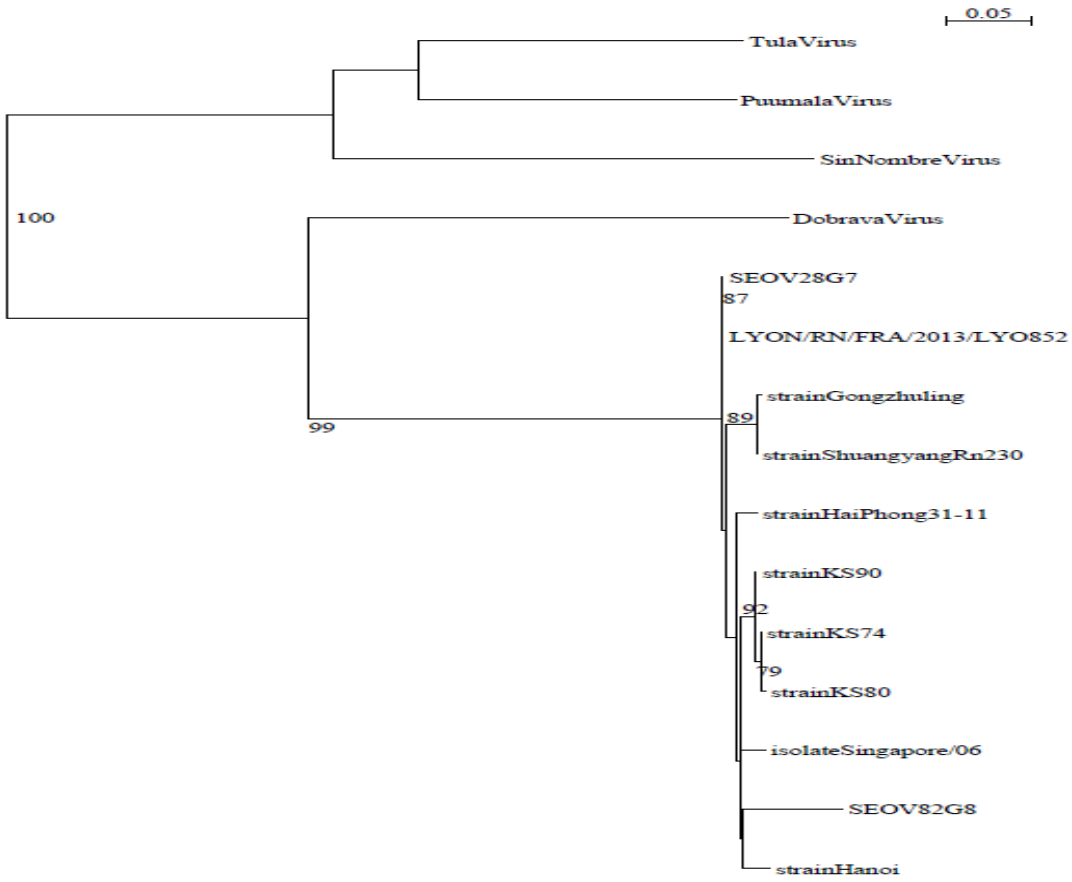


Figure 5.7. Phylogenetic tree produced from the partial sequence of the glycoprotein gene sequenced from the array-positive samples and from other sequences obtained from the NCBI database.
The squared PhyML tree includes other, previously sequenced SEOV strains including four other Hantavirus species (Dobrava, Sin Nombre, Puumala and Tula virus). Bootstrap values >0.75 are shown. The branch scale is shown in the top right corner.

5.4. DISCUSSION

Although the arrays were evaluated as much as possible during the evaluation stages, limited reference material was available for a number of pathogens on the array. The pathogens present in these rodent samples were of unknown quantity, and cross-hybridisation with the probes may have occurred. This is why any new screening tool must be thoroughly evaluated, and any positive results must be confirmed by other methods.

As the samples obtained were opportunistic, in several cases a complete tissue set was not available. For many of the UK samples, only liver tissue was available and no lung or kidney tissue was provided. Therefore, any pathogens commonly present in the kidney or lung (but not necessarily in the liver) may have been missed. This would, for example, make it very difficult to detect hantaviruses which are primarily detectable in the lungs and urine of infected rodents. Care should also be taken when interpreting the data on the prevalence of *Leptospira* in the UK. All 17 of the positive samples were from animals for which both liver and kidney samples were available. As *Leptospira* colonises kidney tissue the nucleic acid detected by the arrays and confirmatory tests is likely to have originated from these tissues.

Initially only the *Yersinia* species, *T. gondii*, HEV and the hantaviruses were to be included on the array. Samples of liver, kidney and lung – requested in the sampling protocol sent to collaborators - would have been suitable for detecting these pathogens. However, with the addition of other pathogens later in the project, other tissue types would have been useful. This is particularly true for *C. jejuni* for example, which is predominantly found in the intestine and faecal material of infected animals. It is likely that several of the pathogens which may not have been present in the samples animals, were not detected because of the tissues sampled. It was therefore difficult to

draw definitive conclusions about the prevalence of a particular pathogen within a population.

This difficulty was further exacerbated by the fact that pooling the extracted nucleic acid likely diluted the amount of the pathogen nucleic acid in each sample, sometimes to an undetectable level. This was clearly demonstrated with the *Leptospira* results: Many of the array negative samples were positive when tested at the source laboratory. Some of these samples, when subsequently tested by qPCR at the University of Nottingham, tested positive. This suggests that by pooling the extracted nucleic acid from three tissues, the sensitivity of the arrays was reduced. However, whilst pooling may have diluted the nucleic acid to undetectable levels, it is also possible that the sample became degraded either during transport, or during dissection and homogenisation (although care was taken to attempt to avoid sample thawing during handling). Another possible reason for the difference could be due to differences in the location of the pathogen in the tissue sampled. Pathogens are not uniformly distributed throughout tissues (Abu-Bakr Abu-Median, personal communication) and the location of the section of tissue taken from each organ used for homogenisation and extraction at the University of Nottingham would have been different to that taken and tested used at other laboratories. It is possible that false negatives could have been seen because the pathogen may not have been present in the tissue section sent for analysis at the University of Nottingham.

A *Leptospira* prevalence of 19.2% was previously reported in *R. norvegicus* in France based on PCR detection (Levieuge et al., 2010). This is slightly lower than the 25.0% found in Lyon using the arrays, though both figures indicate a large proportion of *R. norvegicus* actively carries the pathogen. Reported *Leptospira* prevalence in Canada varies widely, from 31% in Ontario (Jardine et al., 2011) to 66.7% in Vancouver (Pelludat et al., 2009). This is lower than the overall prevalence detected by the arrays, which

was 6.1% in Canada. However, the prevalence even within a city can vary greatly depending on the location. Distinct clusters of infection have been observed in this study population, with significantly different *Leptospira* infection rates (Himsworth, 2013); this can skew the overall estimated prevalence in a location, depending on which areas are sampled for a study.

Several variables (where available) were assessed when looking at the presence of *Leptospira*. These included species, sex, age, weight, size, season, month, and for females, pregnancy status and whether or not the animal was lactating. Data for all of these variables however, were not available from all three countries. Significant relationships with *Leptospira* infection were found for several of these variables. The significant variables were age, weight and the season in which the animals were trapped. It is also interesting to note that the only positive samples for *Leptospira* were from *R. norvegicus*. This finding is similar to what has been reported in the literature, as *R. norvegicus* has been identified as the most common rodent host, though numerous other rodent species including *R. rattus* and *M. musculus* can carry the pathogen (Dutta and Christopher, 2005, Hartskeerl et al., 2011, Carter and Cordes, 1980).

Mature animals were more likely to have a positive *Leptospira* result than immature ones, and this is supported by the logistic regression carried out for the weight of the animals. Weight has been used as a good determination of age in other studies (Davis, 1953, Himsworth et al., 2013). As the weight of the animal increased it was more likely that it would be positive for *Leptospira*. This has been reported frequently and it is likely that animals acquire the organism early in life, with an increasing rate of infection until maturity. The oldest animals become immune and there seems to be a lower prevalence in the most mature animals (Himsworth, 2013).

The season in which the animals were trapped was also important; the majority of positive animals were caught in the autumn. This is consistent with the literature, as in

several species (including *R. norvegicus*), *Leptospira* appears to show a seasonal pattern, and tends to be more common in the autumn or rainy season (Sarkar et al., 2002, Ward, 2002). Due to the greater number of samples available from Canada, the combined data for age, size and season could have been biased, which is why individual analysis by country was also performed. In France the age of the animal was not a significant factor in *Leptospira* infection, however in Canada it was ($p < 0.05$). The size of the animal was a significant factor in both countries. As for the age of the animal, the season was not a significant factor in France but it was in Canada. These differences could be explained by the different sample sizes available from France and Canada.

There were only two positive SEOV samples identified by the array; these were confirmed by both PCR and sequencing (12 additional samples were identified as positive at the source laboratory). However, the importance of SEOV, and of hantaviruses in general, make this an interesting result. There are an increasing number of SEOV infections in humans occurring throughout Europe and the virus can cause severe clinical disease (Jameson et al., 2013, Volokhov et al., 2002, Macé et al., 2013, Dupinay et al., 2014). It is important, therefore, that methods are developed to detect this pathogen quickly. The array could be useful for this purpose: A more dedicated hantavirus array could be developed, with probes specific to individual hantaviruses, and if only lung or kidney tissue were sampled, this would give the best possible chance of detection.

Many studies have looked at these pathogens in a rural setting; however, relatively few have looked at their presence in urban centres, particularly the prevalence within rodent hosts. These species can act as sentinel species for these pathogens and can provide an early warning system for a possible human outbreak. Using an array such as the one used in this project provides the opportunity to screen a large number of samples for many different pathogens of zoonotic importance. However, there are

many variables to consider when designing an appropriate sampling method and array that will be useful in pathogen screening.

CHAPTER SIX

CONFIRMATORY TESTING

6.1 SUMMARY

It was important that any array positive results were confirmed using other, previously validated methods. Confirmation of *Leptospira* and SEOV array positive results, using a variety of methods, have already been described. This chapter describes the confirmatory testing conducted for the remaining array positive pathogens. Several probes repeatedly produced a positive signal on the arrays and it was thought that these were "rogue" probes. For the majority of suspected array positive samples and "rogue" probes, standard PCR and qPCR were used as confirmatory methods. Another - much more sensitive - method (solution based hybridisation capture) was used for some of the *Y. pestis* suspected positive samples.

The suspected "rogue" probes that were seen on the arrays were not positive by any other tests. The signal on the arrays was therefore deemed likely to be the result of non-specific hybridisation. The PCR results were negative for the majority of all the suspected array positives, and for those which tested positive on standard PCR, further analysis using qPCR indicated that the suspected array positives were in fact negative. By contrast, positive qPCR results were obtained when the *pla* (plasminogen activator) gene of *Y. pestis* was used as the target for samples which had hybridised with the *Y. pestis* probes on the arrays. Further PCR with primers for different regions of the *Y. pestis* genome produced negative results on those same samples. The results of the solution based hybridisation capture, which used several different primer sets, indicated that the samples were negative for *Y. pestis*. The qPCR positive results for the *pla* gene on the PcP plasmid in *Y. pestis* may have resulted from the presence of a homologue recently found in *R. rattus*, which had 99% identity with the *pla* gene sequence found in *Y. pestis*.

6.2 INTRODUCTION

The probes on the array had not been fully validated during the array design and evaluation stages, as validation of any new diagnostic test requires thousands of samples and tests. It was necessary therefore, to perform confirmatory tests on all positive samples to ensure that any array results were real positives. Despite the benefits that PCR provides, a drawback of the technology is that even the smallest amount of contaminating DNA can be amplified, which can then hybridise to the probes on the array. Contamination can lead to false-positive results and this could lead to an incorrect diagnosis, which may be critical in a clinical setting (Panti-May et al., 2012). This is more of a problem with sequence-independent amplification than with sequence-specific amplification due to the nature of the random priming used in sequence-independent amplification, which will amplify any DNA present in a sample. Sequence-specific amplification can, however, be affected if there is a sequence or homologue in the host genome that has a high identity with the PCR primers being used (Janse et al., 2010).

Several samples displayed a positive signal for some significant zoonotic pathogens, including Stx-producing *E. coli* (6 positives) and *Y. pestis* (69 positives). Therefore, it was important to determine the specificity of the array in detecting these pathogens. In this chapter the array results are also compared with the results obtained from other laboratories; any discrepancies were noted and tested.

Confirmatory tests were performed in several ways; standard PCR and qPCR were carried out using established primers and protocols. In addition any samples which were suspected to contain the notifiable pathogen *Y. pestis* were tested at the WHO Yersinia Reference Laboratory at the Institut Pasteur, Paris, in addition to being tested at the University of Nottingham. The notifiable pathogen suspected in some of the

samples was *Y. pestis* and the suspected positive samples were sent to the WHO. In addition, a solution based hybridisation capture was used on some of the suspected *Y. pestis* positive samples (Maricic et al., 2010). This was performed by colleagues at the Leibniz Institute for Zoo and Wildlife Research in Berlin. The methods involved in this are outlined in section 9.2 of the Appendix. Sequencing was then performed and the resulting sequences were given PHRED scores. PHRED is a base-calling programme for DNA sequence traces. It reads DNA sequence chromatogram files and analyses the peaks to call bases, assigning quality scores (PHRED scores) to each base call, a higher PHRED score indicates a higher quality base call.

6.2.1 Aims

The hypothesis investigated was that the array had successfully detected a wide range of zoonotic pathogens in rodent samples. The objectives of this work were to:

- Determine the presence of "rogue" probes on the array.
- Confirm the presence of pathogens identified as positive by the array using methods including PCR, qPCR and solution based hybridisation capture.

6.3 RESULTS

6.3.1 Array Results

Several probes on the array produced a positive signal with nearly every sample. These probes (Table 6.1) also hybridised when a negative control sample (water) was used. Therefore these were classified as 'rogue' probes and were considered unlikely to represent an actual positive result. To confirm this, several PCR runs were performed on a number of samples which produced a positive signal for these probes, and all of the results were negative.

Table 6.1. Probes that displayed rogue properties

Cross-hybridising probes	Pathogen	Target gene
E.coliUPS_521	*E. coli*	*stx1*
SalmonellaUPS_709	*Salmonella*	endonuclease
SalmonellaUPS_710	*Salmonella*	endonuclease
SalmonellaUPS_711	*Salmonella*	endonuclease
SalmonellaUPS_712	*Salmonella*	endonuclease
SalmonellaUPS_713	*Salmonella*	endonuclease
SalmonellaUPS_714	*Salmonella*	endonuclease
SalmonellaUPS_715	*Salmonella*	endonuclease
Y.enteroco_Owiz_178	*Y. enterocolitica*	operon protein C
LeptospiraUPS_630	*Leptospira*	LA1029
S.monilUPS_753	*S. moniliformis*	aspartate-semialdehyde dehydrogenase
SEOV_UPS_S15	SEOV	N protein
ToxoplasmaUPS_359	*T. gondii*	B1 gene
ToxoplasmaUPS_361	*T. gondii*	B1 gene

There were other probes that displayed a positive signal following hybridisation with the field samples, but not with the negative control samples. The probes that hybridised and the pathogens that they represent are shown in Table 6.2. Confirmatory testing was performed even if a single probe on the array hybridised. Descriptive statistics for each probe can be seen in the table as well as the number of samples that had a positive result with these probes. This was performed to see how many probes had signal intensity greater than the cut-off point of 0.4, which is suggestive of a

positive result. There were some pathogens for which a positive signal was observed on multiple probes, in particular *T. gondii* and *Y. pestis*, and to a lesser extent *Y. pseudotuberculosis* and generic *Yersinia*. When multiple probes hybridise it is more likely that the pathogen for which the probes were designed to detect would be present in the sample. Some of these probes were observed to show a positive signal in over 40 samples.

Table 6.2. Probes that produced a detectable signal after sample hybridisation with the array. The number of samples and probes is shown. As each probe was printed in duplicate, the signal for each probe on the same array was not always the same. Any probes with a signal <0.4 were not included.

Probe ID	Pathogen	Number of samples	Mean Signal (number of probes)	Standard Deviation	CI (95%)
BartonellaUPS_461	*Bartonella*	3	0.587 (6)	0.078	0.063
BartonellaUPS_462	*Bartonella*	3	0.582 (6)	0.172	0.151
Campylo_Owiz_481	*C. jejuni*	7	0.521 (12)	0.080	0.045
Campylo_Owiz_487	*C. jejuni*	6	0.509 (10)	0.074	0.042
FTH_FSC155_04	*Fransicella sub sp holarctica*	1	0.432 (1)	NA	NA
LCMVUPS_604	LCMV	1	0.473 (1)	NA	NA
LCMVUPS_605	LCMV	1	0.435 (2)	NA	NA
LCMVUPS_616	LCMV	3	0.470 (5)	0.038	0.034
M. microti_02	*M. microti*	1	0.402 (1)	NA	NA
M. microti_04	*M. microti*	1	0.419 (1)	NA	NA
M. tuberc+bovis_84	*M. tuberculosis + M. bovis*	1	0.432 (1)	NA	NA
PUUV_UPS_L38	PUUV	1	0.401 (1)	NA	NA
R.typhi_Owiz_675	*R. typhi*	1	0.411 (1)	NA	NA
R.typhi_Owiz_676	*R. typhi*	1	0.406(1)	NA	NA
S.monilUPS_748	*S. moniliformis*	1	0.415 (1)	NA	NA
S.monilUPS_749	*S. moniliformis*	1	0.407 (1)	NA	NA
SalmonellaUPS_736	*S. typhimurium*	1	0.436 (1)	NA	NA
Toxoplasma_435	*T. gondii*	41	0.546 (62)	0.097	0.023
ToxoplasmaUPS_360	*T. gondii*	23	0.500 (38)	0.062	0.020
ToxoplasmaUPS_362	*T. gondii*	27	0.519 (51)	0.073	0.020
ToxoplasmaUPS_363	*T. gondii*	40	0.556 (66)	0.097	0.023
ToxoplasmaUPS_364	*T. gondii*	33	0.529 (52)	0.086	0.023
Y.pestis_Owiz_117	*Y. pestis*	41	0.559 (65)	0.122	0.030
Y.pestis_Owiz_127	*Y. pestis*	41	0.587 (67)	0.111	0.027
Y.pestisUPS_785	*Y. pestis*	2	0.578 (4)	0.045	0.045
Y.pestisUPS_786	*Y. pestis*	16	0.529 (28)	0.131	0.049
Y.pestisUPS_787	*Y. pestis*	20	0.502 (30)	0.120	0.043
Y.pestisUPS_789	*Y. pestis*	1	0.493 (2)	NA	NA
Y.pestisUPS_791	*Y. pestis*	2	0.600 (3)	0.016	0.018
Y.pseudo_Owiz_216	*Y. pseudotuberculosis*	11	0.537 (19)	0.071	0.032
Y.pseudo_Owiz_218	*Y. pseudotuberculosis*	17	0.615 (32)	0.104	0.036
Y.pseudo_Owiz_219	*Y. pseudotuberculosis*	19	0.633 (36)	0.125	0.041
Yersinia_Owiz_292	*Yersinia*	10	0.491 (15)	0.060	0.030
Yersinia_Owiz_293	*Yersinia*	24	0.546 (41)	0.107	0.033
Yersinia_Owiz_342	*Yersinia*	19	0.657 (34)	0.143	0.048
Yersinia_Owiz_344	*Yersinia*	18	0.640 (25)	0.139	0.054

A comparison was also made between the array results and other test results from the source laboratory, where available. There were two *T. gondii* PCR positive results from France that were not detected on the array (Florence Aryal, personal communication). However, it should be noted that the tissue type used for this detection was heart tissue. The ideal sample locations for all of the pathogens in this study are shown in Table 5.1. Additionally, there were MRSA positive samples from Canada; however, these results were obtained using samples from an oropharyngeal swab and not from the liver, kidney or lung which was tested at the University of Nottingham. Methicillin resistance in the Canadian samples was confirmed at the source laboratory by demonstrating the presence of the penicillin-binding protein 2a antigen using a latex-agglutination test (Oxoid Ltd., Basingstoke, UK). MRSA isolates were characterised by *spa* typing using the Riodom SpaServer (http://SpaServer.ridom.de). The lukF-PV gene in MRSA, encoding the Panton-Valentine leukocidin toxin was detected by qPCR (Himsworth, 2014). In the same study, 367 *E. coli* positive samples were detected by PCR in Canada; however, this result was obtained using faecal samples. Moreover the test was not focussed on *E. coli* O157, or the *stx* gene which is an important virulence factor. The positive *E. coli* samples may, therefore, have been apathogenic strains.

6.3.2 PCR testing

To confirm that the array positive samples were true positives, all of these samples were tested by confirmatory PCR, using previously published primer sequences. The primers used for this end-point PCR testing are given in Table 6.3.

Table 6.3. Primers used for confirmatory PCR testing.

Pathogen	Primer sequence		Target	Amplicon size (bp)	Reference
Bartonella	F	TAACCGATATTGGTTGTGTTGAAG	*ribC*	585	(Johnson et al., 2003)
	R	TAAAGCTAGAAAGTCTGGCAACATAACG			
CPXV	F	ATCAGATGGAATTATCTCTCACCCG	*B9R*	421	(Shchelkunov et al., 2005)
	R	GATAATTTGATCCATCTCGTCCACC			
C. jejuni	F	GAAGAGGGTTTGGGTGGTG	*hip*	735	(Linton et al., 1997)
	R	AGCTAGCTTCGCATAATAACTTG			
E. coli	F	CAGGTCGTCGTGTCTGCTAAA	*eaeA*	1087	(Gannon et al., 1997)
	R	TCAGCGTGGTTGGATCAACCT			
Fransicella	F	TTTATATAGGTAAATGTTTTACCTGTACCA	*RD1*		This study
	R	GCCGAGTTTGATGCTGAAAA			
Generic Yersinia	F	AAAGCCATTTCCGTATGCTG	*YOPH*	514	This study
	R	TGTACTCGCATTTGGCTGAC			
LCMV	F	GATTCTCACGTCTTCTTCCA	Polymerase	678	(Slifka et al., 1995)
	R	CTCTCCGTAATTTGACTCAC			
Leptospira	F	TGAGCCAAGAAGAAACAAGCTACA	*gyrB*	504	(Slack et al., 2006)
	R	ATGGTTCCGCTTTCCGAAGA			
Mycobacteria	F	TGCACTTCGGGATAAGCCTG	16S		This study
	R	TAGCATGTGTGAAGCCCTGG			
PUUV	F	AGTGCCCATTCATAAAGCCTG	S segment	391	(Hörling et al., 1995)
	R	GTTCGACGCAGGTAAGATTGG			
R. typhi	F	CCGCAGGRTTGGTAACTGC	*rOmpB*	833	(De Sousa et al., 2006)
	R	CCTTTTAGATTACCRCCTAA			
SEOV	F	GTGGACTCTTCTTCTCATTATTG	M segment	323	(Plyusnina, 2004)
	R	TGGGCAATCTGGGGGGTTGCA			
S. moniliformis	F	GCTTAACACATGCAAATCTAT	16S	296	(Boot et al., 2002)
	R	AGTAAGGGCCGTATCTCA			
S. Typhimurium	F	CGGTGTTGCCCAGGTTGGTAAT	*fliC*	620	(Pui et al., 2011)
	R	ACTGGTAAAGATGGCT			
T. gondii	F	CGCTGCAGGGAGGAAGACGAAAGTTG	*B1*	529	(Homan et al., 2000)
	R	CGCTGCAGACACAGTGCATCTGGATT			
Y. enterocolitica	F	ACTCGATGATAACTGGGGAG	*ail*	170	(Nakajima et al., 1992)
	R	CCCCCAGTAATCCATAAAGG			
Y. pestis	F	ATCTTACTTTCCGTGAGAAG	*pla*	480	(Hinnebusch et al., 1998)
	R	CTTGGATGTTGAGCTTCCTA			
Y. pseudotuberculosis	F	CGGTACGGCTCAAGTTAATCTG	*inv*	183	(Thoerner et al., 2003)
	R	CCGTTCTCCAATGTACGTATCC			

Nearly all of the results were negative. Results shown in Table 6.4 indicate that out of the 284 array positive results for the pathogens shown in the table, only 7 of these were confirmed by PCR.

Figures 6.1, 6.2, 6.3, 6.4, 6.5 and 6.6 show the results of confirmatory testing with end-point PCR. Figure 6.7 shows the negative controls. It was noted that the genomic ladders had merged at the bottom of the gel seen in Figure 6.1, and it was concluded that the gel had not set correctly the first time. Rows 7-12 from this gel were therefore re-run (see Figure 6.2). For each figure a table is included beneath that shows the layout of the gel and the identity of the pathogen being tested in each well. The positive results are indicated in each image by a box around the band produced. The positives are indicated in the table by a '*'. Other than the *Leptospira* and SEOV positive results that were already discussed in Chapter 5, there were positive PCR results for two *T. gondii*, three *C. jejuni*, one *Mycobacterium* and one generic *Yersinia* samples (Table 6.3). A 1% agarose gel was used in each figure. The positive PCR results were sent off for sequence analysis at the Institute of Zoology, Oxford; however no sequences could be obtained for *T. gondii, C. jejuni, Mycobacterium* or the generic *Yersinia* samples.

Table 6.4. Results of end-point confirmatory PCR testing

Pathogen	Number of array positives	Number of PCR positives	Number of PCR negatives
T. gondii	72	2	70
Y. pestis	69	0	69
Generic Yersinia	63	1	62
Y. pseudotuberculosis	23	0	23
C. jejuni	21	3	18
LCMV	9	0	9
Bartonella	8	0	8
E. coli	6	0	6
CPXV	5	0	5
Mycobacterium	3	1	2
R. typhi	2	0	2
PUUV	2	0	2
S. typhimurium	2	0	2
Fransicella	2	0	2
S. moniliformis	1	0	1
MRSA	1	0	1

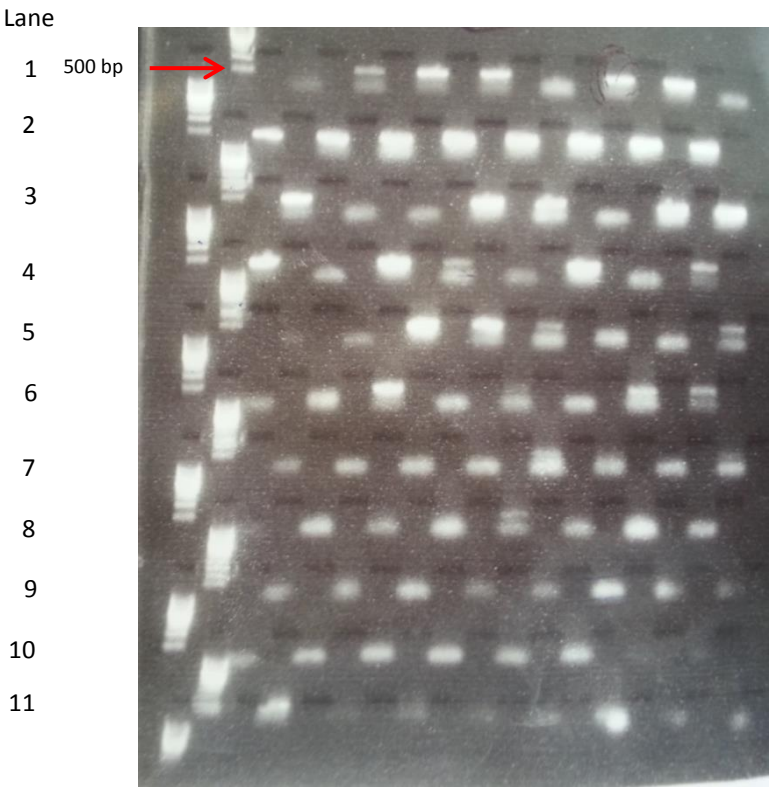


Figure 6.1. Agarose gel electrophoresis of the first confirmatory 96 PCR test (96 well plate format). 1 kbp DNA ladders are also shown with the 500 bp marker indicated by an arrow for the uppermost ladder.

Table 6.5. A summary of the agarose gel electrophoresis carried on the first set of 96 confirmatory PCR samples. Positive results are highlighted in yellow. The legend for the pathogens in the table is as follows with the expected product size in bp shown in parentheses: GY=Generic *Yersinia* (514); Lep=*Leptospira* (504); T.gon=*T.gondii* (529); SEO=SEOV (323); LCM=LCMV (678); Y.pse=*Y. pseudotuberculosis* (183); R.ty=*R. typhi* (833); Y.pes=*Y. pestis* (480); CPX=CPXV (421).

Lane	Pathogen							
1	GY	**Lep**	**Lep**	**Lep**	T.gon	**SEO**	**Lep**	LCM
2	**Lep**	**Lep**	**Lep**	**Lep**	**Lep**	**Lep**	**Lep**	**Lep**
3	**Lep**	Y.pse	GY	**Lep**	**Lep**	Lep	**Lep**	**Lep**
4	**Lep**	T.gon	**Lep**	**SEO**	GY	Lep	**GY**	**Lep**
5	GY	R.ty	**Lep**	**Lep**	**Lep**	T.gon	Lep	**Lep**
6	Y.pes	T.gon	**Lep**	Lep	**Lep**	Lep	**Lep**	**Lep**
7	T.gon	Lep	Lep	Lep	**Lep**	**Lep**	Lep	Lep
8	Y.pes	T.gon	**Lep**	Y.pes	**Lep**	CPX	T.gon	T.gon
9	Lep	Lep	**T.gon**	Lep	Fra	T.gon	T.gon	T.gon
10	Lep	T.gon	T.gon	T.gon	Lep	Lep	Y.pes	Lep
11	**Lep**	Y.pes	SEO	GY	GY	Y.pes	GY	Fra
12	LCM	R.ty	GY	T.gon	T.gon	Y.pes	Y.pes	LCM

Table 6.6. A summary of the agarose gel electrophoresis carried on the second set of 96 confirmatory PCR samples.
Positive results are highlighted in yellow. The legend for the pathogens in the table is as follows: Y.pes=*Y.pestis*; Bar=*Bartonella*; GY=Generic *Yersinia*; LCM=LCMV; Lep=*Leptospira*; T.gon=*T.gondii*; CPX=CPXV; PUU=PUUV; Y.pse=*Y.pseudotuberculosis*; C.jej=*C.jejuni.*

Lane	Pathogen							
1	Y.pes	Bar	Y.pes	GY	LCM	Lep	Lep	Y.pes
2	T.gon	T.gon	GY	Bar	Y.pes	Y.pes	Y.pes	Y.pes
3	T.gon	Y.pes	Y.pes	Y.pes	Y.pse	GY	Bar	Y.pes
4	T.gon	GY	Lep	CPX	Y.pes	Y.pse	GY	PUU
5	Y.pse	GY	Y.pes	Y.pes	GY	Y.pes	Y.pse	GY
6	Y.pes	Lep	Lep	Y.pes	C.jej	E.coli	T.gon	Lep
7	T.gon	Lep	Y.pes	GY	T.gon	Lep	C.jej	Y.pes
8	Y.pes	T.gon	Y.pes	T.gon	Y.pes	T.gon	Y.pes	T.gon
9	Y.pes	Y.pes	GY	Y.pes	GY	Lep	Y.pes	GY
10	T.gon	T.gon	Y.pes	C.jej	Y.pse	GY	T.gon	Y.pes
11	Y.pse	GY	Y.pes	C.jej	T.gon	T.gon	T.gon	Y.pes
12	Y.pse	GY	T.gon	Y.pes	GY	T.gon	Y.pes	Y.pes

Table 6.7. A summary of the agarose gel electrophoresis carried on the third set of 96 confirmatory PCR samples.
Positive results are highlighted in yellow. The legend for the pathogens in the table is as follows with the expected product size in bp shown in parentheses: T.gon=*T.gondii*; GY=Generic *Yersinia*; S.ty=*S.*Typhimurium; Bar=*Bartonella*; C.jej=*C.jejuni*; Y.pes=*Y.pestis*; Y.pse=*Y.pseudotuberculosis*; CPX=CPX; LCM=LCMV; Pos=*Y. pestis* positive control; MR=MRSA.

Lane	Pathogen							
1	T.gon	GY	T.gon	GY	S.ty	GY	Bar	C.jej
2	T.gon	GY	Y.pes	T.gon	Y.pse	GY	Lep	GY
3	Y.pes	CPX	T.gon	Y.pes	GY	T.gon	LCM	Y.pes
4	T.gon	Y.pes	Y.pes	GY	Y.pes	GY	Y.pes	C.jej
5	T.gon	T.gon	**Lep**	Y.pes	CPX	E.coli	Y.pes	C.jej
6	Y.pes	GY	T.gon	C.jej	Y.pes	GY	C.jej	Lep
7	Y.pes	T.gon	T.gon	Y.pes	GY	T.gon	T.gon	Bar
8	Y.pes	GY	T.gon	Y.pes	T.gon	GY	T.gon	LCM
9	Y.pes	HEV	Lep	**Pos**	T.gon	Y.pes	Y.pes	T.gon
10	HEV	Lep	GY	Y.pes	Lep	GY	MR	E.coli
11	Y.pes	**C.jej**	**Lep**	LCM	T.gon	Lep	**Lep**	Y.pse
12	GY	Y.pes	E.coli	C.jej	T.gon	MR	Han	C.jej

Table 6.8. A summary of the agarose gel electrophoresis carried on the fourth set of 96 confirmatory PCR samples.
Positive results are highlighted in yellow. The legend for the pathogens in the table is as follows with the expected product size in bp shown in parentheses: T.gon=*T.gondii*; C.jej=*C. jejuni*; Y.pse=*Y.pseudotuberculosis*; GY=Generic *Yersinia*; Lep=*Leptospira*; Bar=*Bartonella*; Y.pes=*Y.pestis*; LCM=LCMV; PUU=PUUV; S.mon=*S. monoliformis*; Fra=*Fransicella*; S.ty=*S*. Typhimurium; CPX=CPXV; Myc=*Mycobacteria*.

Lane	Pathogen							
1	T.gon	C.jej	Y.pse	GY	GY	GY	T.gon	**Lep**
2	T.gon	T.gon	Lep	E.coli	Lep	T.gon	**C.jej**	**Lep**
3	Bar	Y.pes	LCM	Y.pse	GY	Lep	T.gon	T.gon
4	Y.pse	GY	C.jej	Y.pes	GY	Lep	Y.pes	T.gon
5	PUU	S.mon	GY	T.gon	**Lep**	GY	Y.pes	Han
6	Y.pse	GY	E.coli	T.gon	Y.pse	GY	Y.pse	GY
7	T.gon	C.jej	GY	Lep	Fra	S.ty	T.gon	Lep
8	LCM	**Lep**	C.jej	Y.pes	**C.jej**	Lep	CPX	Y.pse
9	GY	Bar	**Lep**	**Lep**	**C.jej**	**Lep**	**Lep**	**Lep**
10	**Lep**	Fra	**Lep**	**Lep**	Y.pse	GY	Bar	GY
11	Myc	Y.pse	GY	Y.pes	GY	Y.pse	GY	**Lep**
12	**Lep**	**Lep**	Myc	T.gon	Y.pse	GY	Y.pse	GY

Table 6.9. A summary of the agarose gel electrophoresis carried on the fifth set of confirmatory PCR samples.
Positive results are highlighted in yellow. The legend for the pathogens in the table is as follows with the expected product size in bp shown in parentheses: Lep=*Leptospira*; Y.ent=*Y. enterocolitica*; GY=Generic *Yersinia*; C.jej=*C.jejuni*; Y.pes=*Y.pestis*; Y.pse=*Y. pseudotuberculosis*; Myc=*Mycobacteria*.

Lane	Pathogen							
1	**Lep**	Y.ent	GY	C.jej	Y.pes	Y.pse	GY	**Lep**
2	**Myc**	**Lep**	**Lep**					

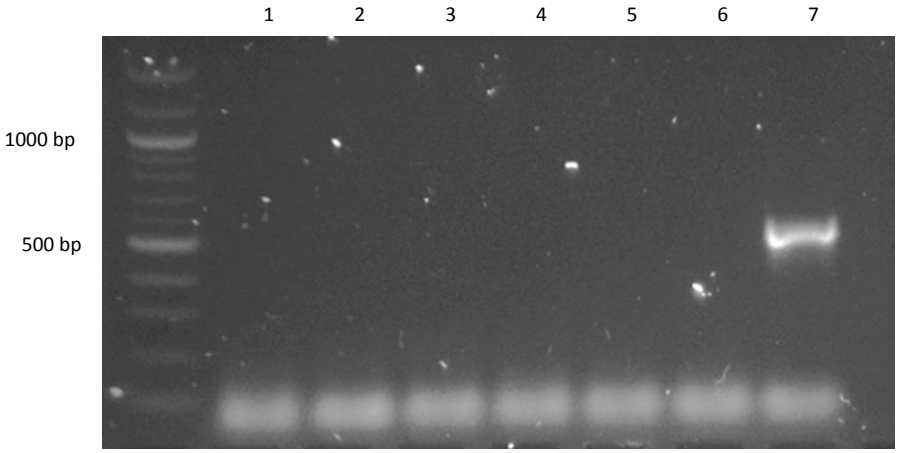


Figure 6.2. Agarose gel electrophoresis of the negative control samples for each confirmatory test run.
Lanes 1-5 show the negative control sample from each of the confirmatory PCR tests shown in Tables 6.5-6.9. Lane 6 is an additional negative control and Lane 7 shows a positive control with *Y. pestis* as the reference material. The product is shown at just above 500 bp which is expected (529 bp).

6.3.3 qPCR TESTING

In addition to standard, end-point PCR, qPCR was also performed. The result of qPCR amplification on these samples is given in Table 6.5. The standard PCR was negative for all the *Y. pestis* samples. However, 12 of the samples were positive for *Y. pestis* on qPCR. All of the remaining samples were negative for the screened pathogens. This included the three *C. jejuni* and two *T. gondii* results that were positive by end-point PCR.

Table 6.10. qPCR amplification results

Pathogen	Number of array positives	Number of qPCR positives	Number of qPCR negatives
E. coli	6	0	6
LCMV	9	0	9
Bartonella	8	0	8
CPXV	5	0	5
T. gondii	72	0	42
C. jejuni	21	0	21
Y. pestis	69	12	11

6.3.4 *Y. PESTIS*

12/23 of the *Y. pestis* array positive results that were tested by qPCR were identified as positive for the *pla* gene. Two of these are shown as an example in Figure 6.8. The first column of images shows the curve produced with a suspected positive *Y. pestis* sample. The second column shows the positive control (*Y. pestis* reference material) and the third column shows the negative control (water replaced the DNA template). For both samples the ct value was approximately 36. This was a similar value to that observed for the remaining ten samples. The amplification of DNA in the samples was also low, as the low curve shows. The ct value reached when the positive control was used was between 28 and 32, while for the negative control no amplification took place that crossed the automatic threshold value of 0.2.

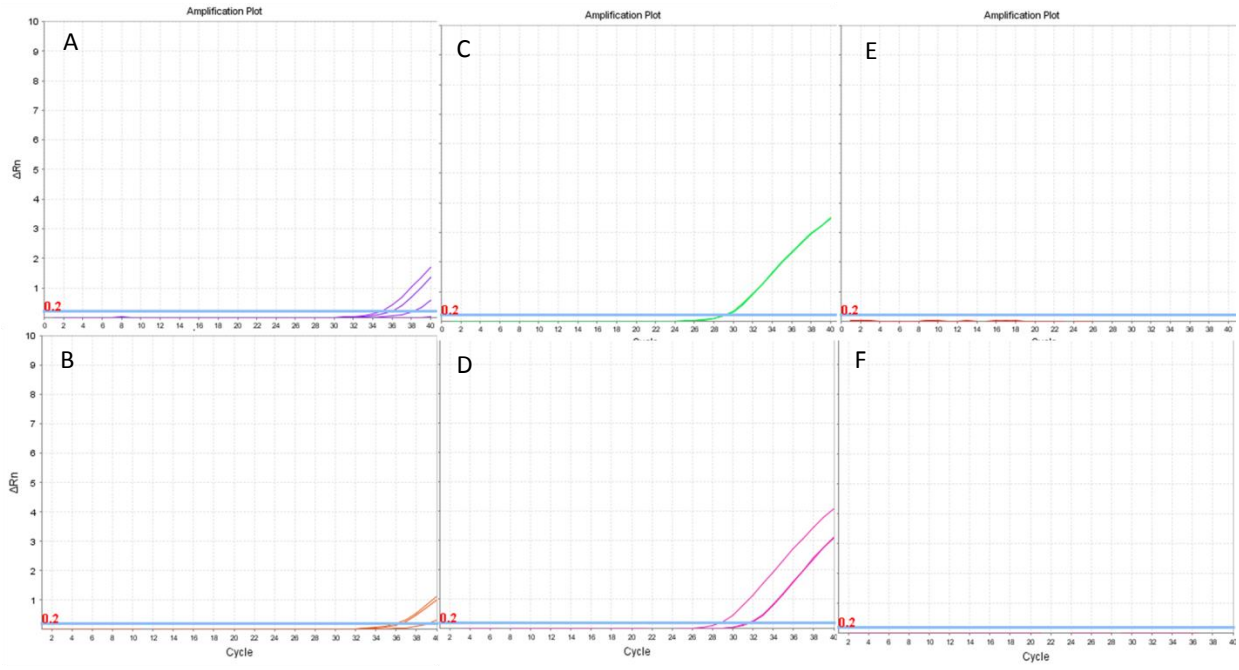


Figure 6.3. *Y. pestis* qPCR amplification plots

A. Suspected *Y. pestis* positive sample.
B. Suspected *Y. pestis* positive sample.
C. Positive reference sample for *Y. pestis.*
D. Positive reference sample for *Y. pestis.*
E. Negative control sample (water replaced sample).
F. Negative control sample (water replaced sample).

The qPCR primers Y.pes_pPCP_F and Y.pes_pPCP_R were used to identify a particular region (*pla* gene) on the PcP plasminogen activator plasmid of the *Y. pestis* genome, which confers virulence. This region has since been discovered to have a homologue; this was demonstrated in 8/11 samples from *R. rattus* and 2/4 samples from *R. norvegicus* trapped in the Netherlands in 2009 (Janse et al., 2010). An alignment of the rat homologue (HQ606074) previously sequenced with 5 different *Y. pestis* isolates is shown in Figure 6.9. The alignment indicates that the sequence for the *pla* gene is almost identical to the homologue, with only 8/750 differences between the rat homologue and the sequence from the *Y. pestis* isolates. Further testing with the primers shown in Table 2.12 from Chapter 2, which targeted a different region of the *Y.*

pestis genome indicated that the samples were actually negative for *Y. pestis*, and did not produce any amplification on the qPCR curve. The Institut Pasteur, a WHO Reference Laboratory for *Y. pestis*, tested the positive samples with an F1 antigen dipstick and through phage lysis on cultures. The results of this testing were also negative for *Y. pestis*. The results of the solution-based hybridisation capture and following analysis by Dr Tom Giles at the University of Nottingham are shown in section 9.2.2 of the Appendix.

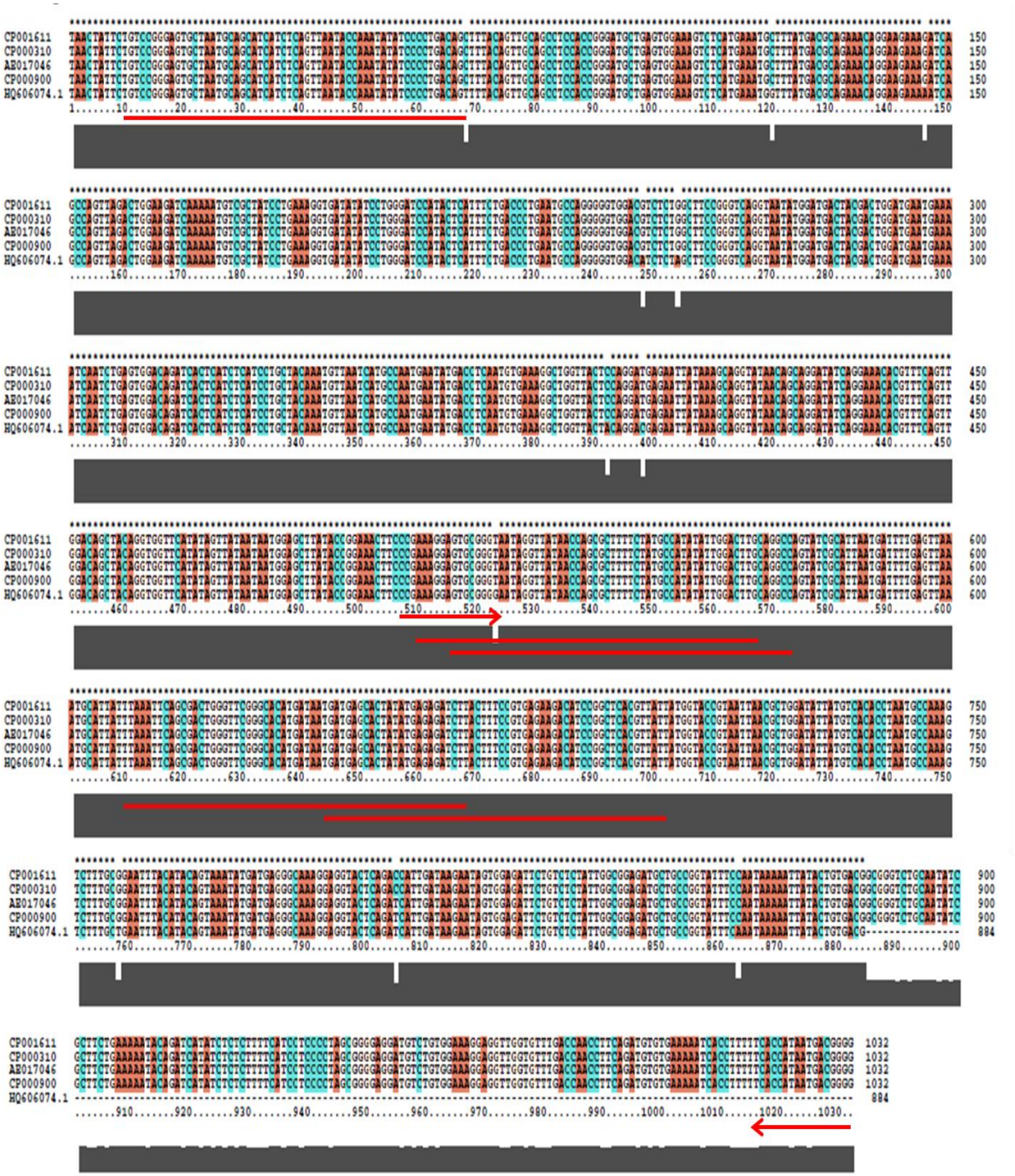


Figure 6.4. Multiple sequence alignment of the *Y. pestis pla* gene and the homologue in rats (HQ606074).
The sequence identity is shown on the left and each different base is represented by a symbol G, A, C, and T. A * above each nucleotide position in the alignment, represents an identical base for all samples. Oligonucleotide probe positions are indicated by solid red bars. The positions of the forward and reverse primers are indicated by arrows.

6.4 DISCUSSION

The array was successful in detecting *Leptospira* and SEOV positive samples, which was confirmed by various methods, as discussed in Chapter 5. It was necessary to confirm the remaining array-positive results in Table 6.2 using other testing methods. The majority of these samples were negative on the confirmatory tests. The reason for positive signals seen on the array remains unknown, though for most of these, it seems likely to be a result of non-specific hybridisation.

For several samples, there were multiple probes that displayed a positive signal. This was particularly the case for *T. gondii* and *Y. pestis*. There were five probes commonly positive for *T. gondii* and seven for *Y. pestis*. The mean signal for both *T. gondii* and *Y. pestis* probes was between 0.3 and 0.4 on several occasions, which was regarded as an inconclusive result. When multiple probes display a signal, even if the signal is low, it is suggestive of a target sequence in the sample. The threshold value for a positive signal on arrays can be variable, and there is no standardised value required to declare a sample positive or negative (Tsien et al., 2001). The lower signal observed may simply have been the result of a low pathogen load in the sample, or an indication that the probes were not performing as efficiently as other, more strongly hybridising probes. Therefore it is important to perform subsequent tests to check inconclusive results. However, subsequent negative results on PCR and qPCR suggested that these were likely to be negative samples, and the signal produced was from hybridisation with non-specific sequences.

There were conflicting results for some tests. Specifically, from the samples which tested positive on the array, a number were also positive by endpoint PCR: 2/72 *T. gondii*, 3/21 for *C. jejuni*, 1/3 for *Mycobacterium* and 1/63 for a generic *Yersinia*. However, both sequencing of the PCR products, and additional testing by qPCR yielded negative results for these samples. Quantitative PCR has been shown to be more

specific than standard PCR; this may explain why these samples were negative on qPCR but positive on standard PCR and on the array, which used PCR as the method of amplification (Bryant et al., 2004, Charrel and de Lamballerie, 2003, Tatineni et al., 2008, Koskinen et al., 2009). The low mean signal on the array probes for these pathogens could also be consistent with a negative result. It is possible that some non-specific hybridisation occurred with these probes, which could have been responsible for the low signal seen. In one case, the resulting PCR amplicons was not what was expected; specifically there was a double amplicon produced for the PCR positive *Mycobacterium* sample (Figure 5.6). For the primer set used, only one amplicon was expected (Abu-Bakr Abu-Median, personal communication). This further casts doubt on the possibility that this sample was genuinely positive.

There were several samples for which one of the duplicated probes produced a high signal, yet the other probe had low signal intensity. The pathogens for which this occurred included *C. jejuni*, Stx producing *E. coli, T. gondii* and *Y. pestis*; and the probes are shown in Table 6.13. One source of variation which can be introduced in microarray experiments is the hybridisation step, which includes each washing step (Russell et al., 2009). The image recorded after two separate hybridisations on different arrays can often have considerable levels of unpredictable variation in the signal. Whilst automation of the hybridisation process can help eliminate variation (as it can eliminate potential manual handling errors), uneven hybridisation and washing cannot be totally eliminated (Russell et al., 2009). This may explain some of the variation seen with some of the probes. For this reason, some researchers suggest using multiple replicates of each probe on the array surface (Smyth et al., 2005). However, the additional replicates would reduce the number of unique probes which could fit on the array.

The probes that displayed rogue-like properties, i.e. those that produced a signal even when water was used instead of a DNA sample, could be probes that had been printed

at a higher concentration than the other probes on the array (Abu-Bakr Abu-Median, personal communication). Spot homogeneity depends on the concentration of the oligonucleotide within the spot. A high oligonucleotide concentration can result in the accumulation of the oligonucleotide at several points on the spot (ProMED-mail, 2014). These can result from anomalies during the production and printing of the arrays, in particular the drying step after spotting. Any hybridisation on the probe does not necessarily mean the result is a false positive as they do sometimes give true positives.

The difference in results between the source laboratory and the University of Nottingham highlights the importance of standardising the selection and processing of the sample to be tested. The use of heart tissue for *T. gondii*, oropharyngeal swabs for MRSA, and faecal samples for *E. coli* at the source laboratory (Tsangaras et al., 2014) were thought likely to explain the difference in results from those obtained at the University of Nottingham where a liver, kidney and lung homogenate were used. Detection of *E. coli* in faecal samples and MRSA in nasal swabs, are much more common than from liver, kidney or lung (Inglesby et al., 2000b, Ziwa et al., 2013). The sensitivity of a test clearly can be greatly affected by the appropriacy of the site selected for sampling.

Quantitative PCR analysis of samples which were array positive for *Y. pestis* indicated that a number of these were positive for the *pla* gene. Further analysis, with additional primers specific to other regions of the *Y. pestis* genome, suggested that the samples were not truly positive on qPCR for *Y. pestis*, but instead, might have been positive for a *pla* homologue which has been detected previously in rat species (Janse et al., 2010). Even though amplification of the *pla* gene was seen for 12 of the 23 samples tested by qPCR, the amplification was very low (Figure 5.7). The curve produced was comparable to that seen during the sensitivity testing of *Y. pestis,* when the concentration of DNA was at 5.69 $^{x10-8}$ ng/µl (Chapter 3). The sequence of the *pla* gene from the *Y. pestis* PcP

plasmid, was aligned with a sequence from the homologue found in *R. rattus* samples (both were obtained from the NCBI database). The sequence from the *R. rattus* sample was almost identical (99%) to that of *Y. pestis* (Figure 5.8). This particular gene had been chosen for probe design due to its widespread use in PCR detection of *Y. pestis* (Riehm et al., 2011, Tomaso et al., 2003, Loïez et al., 2003, Raoult et al., 2000).

Samples were sent to Berlin for solution based hybridisation capture. The data suggests that the rodents were not harbouring any bacteria which may have cross-hybridised with the probes. The majority of the sequences detected at the laboratory in Berlin aligned with the rat genome. The results suggested that DNA associated with rat and mouse sequences was highly prevalent in the samples. Most of the reads that did not align to the rat genome were likely reads associated with rat DNA that is not currently a part of the *R. norvegicus* genome build, as BLAST results did not show significant alignment with pathogen species following a De Novo assembly and BLAST at NCBI.

The evidence suggests that the array detected a homologue of the *pla* gene from the PcP plasmid of *Y. pestis*. The amplification of this region highlights the fact that the *pla* gene alone is not a sufficient diagnostic marker of *Y. pestis* and that it is important that other gene targets are also used for a clinical diagnosis.

It is interesting to note that several of the *Y. pestis pla* positive samples came from mice species (*M. musculus*), while this homologue had previously only been reported in rat species. At present, there is no additional information available in the literature, and further work is required to understand these results. The PcP region, and the *pla* gene in particular, is involved in transmission of *Y. pestis* (Broekhuijsen et al., 2003). The acquisition by *Y. pestis* of PcP, and of another virulence plasmid, the pMT, is thought to have contributed to the evolutionary transformation of *Y. pestis* from a mainly gut-associated *Y. enterocolitica*, into a highly host-adapted mammalian blood-borne

pathogen (Eppinger et al., 2010). The plasminogen activator encoded by the virulence-associated *pla* gene is associated with increasing the likelihood of the bubonic form of the disease by assisting bacterial distribution at the site of a flea bite. It does this through a combination of proteolytic, adhesive, and invasive functions (Zhan et al., 2008). It has been hypothesised that these activities disrupt the ability of the host to contain the bacteria at peripheral locations (Sebbane et al., 2006). These functions also encourage bacterial proliferation in the respiratory system during pneumonic plague (Schuenemann et al., 2011). These findings indicate that the *pla* gene is used in bacterial distribution and proliferation, something that is of importance to many bacterial species. Therefore, it is reasonable to suggest that after horizontal gene transfer, this gene may have been utilised for similar purposes in other bacteria.

As suggested by Janse et al, it is likely that a homologue to the *pla* gene of *Y. pestis* is present in uncharacterised bacteria, which have since been found in rat tissue samples (Janse et al., 2013), as well as mice tissue samples in this study. When the sequence of the homologue (HQ606074) was entered onto BLAST, there was a high degree of identity (100% and 99% respectively) with a *Citrobacter koseri* genome assembly (LK931337) and an *Escherichia coli* genome assembly (LM995843).

CHAPTER 7

DISCUSSION

7.1 GENERAL DISCUSSION

This thesis outlines the steps required to design and develop a microarray to screen for a number of pathogens of zoonotic origin. The justification for such a study was that the majority of microbial diseases affecting humans are of zoonotic origin. Furthermore, 75% of emerging zoonotic pathogens are zoonotic, and wildlife is considered a major source of infection (Taylor et al., 2001). Increasing global urbanisation may result in an increased threat to human health, from emerging and re-emerging infectious diseases (Gratz, 1999). Deforestation and encroachment into the natural environment can bring humans into increased contact with wildlife and this can increase the potential for zoonotic pathogens to cross the species barrier into humans (Berkelman et al., 1994). The ever-increasing size and density of modern day cities has also made them the "perfect distribution centre for pathogens" (Morse, 1995).

The speed at which people can now travel very large distances has created varied opportunities for pathogens to rapidly expand their geographic host range, not to mention the potential for new pandemics to occur. It is not only the pathogen itself which has the ability to travel quickly. News of the pathogen and rumour of infection, can be spread much faster via social media and the internet, and can be almost as troublesome as the pathogen due to the panic and fear it could cause (Grein et al., 2000).

Proactive monitoring of wildlife populations and disease is essential in order to provide baseline data for epidemiological analyses and design of surveillance programmes and control (Böhm et al., 2006). Microarrays are a potentially useful tool, as samples can be screened for numerous pathogens simultaneously in a high-throughput capacity. Surveillance strategies that utilise new technologies such as

microarrays and NGS are rarely combined with risk analyses, yet this could help to identify, in detail, current and future disease threats (Hutchings, 2006).

Altogether, this thesis provides new information on the design and development of a microarray for use as a screening tool. A microarray approach was developed as a potential alternative to existing surveillance tools (Sachse et al., 2005). The assumption was that if several pathogens were present in one sample, a microarray would be more useful as a screening tool as it can test for multiple pathogens at once rather than employing multiple tests which were each designed to detect individual pathogens. Individual tests would require more reagents and more sample, which may not always be available. Although the choice of array platforms or probe types can be challenging, it has been suggested that when after thorough design and evaluation, the results obtained with different platforms are likely to be comparable (Russell et al., 2009). After a series of pilot arrays were manufactured and tested, a screening chip was developed and rodent samples were screened for multiple pathogens. The platform chosen for use in this project was the Alere ArrayStrip, which has the advantage of being able to screen 96 samples simultaneously. The Alere ArrayTube and ArrayStrip are more economical microarrays than the glass slide-based microarray which have thousands of spots on them and can be expensive (Sachse et al., 2005). The disadvantage of the ArrayTube and ArrayStrip however, is that they are lower density arrays with up to a maximum of approximately 600 probes available on the chip. For the current project, the aim of which was to design a pathogen screening tool, it was only necessary to have a limited number of probes per pathogen. For screening purposes a lower number of probes are acceptable, for diagnostic purposes a larger number of probes per pathogen would be required (Abu-Bakr Abu-Median, personal communication). This is because as a screening tool, a positive result would then be confirmed with a validated test. A diagnostic tool should be sufficient to provide a diagnosis with no further testing, and as

such should be much more accurate than a screening tool. After a comparison of the advantages and disadvantages of both approaches, it was decided to use the Alere microarrays.

New diagnostic tools need thorough evaluation and, if possible, full validation before the results obtained from them can be used definitively without confirmatory tests. Because array development was part of the current project, confirmatory tests were included in the study to assess array performance in comparison to previously established methods.

Potential genomic locations from which to design probes were identified for each of the target pathogens. These regions formed the basis for the design of the oligonucleotide probes (Chapter 3). This is a key part of any study which involves microarrays, and the success of the array hinges on how these probes perform in an experimental setting.

Once the probes were designed, evaluation took place in several stages using, where possible, a variety of samples (Chapter 4). The ideal would be to obtain nucleic acid samples of pathogens obtained from culture, from experimentally-infected animals, and from animals that were infected naturally. This range of sample types is optimal as it shows how the probes would perform in different scenarios, and can give an idea of how the probe signal may be affected if different sample types were used. It would be expected that higher signal intensity would be observed with a sample from pure culture than from one obtained from a naturally-infected animal. Unfortunately, however, pure culture samples were unavailable for some of the pathogens. Furthermore, samples from experimentally-infected or naturally-infected animals were only available for a small number of pathogens. Therefore, the performance of each pathogen on the array could not be fully assessed as the project was restricted by the samples available (Table 2.2).

Once a final set of probes had been evaluated using the reference samples that were available, these probes were used on a finalised array. This array was used with a total of more than a thousand field samples to screen for the pathogens of interest. The probes were designed to be approximately 60 nucleotide base pairs long, as it has been suggested that this offers the best compromise between specificity and sensitivity (Kane et al., 2000, Relogio et al., 2002, Wang et al., 2002a). Specifically, the probe length was between 57 and 60 bp long. Although consistent probe length is preferred in order to achieve a better uniformity during hybridisation, it is more important to have a narrow melting temperature distribution, which is dependent on the GC content (Haque et al., 2007). A narrow melting temperature range is preferable as the hybridisation step in the microarray experiment occurs at a single temperature. By designing probes that have a similar melting temperature it was hoped that the optimal conditions for hybridisation could be reached.

Three ArrayTube types were produced during the study (Hanta_HEV_01, Yersinia_01, and Toxoplasma_01) and two ArrayStrip types (WT_Rodent_Chip_02 and Rodent_Chip_03). The final ArrayStrip (Rodent_Chip_03) incorporated the results of array evaluation and included the probes which performed best for each pathogen, and had been tested on both the ArrayTubes and the pilot ArrayStrip. Over 800 probes were designed, though not all of these were spotted on the arrays and evaluated, as suitable probes had already been identified. For the pathogens for which no reference material was available, probe evaluation could not take place, but their probes were still spotted on the array so that if any positive results were obtained confirmatory work could be performed.

There is a degree of variability between probes in any microarray experiment (Russell et al., 2009). As stated in Chapter 2, each probe was printed in duplicate on the array. There were times when there was a large difference in the recorded signal between

duplicate probes on the same array. This highlights the importance of printing replicates of each probe. It is advantageous to have multiple probe copies on the same array; however this may not be possible in all circumstances due to density constraints.

In addition to the development of a new microarray, a new PCR approach was evaluated. This involved the use of biotin-labelling combined with multiplex PCR. Both sequence-independent and sequence-specific amplifications were attempted with the arrays, and it was found that sequence-specific amplification resulted in hybridisation with probes showing signal at greater intensities. The results from this study suggested that sequence-independent amplification was less sensitive than sequence-specific amplification when attempting to detect specific pathogens on the arrays; lower signal intensity on the arrays was generally observed with sequence-independent amplification. An advantage of sequence-independent amplification is that all the nucleic acid in a sample is amplified, and novel pathogens may be identified (Wang et al., 2003a, Wang et al., 2002b), however, this also requires that the probes on the array are designed to detect pathogens at a genus level, as well as at the species and, if possible, strain level. However, singleplex sequence-specific amplification can be a laborious process to amplify each potential target separately. Not only can this be time-consuming, but also requires large quantities of reagents and sample. Multiplex PCR offers highly sensitive and specific PCR, but is not limited to identifying one target per sample, as in singleplex sequence-specific PCR. Many genome regions can be targeted simultaneously such as the detection of six virulence and regulatory genes in differentiating between pathogenic and non-pathogenic *Vibrio cholera* and *Vibrio mimicus* strains (Singh et al., 2002), or differentiating between members of the same species, e.g. between species of the *Listeria* genus (Volokhov et al., 2002).

Multiplex PCR was used to amplify pathogenic material in a sample prior to array hybridisation. When used in combination with biotin-labelled primers, this shortened

the total PCR reaction by several hours as an additional biotin-labelling step of the product was not required. The number of reagents required was also reduced, which made it more cost and time effective. This method increased the number of pathogens that could be tested in a single PCR reaction, and when combined with microarray analysis, allowed a high-throughput approach. When rodent tissue was spiked with a mixture of the available reference pathogen nucleic acid, all of the pathogens were identified by the array. This demonstrates the potential of this multiplex approach combined with microarrays. Results indicated that the microarray had a sensitivity that is comparable to PCR, as shown in Section 4.3.4. However, tests showed that the array was not as sensitive as qPCR; since a microarray is reliant on standard PCR to amplify a product, this was not surprising.

Despite repeated attempts to source reference material from several institutions, reference material was unavailable for a number of pathogens present on the array. As a result, it is possible that some of the probes for these pathogens did not work, and were negative despite the presence of the pathogen in the sample.

Samples of kidney, liver and lung came from *R. rattus, R. norvegicus, M. musculus* and *A. sylvaticus* from the UK, France and Canada. Urban rodents were the main focus of the screening study; less is known about rodent disease in urban settings, as most work has been done on rural rodents. In the UK, animals were sampled from Liverpool, in France they were from Lyon and in Canada, they came from Vancouver.

All three tissue types were not available for each animal sampled. This is likely to have had a negative impact on the ability to detect the target pathogens in the screening study, as some of the pathogens may not have been present in all three of these tissues in an infected host. Therefore, if the single tissue available did not have the pathogen present, the animal may still be positive for that pathogen, impossible to detect with the available sample. Furthermore, the study was initially designed to

screen for a small number of pathogens (*Y. pestis, Y. enterocolitica, Y. pseudotuberculosis, T. gondii*, SEOV, PUUV and HEV), and for those pathogens, samples from the liver, kidney and lung would have been sufficient to detect any of these pathogens if they were present (Table 5.1). Using this information, an SOP (shown in the Appendix) was developed by members of the WildTech consortium and sent to collaborating partners who trapped the rodents and collected tissue samples, which were then sent to the University of Nottingham. However, after a discussion with collaborating partners, the range of pathogens on the array was increased. However, some of these pathogens were unlikely to be detectable in the liver, kidney or lung samples that had been collected, as they were more likely to be detectable in other organs. Nevertheless, probes for these pathogens were included on the array, as the array was being designed for future use, and has the potential to be used with a wide variety of samples, not just the three tissue types used in this particular study. Thus, although the array did not detect any *Salmonella* or *C. jejuni* in any of the tested samples, this does not mean that these animals were negative for these pathogens. To detect these pathogens faecal or intestine samples would be preferable (Table 5.1).

From the screening that was performed, the only confirmed positive array results were those for *Leptospira* and SEOV. *Leptospira* was found in samples from all three countries, whereas, SEOV was only detected in samples from France. Numerous reports have indicated that *Leptospira* is one of the most common pathogens associated with rodents; the pathogen has a global distribution and can be found wherever its host can be found (Bharti et al., 2003). Whilst SEOV is not a notifiable disease in France, it is attracting increasing attention as more cases have been identified over the past decade (European Centre for Disease Prevention and Control, 2014).

There were only two positive results for SEOV, so statistical analyses could not be performed on the results; however, both samples were from *R. norvegicus*, in which

SEOV has previously been detected in France (Artois et al., 2007, Heyman et al., 2004). The two positive samples were sequenced and found to have a high degree of identity when aligned with other SEOV isolates obtained from the NCBI database. The analyses carried out by the collaborating partner (Florence Aryal, University of Lyon) indicated that 14 of the rodents trapped in France were positive for SEOV. The array failed to detect 12 of these SEOV positive rodents. Subsequent qPCR also failed to identify the positive samples. It was therefore hypothesised that pooling the extracted material from liver, kidney and lung prior to PCR analysis may have diluted the pathogen load within the sample to undetectable levels, even for extremely sensitive methods such as qPCR. Another possible explanation relates to the location of pathogen within the tissue, as it is not homogeneously distributed throughout. The precise section of tissue sampled was, by necessity, different between the source laboratory and the University of Nottingham. If the pathogen was only present in a particular area of the tissue (that which was tested at the source laboratory) this could also explain the difference in results observed between laboratories. Another possibility is that sample degradation occurred in transit.

On the other hand, many samples were positive for *Leptospira*, and after a test for normality, statistical analysis was performed on the results. Of the variables examined, only age, size and season trapped had any significance. Sex, weight, and whether the females were pregnant or lactating were not significant. Older and larger animals were found to be more likely to carry *Leptospira*, and autumn was the season when the largest percentage of animals was positive. These findings are in agreement with reports from the literature; it has been found that animals are more likely to be infected as they age, and an increased number of *Leptospira*-positive animals are trapped in the autumn (Sarkar et al., 2002, Ward, 2002).

Other array positive samples subsequently tested negative on PCR and qPCR analysis. One particularly interesting finding was the number of samples that were positive for the *pla* gene of *Y. pestis*. After extensive testing, it was determined that the samples did not contain pathogenic *Y. pestis* (see Appendix for further detail). However, the *pla* gene was confirmed to be present in these samples. There was a report from a previous study that this region had also been identified in *R. rattus* samples (Janse et al., 2010). The results from this study, however, indicated that the *pla* homologue was present in *R. rattus, R. norvegicus* and *M. musculus* samples. This raises some interesting questions: How widespread is the homologue, and what bacterial species contain the homologue? The answers to these questions were beyond the scope of this study; however, further work could be undertaken to explore these questions. If the homologue is widespread in these populations, one would have expected it to be detected in most, if not all, of the sampled animals.

There were some anomalous results observed with the Alere ArrayTubes and ArrayStrips. This was apparent on the Yersinia_01 ArrayTube when some probes showed inconsistent hybridisation with the same sample on different arrays (see section 4.3.1.7.). Rogue-like properties were also observed on the Rodent_Chip_03 array, which was the array used with the screening samples. Specifically a number of probes showed constant signal intensities on nearly all samples used, including the negative control samples with water replacing template nucleic acid. This could have indicated contamination of some of the reagents, though it would be expected that if this were the case, then other probes for the same pathogens would also have hybridised, instead of just a select few. The results were also checked by PCR, and no amplicons were produced when using primers that were specific to these pathogens. These potential rogue probes could have been erroneously printed at a higher concentration than the other probes on the array which may have led to this aberrant performance on the

array, as higher concentrations of probe have been reported to cause a saturation in signal (Leek et al., 2010, Russell et al., 2009).

7.2 FUTURE WORK

Microarrays are a potentially useful tool for detecting multiple pathogens in a single sample. However, several observations were made during this study, which could improve and assist in future array work. One concern was the difference between results from the arrays and the tests conducted at the source laboratories. As stated in Chapter 5, qPCR was performed on several array-negative results and a positive qPCR result was obtained for some of these pathogens in some samples. One possible explanation for this finding is that the array is less sensitive than qPCR. In addition, homogenising the tissue and then pooling the extracted NA for PCR amplification may also have reduced the sensitivity of the array. An alternative approach would be to amplify the NA extracted from each tissue and then pool the PCR products for use on the arrays. However, this would increase the cost of the test, as more PCR reagents would be required. Time was not sufficient to trial this approach and determine how this would have affected the results; however, for the future this approach should be considered and compared to the one already used.

The objective of pooling the tissue was to decrease the cost of the array work. However, pooling the tissue was likely to have decreased the sensitivity of the test. One alternative would be the method mentioned above. Another option is to use tissue-specific, syndromic arrays. The pathogens of interest in this project may be found in a number of tissues throughout the body, and the initial pathogens to be screened for were more likely to be present in the liver, kidney and lung. The pathogens added later in the project were likely to be found in other tissues. A tissue-specific array would be more appropriate when screening for pathogens that are only found in specific tissues.

In these circumstances it would not be necessary to screen other tissues for the presence of the pathogen. Syndromic arrays could be useful in a diagnostic setting where a patient (human/livestock/wild animal) presents specific symptoms and a microarray could be used to identify the pathogen present.

Results indicated that a sequence-independent approach was not as sensitive as a sequence-specific approach. This limited the number of target pathogens in the study, because this was restricted by the number of primers which could be used in the multiplex PCR. A sequence-independent approach would have been a less restrictive solution, as probes for a large number of pathogens can be spotted onto the array, and all of them could then be tested. In this project, a multiplex approach was used which involved the use of 24 primer pairs in the primer mix, and screened for 23 pathogens with 327 probes. In most cases, there was only one primer pair per pathogen. It would have been better to have several primer pairs per pathogen (but this would have increased the number of primer pairs required in the mix), or to have additional PCR steps with separate primer mixes which could then be pooled and hybridised with the array.

Probes set at genus and species levels can differentiate between members of the same genus, yet if probes are positive for the genus level probes, but not for any of the species specific probes then this is suggestive of a novel species (Long et al., 2004). This approach might have been helpful for more accurate detection of Y. pestis in the study. The decision to use the *pla* gene for identification of *Y. pestis* was based on the widespread use of this gene for *Y. pestis* detection (Schuenemann et al., 2011, Skottman et al., 2006, Wiechmann and Grupe, 2004), without knowledge of the presence of the homologue for this gene. Additional probes designed for another region specific to *Y. pestis* would have been useful on the array, as these would have been negative when the others were positive.

Because of the extensive validation required before a microarray could be used as a definitive diagnostic tool, it is less likely that they will be used for this purpose. They can however, be very useful in screening a sample for a wide range of pathogens, as shown in this thesis. If array results suggest there are positive samples, then confirmatory tests could be carried out on a smaller subset of positive samples to validate the array results. Confirmatory tests could include standard PCR, qPCR or sequencing to verify and more accurately determine the identity of the organism.

In conclusion, this study was undertaken to design and develop a microarray to screen for zoonotic pathogens in urban rodents. The array correctly identified two important zoonotic pathogens in rodent species; although there were caveats and limitations to the findings, the results demonstrate the potential of this tool for disease surveillance. This array is novel in that it is the first tool that has been designed to screen for a range of zoonotic pathogens, including bacteria, viruses and parasites from rodents, an important reservoir species.

REFERENCES

ACKE, E., MCGILL, K., LAWLOR, A., JONES, B. R., FANNING, S. & WHYTE, P. 2010. Genetic diversity among *Campylobacter jejuni* isolates from pets in Ireland. *Veterinary Record,* 166**,** 102-106.

ADAK, G. K., LONG, S. M. & O'BRIEN, S. J. 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut,* 51**,** 832-841.

ADÉKAMBI, T. & DRANCOURT, M. 2004. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, hsp65, sodA, recA and rpoB gene sequencing. *International Journal of Systematic and Evolutionary Microbiology,* 54**,** 2095-2105.

ADESIJI, Y. O., DEEKSHIT, V. K. & KARUNASAGAR, I. 2014. Antimicrobial-resistant genes associated with *Salmonella* spp. isolated from human, poultry, and seafood sources. *Food Science & Nutrition,* 2**,** 436-442.

ADLER, B. & DE LA PEÑA MOCTEZUMA, A. 2010. *Leptospira* and leptospirosis. *Veterinary Microbiology,* 140**,** 287-296.

AGGARWAL, R. & NAIL, S. 2009. Epidemiology of hepatitis E: Current status. *Journal of Gastroenterology and Hepatology,* 24**,** 1484-1493.

ALBARIÑO, C. G., PALACIOS, G., KHRISTOVA, M. L., ERICKSON, B. R., CARROLL, S. A., COMER, J. A., HUI, J., BRIESE, T., GEORGE, K. S., KSIAZEK, T. G., LIPKIN, W. I. & NICHOL, S. T. 2010. High Diversity and Ancient Common Ancestry of Lymphocytic Choriomeningitis Virus. *Emerging Infectious Diseases,* 16**,** 1093-1100.

ALBEDWAWI, S., LEBLANC, C., SHOW, A. & SLINGER, R. W. 2006. A teenager with fever, rash and arthritis. *Canadian Medical Association Journal,* 175**,** 354.

ALZHANOVA, D. & FRÜH, K. 2010. Modulation of the host immune response by cowpox virus. *Microbes and Infection,* 12**,** 900-909.

ANDERSON, B. E. & NEUMAN, M. A. 1997. *Bartonella* spp. as Emerging Human Pathogens. *Clinical Microbiology Reviews,* 10**,** 203-219.

ANDERSON, T. P., WERNO, A. M., BARRATT, K., MAHAGAMASEKERA, P., MURDOCH, D. R. & JENNINGS, L. C. 2013. Comparison of four multiplex PCR assays for the detection of viral pathogens in respiratory specimens. *Journal of Virological Methods,* 191**,** 118-121.

ARANGO-JARAMILLO, S., WISSEMAN, C. L. & AZAD, A. F. 1988. Newborn rats in the murine typhus enzootic infection cycle: studies on transplacental infection and passively acquired maternal anti-rickettsial

antibodies. *American Journal of Tropical Medicine and Hygiene,* 39**,** 391-397.

ARIKAWA, J., YOSHIMATSU, K., THANG, T. U. & NINH, T. U. 2007. Hantavirus Infection - typical rodent-borne viral zoonosis. *Tropical Medicine and Health,* 35**,** 55-59.

ARTOIS, M., COCHEZ, C., MELE, R. V. & HEYMAN, P. 2007. Genetic evidence of Puumala and Tula Hantaviruses in rodents in the Jura region, France - preliminary results. *Eurosurveillance,* 12**,** pii3226.

ASHFORD, R. W. 2003. When Is a Reservoir Not a Reservoir? *Emerging Infectious Diseases,* 9**,** 1495-1496.

AVIAT, F., BLANCHARD, B., MICHEL, V., BLANCHET, B., BRANGER, C., HARS, J., MANSOTTE, F., BRASME, L., DE CHAMPS, C., BOLUT, P., MONDOT, P., FALIU, J., ROCHEREAU, S., KODJO, A. & ANDRE-FONTAINE, G. 2009. *Leptospira* exposure in the human environment in France: A survey in feral rodents and in fresh water. *Comparative Immunology, Microbiology and Infectious Diseases,* 32**,** 463-476.

AZAD, A. F. 1990. Epidemiology of Murine Typhus. *Annual Reviews in Entomology,* 35**,** 553-570.

AZAD, A. F. & RADULOVIC, S. 2003. Pathogenic *Rickettsiae* as Bioterrorism Agents. *Annals of the New York Academy of Sciences,* 990**,** 734-738.

AZAD, A. F., RADULOVIC, S., HIGGINS, J. A., NODEN, B. H. & TROYER, J. M. 1997. Flea-borne Rickettsioses: Ecologic Considerations. *Emerging Infectious Diseases,* 3**,** 319-327.

AZZARI, C., MORIONDO, M., INDOLFI, G., CORTIMIGLIA, M., CANESSA, C., BECCIOLINI, L., LIPPI, F., DE MARTINO, M. & RESTI, M. 2010. Realtime PCR Is More Sensitive than Multiplex PCR for Diagnosis and Serotyping in Children with Culture Negative Pneumococcal Invasive Disease. *PLoS one,* 5**,** e9282.

BACKHANS, A., FELLSTRÖM, C. & LAMBERTZ, S. T. 2010. Occurence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in small wild rodents. *Epidemiology and Infection,* 15.

BAHL, A., DAVIS, P. H., BEHNKE, M., DZIERZINSKI, F., JAGALUR, M., CHEN, F., SHANMUGAM, D., WHITE, M. W., KULP, D. & ROOS, D. S. 2010. A novel multifunctional oligonucleotide microarray for *Toxoplasma gondii*. *BMC Genomics,* 11.

BAR-OR, C., CZOSNEK, H. & KOLTAI, H. 2007. Cross-species microarray hybridisations: a developing tool for studying species diversity. *Trends in Genetics,* 23**,** 200-207.

BARBA, M., CZOSNEK, H. & HADIDI, A. 2014. Historical Perspective, Development and Applications of Next-Generation Sequencing in Plant Virology. *Viruses,* 6**,** 106-136.

BARTON, L. L. & METS, M. B. 2001. Congenital Lymphocytic Choriomeningitis Virus Infection: Decade of Rediscovery. *Clinical Infectious Diseases,* 33**,** 370-374.

BARZON, L., LAVEZZO, E., COSTANZI, G., FRANCHIN, E., TOPPO, S. & PALÙ, G. 2013. Next-generation sequencing technologies in diagnostic virology. *Journal of Clinical Virology,* 58**,** 346-350.

BASSO, W., HARTNACK, S., PARDINI, L., MAKSIMOV, P., KOUDELA, B., VENTURINI, M. C., SCHARES, G., SIDLER, X., LEWIS, F. I. & DEPLAZES, P. 2013. Assessment of diagnostic accuracy of a commercial ELISA for the detection of Toxoplasma gondii infection in pigs compared with IFAT, TgSAG1-ELISA and Western blot, using a Bayesian latent class approach. *International Journal for Parasitology,* 43**,** 565-570.

BASTIEN, P., PROCOP, G. W. & REISCHL, U. 2008. Quantitative Real-Time PCR Is Not More Sensitive than "Conventional" PCR. *Journal of Clinical Microbiology,* 46**,** 1897-1900.

BECKER, C., KURTH, A., HESSLER, F., KRAMP, H., GOKEL, M., HOFFMANN, R., KUCZKA, A. & NITSCHE, A. 2009. Cowpox Virus Infection in Pet Rat Owners: Not Always Immediately Recognised. *Deutsches Ärzteblatt International,* 106**,** 329-334.

BEGON, M., TELFER, S., BURTHE, S., LAMBIN, X., SMITH, M. J. & PATERSON, S. 2009. Effects of abundance on infection in natural populations: Field voles and cowpox virus. *Epidemics,* 1**,** 35-46.

BERKELMAN, R. L. & HUGHES, J. M. 1993. The Conquest of Infectious Diseases: Who Are We Kidding? *Annals of Internal Medicine,* 119**,** 426-428.

BERKELMAN, R. L., R.T., B., OSTERHOLM, M. T., LEDUC, J. W. & HUGHES, J. M. 1994. Infectious Disease Surveillance: A Crumbling Foundation. *Science,* 264**,** 368-370.

BERNABEAU-WITTEL, M., PACHÓN, J., ALCARÓN, A., LÓPEZ-CORTÉZ, L. F., VICIANA, P., JIMINÉZ-MEJÍAS, M. E., VILLANUEVA, J. L., TORRONTERAS, R. & CABALLERO-GRANADO, F. J. 1999. Murine Typhus as a Common Cause of Fever of Intermediate Duration: A 17-Year Study in the South of Spain. *Archives of Internal Medicine,* 159**,** 872-876.

BERRANG, M. E., BUHR, R. J., CASON, J. A. & DICKENS, J. A. 2001. Broiler carcass contamination with *Campylobacter* from faeces during defeathering. *Journal of Food Protection,* 64**,** 2063-2066.

BESSELSEN, D. G., WAGNER, A. M. & LOGANBILL, J. K. 2003. Detection of Lymphocytic Choriomeningitis Virus by Use of Fluorogenic Nuclease Reverse Transcriptase-Polymerase Chain Reaction Analysis. *Comparative Medicine,* 53**,** 65-69.

BHARTI, A. R., NALLY, J. E., RICALDI, J. N., MATTHIAS, M. A., DIAZ, M. M., LOVETT, M. A., LEVETT, P. N., GILMAN, R. H., WILLIG, M. R., GOTUZZO, E. & VINETZ, J. M. 2003. Leptospirosis: a zoonotic diease of global importance. *The Lancet Infectious Diseases,* 3**,** 757-771.

BIDAISEE, S. & MACPHERSON, C. N. L. 2014. Zoonoses and One Health: A Review of the Literature. *Journal of Parasitology Research,* 2014**,** 8.

BIGGINS, D. E. & KOSOY, M. Y. 2001. Influences of introduced plague on North American mammals: implications from ecology of plague in Asia. *Journal of Mammalogy,* 82**,** 906-916.

BIHL, F. & NEGRO, F. 2010. Hepatitis E virus: a zoonosis adapting to humans. *Journal of Antimicrobial Chemotherapy,* 65**,** 817-821.

BIRDSELL, D. N., VOGLER, A. J., BUCHHAGEN, J., CLARE, A., KAUFMAN, E., NAUMANN, A., DRIEBE, E., WAGNER, D. M. & KEIM, P. S. 2014. TaqMan Real-Time PCR Assays for Single-Nucleotide Polymorphisms Which Identify <italic>Francisella tularensis</italic> and Its Subspecies and Subpopulations. *PLoS ONE,* 9**,** e107964.

BLALOCK, E. 2003. *A Beginner's Guide to Microarrays,* Norwell, Massachussetts, Kluwer Academic Publishers.

BLASDELL, K., HERBRETEAU, V., HENTTONEN, H., DAROUNY, P., HUGOT, J. P. & BUCHY, P. 2008a. Rodent-Borne Zoonotic Viruses in Southeast Asia. *Natural Science,* 43**,** 94-105.

BLASDELL, K. R., BECKER, S. D., HURST, J., BEGON, M. & BENNETT, M. 2008. Host range and genetic diversity of arenaviruses in rodents, United Kingdom. *Emerging Infectious Diseases,* 14**,** 1455-1458.

BÖHM, M., WHITE, P. C. L., DANIELS, M. J., ALLCROFT, D. J., MUNRO, R. & HUTCHINGS, M. R. 2006. The health of wild red deer and sika deer in Scotland: An analysis of key endoparasites and reccomendations for monitoring disease. *The Veterinary Journal,* 171**,** 287-294.

BOOT, R., OOSTERHUIS, A. & THUIS, H. C. W. 2002. PCR for the detection of *Streptobacillus moniliformis*. *Laboratory Animals,* 36**,** 200-208.

BOTELHO-NEVERS, E. & RAOULT, D. 2011. Host, pathogen and treatment-related prognostic facots in rickettsioses. *European Journal of Clinical Microbiology and Infectious Diseases,* 30**,** 1139-1150.

BOTTONE, E. J. 1997. *Yersinia enterocolitica*: The Charisma Continues. *Clinical Microbiology Reviews,* 10**,** 257-276.

BOULOUIS, H.-J., CHANG, C.-C., HENN, J. B., KASTEN, R. W. & CHOMEL, B. B. 2005. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. *Veterinary Research,* 36**,** 383-410.

BOURHY, P., BREMONT, S., ZININI, F., GIRY, C. & PICARDEAU, M. 2011. Comparison of Real-Time PCR Assays for Detection of Pathogenic *Leptospira* spp. in Blood and Identification of Variations in Target Sequences. *Journal of Clinical Microbiology,* 49**,** 2154-2160.

BRADLEY, C. A. & ALTIZER, S. 2006. Urbanization and the ecology of wildlife diseases. *Trends in Ecology and Evolution,* 22**,** 95-102.

BREITSCHWERDT, E. B. & KORDICK, D. L. 2000. *Bartonella* Infection in Animals: Carriership, Reservoir Potential, Pathogenicity, and Zoonotic Potential for Human Infection. *Clinical Microbiology Reviews,* 13**,** 428-438.

BROEKHUIJSEN, M., LARSSON, P., JOHANSSON, A., BYSTRÖM, M., ERIKSSON, U., LARSSON, E., PRIOR, R. G., SJÖSTEDT, A., TITBALL, F. W. & FORSMAN, M. 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent F. tularensis subsp. tularensis. *Journal of Clinical Microbiology,* 41**,** 2924-2931.

BROUQUI, P., LASCOLA, B., ROUX, V., PHARM, D. & RAOULT, D. 1999. Chronic *Bartonella quintana* bacteremia in homeless patients. *The New England Journal of Medicine,* 340**,** 184-189.

BRYANT, P. A., VENTER, D., ROBINS-BROWNE, R. & CURTIS, N. 2004. Chips with everything: DNA microarrays in infectious diseases. *The Lancet,* 4**,** 100-111.

BURTON, J. E., OSHOTA, O. J., NORTH, E., HUDSON, M. J., POLYANSKAYA, N., BREHM, J., LLOYD, G. & SILMAN, N. J. 2005. Development of a multipathogen oligonucleotide microarray for detection of *Bacillus anthracis*. *Molecular and Cellular Probes,* 19**,** 349-357.

BUTLER, T. 1983. *Plague and other Yersinia infections,* New York, Plenum Press.

BUXTON, A. & FRASER, G. 1977. *Animal Microbiology Volume 1*, Wiley-Blackwell.

CACCIAPOUTI, B., CICERONI, L., PINTO, A., APOLLINI, M., RONDINELLA, V., BONOMI, U., BENEDITTI, E., CINCO, M., DESSI, S., DETTORI, G., GRILLO, R., FALOMO, R., MANSUETO, S., MICHELI, D., MARUCCIO, L., MARUCCIO, C., PIZZOCARO, P., SCHIVO, S. L., VARALDO, E., LUPIDI, R., IOLI, A., MARZOLINI, A. & ROSMINI, F. 1994. Survey on the prevalence of leptospira infections in the Italian population. *European Journal of Epidemiology,* 10**,** 173-180.

CALIENDO, A. M. 2011. Multiplex PCR and Emerging Technologies for the Detection of Respiratory Pathogens. *Clinical Infectious Diseases,* 52**,** S326-S330.

CANNON, G. A., CARR, M. J., YANDLE, Z., SCHAFFER, K., KIDNEY, R., HOSNY, G., DOYLE, A., RYAN, J., GUNSON, R., COLLINS, T., CARMAN, W. F., CONNELL, J. & HALL, W. W. 2010. A low density oligonucleotide microarray for the detection of viral and atypical bacterial respiratory pathogens. *Journal of Virological methods,* 163**,** 17-24.

CARROLL, D. S., EMERSON, G. L., LI, Y., SAMMONS, S., OLSON, V., FRACE, M., NAKAZAWA, Y., CZERNY, C. P., TRYLAND, M., KOLODZIEJEK, J., NOWOTNY, N., OLSEN-RASMUSSEN, M., KHRISTOVA, M., GOVIL, D., KAREM, K., DAMON, I. K. & MEYER, H. 2011. Chasing Jenner's Vaccine: Revisiting *Cowpox Virus* Classification. *PLoS one,* 6.

CARTER, M. E. & CORDES, D. O. 1980. Leptospirosis and other infections of *Rattus rattus* and *Rattus norvegicus*. *New Zealand Veterinary Journal,* 28**,** 45-50.

CARTER, P. B. 1975. Pathogenicity of *Yersinia enterocolitica* for mice. *Infection and Immunity,* 11**,** 164-170.

CDC 2005. Cases of HIV infection and AIDS in the United States, 2004. *In:* US DEPARTMENT OF HEALTH AND HUMAN SERVICES, C. (ed.) *HIV/AIDS surveillance report.* Atlanta.

CDC 2006. Reported tuberculosis in the United States, 2005. *In:* US DEPARTMENT OF HEALTH AND HUMAN SERVICES, C. (ed.). Atlanta.

CDC 2008. Salmonella Surveillance: Annual Summary 2006. *In:* US DEPARTMENT OF HEALTH AND HUMAN SERVICES, C. (ed.). Atlanta.

CDC 2013. Tularemia - United States, 2001-2010. *Morbidity and Mortality Weekly Report,* 62**,** 963-966.

CDC. 2014. *vCJD (Variant Creutzfeldt-Jakob Disease)* [Online]. [Accessed 23/04/2015 2015].

CELLI, J. & ZAHRT, T. C. 2013. Mechanisms of *Francisella tularensis* Intracellular Pathogenesis. *Cold Spring Harbor Perspectives in Medicine,* 3.

CHANDRA, V., TANEJA, S., KALIA, M. & JAMEEL, S. 2008. Molecular Biology and pathogenesis of hepatitis E virus. *Journal of Biosciences,* 33**,** 451-464.

CHANNON, D., CHANNON, E., ROBERTS, T. & HAINES, R. 2006. Hotspots: are some areas of sewer network prone to re-infestation by rats (*Rattus norvegicus*) year after year? *Epidemiology and infection,* 134**,** 41-48.

CHANTEAU, S., RAHALISON, L., RALAFIARISOA, L., FOULON, J., RATSITORAHINA, M., RATSIFASOAMANANA, L., CARNIEL, E. & NATO, F. 2003. Development and testing of a rapid diagnostic test for bubonic and pneumonic plague. *The Lancet,* 361**,** 211-216.

CHANTREY, J., MEYER, H., BAXBY, D., BEGON, M., BOWN, K. J., HAZEL, S. M., JONES, T., MONTGOMERY, W. I. & BENNETT, M. 1999. Cowpox: reservoir hosts and geographic range. *Epidemiology and Infection,* 122**,** 455-460.

CHARREL, N. M. & DE LAMBALLERIE, X. 2003. Arenaviruses other than Lassa virus. *Antiviral Research,* 57**,** 89-100.

CHEN, H. D. & FRANKEL, G. 2004. Enteropathogenic *Escherichia coli:* unravelling pathogenesis. *FEMS Microbiology Reviews,* 29**,** 83-98.

CHEN, P.-L., LEE, N.-Y., YAN, J.-J., YANG, Y.-J., CHEN, H.-M., CHANG, C.-M., LEE, S.-C., KO, N.-Y., LAI, C.-H. & KO, W.-C. 2007. Prosthetic Valce Endocarditis Caused by *Streptobacillus moniliformis*: a Case of Rat Bite Fever. *Journal of Clinical Microbiology,* 45**,** 3125-3126.

CHEN, S. H., LO, C. Z., SU, S. Y., KUO, B. H., HSIUNG, C. A. & LIN, C. Y. 2010. UPS 2.0: unique probe selector for probe design and oligonucleotide microarrays at the pangenomic/genomic level. *BMC Genomics,* 11.

CHILDS, J., SHOPE, R. E., FISH, D., MESLIN, F. X., PETERS, C. J., JOHNSON, K., DEBESS, E., DENNIS, D. & JENKINS, S. 1998. Emerging Zoonoses. *Emerging Infectious Diseases,* 4**,** 453-454.

CHILDS, J. E., GLASS, G. E., KORCH, G. W., KSIAZEK, T. G. & LEDUC, J. W. 1992a. Lymphocytic choriomeningitis virus infection and house mouse (*Mus musculus*) distribution in urban Baltimore. *American Journal of Tropical Medicine and Hygiene,* 47**,** 27-34.

CHILDS, J. E., MCLAFFERTY, S. L., SADEK, R., MILLER, G. L., KHAN, A. S., DUPREE, E. R., ADVANI, R., MILLS, J. N. & GLASS, G. E. 1998b. Epidemiology of Rodent Bites and Prediction of Rat Infestation in New York City. *American Journal of Epidemiology,* 148**,** 78-87.

CHILDS, J. E., SCHWARTZ, B. S., KSIAZEK, T. G., GRAHAM, R. R., LEDUC, J. W. & GLASS, G. E. 1992b. Risk factors associated with antibodies to leptospires in inner-city residents of Baltimore: a protective role for cats. *American Journal of Public Health,* 82**,** 597-599.

CHIU, C. Y. 2013. Viral pathogen discovery. *Current Opinion in Microbiology,* 16**,** 468-478.

CHOMEL, B. B. 2004. *Bartonellaceae, in: Veterinary Microbiology,* Ames, IA, USA, Blackwell Publishing.

CHOMEL, B. B., ABBOTT, R. C., KASTEN, R. W., FLOYD-HAWKINS, K. A., KASS, P. H., GLASER, C. A., PEDERSEN, N. C. & KOEHLER, J. E. 1995. *Bartonella henselae* prevalence in domestic cats in California: risk factors and association between bacteremia and antibody titers. *Journal of Clinical Microbiology,* 33**,** 2445-2450.

CHOMEL, B. B., BELOTTO, A. & MESLIN, F. X. 2007. Wildlife, Exotic Pets and Emerging Zoonoses. *Emerging Infectious Diseases,* 13**,** 6-11.

CHOMEL, B. B., KASTEN, R. W., FLOYD-HAWKINS, K., CHI, B., YAMAMOTO, K., ROBERTS-WILSON, J., GURFIELD, A. N., ABBOTT, R. C., PEDERSEN, N. C. & KOEHLER, J. E. 1996. Experimental Transmission of *Bartonella henselae* by the Cat Flea. *Journal of Clinical Microbiology,* 34**,** 1952-1956.

CHOU, C. C., CHEN, C. H., LEE, T. T. & PECK, K. 2004. Optimisation of probe length and the number of probes per gene for optimal microarray analysis of gene expression. *Nucleic Acids Research,* 32**,** e99.

CHRISTOVA, I. & GLADNISHKA, T. 2005. Prevalence of infection with *Francisella tularensis, Borrelia burgdorferi sensu lato* and *Anaplasma phagocytophilum* in rodents from an endemic focus of tularemia in Bulgaria. *Annals of agricultural and environmental medicine : AAEM,* 12**,** 149-152.

CHUA, K. B., KOH, C. L., HOOI, P. S., WEE, K. F., KHONG, J. H., CHUA, B. H., CHAN, Y. P., LIM, M. E. & LAM, S. K. 2002. Isolation of Nipah virus from Malaysian Island flying-foxes. *Microbes and Infection,* 4**,** 145-151.

CIVEN, R. & NGO, V. 2008. Murine Typhus: An Unrecognised Suburban Vectorborne Disease. *Clinical Infectious Diseases,* 46**,** 913-918.

CLARKE, K.-A. R., FITZGERALD, S. D., ZWICK, L. S., CHURCH, S. V., KANEENE, J. B., WISMER, A. R., BOLIN, C. A., HATTEY, J. A. & YUZBASIYAN-GURKAN, V. 2007. EXPERIMENTAL INOCULATION OF MEADOW VOLES (MICROTUS PENNSYLVANICUS), HOUSE MICE (MUS MUSCULUS), AND NORWAY RATS (RATTUS NORVEGICUS) WITH MYCOBACTERIUM BOVIS. *Journal of Wildlife Diseases,* 43**,** 353-365.

COBURN, B., GRASSL, G. A. & FINLAY, B. B. 2007. *Salmonella*, the host and disease: a brief review. *Immunology and Cell Biology,* 85**,** 112-118.

CODONY, F., AGUSTÍ, G. & ALLUÉ-GUARDIA, A. 2015. Cell membrane integrity and distinguishing between metabolically active and inactive cells as a means of improving viability PCR. *Molecular and Cellular Probes*.

COLTON, L., KABEYA, H. & KOSOY, M. 2012. Experimental infection of three laboratory mouse stocks with a shrew origin *Bartonella elizabethae* strain: an evaluation of bacterial host switching potential. *Infection Ecology and Epidemiology,* 2.

COMER, J. A., FLYNN, C., REGNERY, R. L., VLAHOV, D. & CHILDS, J. E. 1996. Antibodies to *Bartonella* species in inner-city intravenous drug users in Baltimore. *Archives of Internal Medicine,* 156**,** 2491-2495.

COOPER, K., HUANG, F. F., BATISTA, L., RAYO, C. D., BEZANILLA, J. C., TOTH, T. E. & MENG, X. J. 2005. Identification of Genotype 3 Hepatitis E Virus (HEV) in Serum and Fecal Samples from Pigs in Thailand and Mexico, Where Genotype 1 and 2 HEV Strains Are Prevalent in the Respective Human Populations. *Journal of Clinical Microbiology,* 43**,** 1684-1688.

CORDEY, S., SAHLI, R., MORAZ, M.-L., ESTRADE, C., MORANDI, L., CHERPILLOD, P., CHARREL, R. N., KUNZ, S. & KAISER, L. 2011. Analytical validation of a lymphocytic choriomeningitis virus real-time RT-PCR assay. *Journal of Virological Methods,* 177**,** 118-122.

CORNELIUS, A. J., GILPIN, B., CARTER, P., NICOL, C. & ON, S., L.W. 2010. Comparison of PCR Binary Typing (P-BIT), a New Approach to Epidemiological Subtyping of *Campylobacter jejuni*, with Serotyping, Pulsed-Field Gel Electrophoresis, and Multilocus Sequence Typing Methods. *Applied and Environmental Microbiology,* 76**,** 1533-1544.

CZERNY, C.-P., EIS-HÜBINGER, A. M., MAYR, A., SCHNEWEIS, K. E. & PFEIFF, P. 1991. Animal poxviruses transmitted from cat to man: current event with lethal end. *Journal of Veterinary Medicine,* 38**,** 421-431.

DANIELS, M. J., HUTCHINGS, M. R. & GREIG, A. 2003. The risk of disease transmission to livestock posed by contamination of farm stored feed by wildlife excreta. *Epidemiology and Infection,* 130**,** 561-568.

DASZAK, P., CUNNINGHAM, A. A. & HYATT, A. D. 2000. Emerging infectious diseases of wildife - threats to biodiversity and human health. *Science,* 287**,** 443-449.

DASZAK, P., CUNNINGHAM, A. A. & HYATT, A. D. 2001. Anthropogenic environmental change and the emergence of infectious disease in wildlife. *Acta tropica,* 78**,** 103-116.

DAVIS, D. E. 1953. The characteristics of rat populations. *Quarterly Review of Biology,* 28**,** 373-401.

DAY, N. P., SCOTLAND, S. M., CHEASTY, T. & ROWE, B. 1983. *Escherichia coli* O157:H7 associated with human infections in the United Kingdom. *Lancet,* 1**,** 825.

DE BARCELLOS, D. E. & DE CASTRO, A. F. 1981. Isolation of *Yersinia pseudotuberculosis* from diarrhoea in pigs. *British Veterinary Journal,* 137**,** 95-96.

DE SOUSA, R., EDUOUARD-FOURNIER, P., SANTOS-SILVA, M., AMARO, F., BACELLAR, F. & RAOULT, D. 2006. Molecular detection of *Rickettsia felis, Rickettsia typhi* and two genotypes closely related to *Bartonella Elizabethae*. *American Journal of Tropical Medicine and Hygiene,* 75**,** 727-731.

DE SOUZA, F. M., DO PRADO, T. N., PINHEIRO, J. D. S., PERES, R. L., LACERDA, T. C., LOUREIRO, R. B., CARVALHO, J. A., FREGONA, G., DIAS, E. S., COSME, L. B., RODRIGUES, R. R., RILEY, L. W. & MACIEL, E. L. N. 2014. Comparison of Interferon-γ Release Assay to Two Cut-Off Points of Tuberculin Skin Test to Detect Latent *Mycobacterium tuberculosis* Infection in Primary Health Care Workers. *PLoS ONE,* 9**,** e102773.

DEARING, D. M. & DIZNEY, L. 2010. Ecology of hantavirus in a changing world. *Annals of the New York Academy of Sciences,* 1195**,** 99-112.

DELONG, J. P., BURGER, O. & HAMILTON, M. J. 2010. Current Demographics Suggest Future Energy Supplies Will Be Inadequate to Slow Human Growth. *PLoS ONE,* 5.

DEMERS, R. Y., THIERMANN, A., DEMERS, P. & FRANK, R. 1983. Exposure to *Leptospira* icterohaemorrhagiae in inner-city and suburban children: a serologic comparison. *The Journal of Family Practice,* 17**,** 1007-1011.

DENNIS, D. T., GAGE, K. L., GRATZ, N., POLAND, J. & TIKHORIMOV, E. 1999. *Plague manual: epidemiology, distribution, surveillance and control,* Geneva.

DENNIS, D. T., INGLESBY, T. V., HENDERSON, D. A. & ET AL. 2001. Tularemia as a biological weapon: Medical and public health management. *JAMA,* 285**,** 2763-2773.

DESVARS, A., FURBERG, M., HJERTQVIST, M., VIDMAN, L., SJÖSTEDT, A., RYDÉN, P. & JOHANSSON, A. 2015. Epidemiology and Ecology of Tularemia in Sweden, 1984–2012. *Emerging Infectious Diseases,* 21**,** 32-39.

DEVANE, M. L., NICOL, C., BALL, A., KLENA, J. D., SCHOLES, P., HUDSON, J. A., BAKER, M. G., GILPIN, B. J., GARRETT, N. & SAVILL, M. G. 2005. The occurrence of *Campylobacter* subtypes in environmental reservoirs and potential transmission routes. *Journal of Applied Microbiology,* 98**,** 980-990.

DONG, J., OLANO, J. P., MCBRIDE, J. W. & WALKER, D. H. 2008. Emerging Pathogens: Challenges and Successes of Molecular Diagnostics. *Journal of Molecular Diagnostics,* 10**,** 185-197.

DRAGHICI, S., KHATRI, P., EKLUND, A. C. & SZALLASI, Z. 2006. Reliability and reproducibilty issues in DNA microarray measurements. *Trends in Genetics,* 22**,** 101-109.

DUBEY, J. P. 2004. Toxoplasmosis - a waterborne zoonosis. *Veterinary Parasitology,* 126**,** 57-72.

DUBEY, J. P. 2008. The History of *Toxoplasma gondii* - The First 100 Years. *Journal of Eukaryotic Microbiology,* 55**,** 467-475.

DUOCOURNAU, C., FERRIER-REMBERT, A., FERRARIS, O., JOFFRE, A., FAVIER, A. L., FLUSIN, O., VAN CAUTEREN, D., KECIR, K., AUBURTIN, B., VEDY, S., BESSAUD, M. & PEYREFITTE, C. N. 2013. Concomitant Human Infections with 2 Cowpox Virus Strains in Related Cases, France, 2011. *Emerging Infectious Diseases,* 19**,** 1996-1999.

DUPINAY, T., POUNDER, K. C., AYRAL, F., LAABERKI, M. H., MARSTON, D. A., LACÓTE, S., REY, C., BARBET, F., VOLLER, K., NAZARET, N., ARTOIS, M., MARIANNEAU, P., LACHUER, J., FOOKS, A. R., PÉPIN, M., LEGRAS-LACHUER, C. & MCELHINNEY, L. M. 2014. Detection and genetic characterisation of Seoul Virus from commensal brown rats in France. *Virology,* 11.

DURNEZ, L., KATAKWEBA, A., SADIKI, H., KATHOLI, C. R., KAZWALA, R. R., MACHANG'U, R. R., PORTAELS, F. & LEIRS, H. 2011. *Mycobacteria* in small Terrestrial Small Mammals on Cattle Farms in Tanzania. *Veterinary Medicine International,* 2011.

DURNEZ, L., KATAKWEBA, A., SADIKI, H., KATHOLI, C.R., KAZWALA, R.R., MACHANG'U, R.R., PORTAELS, F., LEIRS, H. 2011. *Mycobacteria* in Terrestial Small Mammals on Cattle Farms in Tanzania. *Veterinary Medicine International,* 2011.

DUTTA, T. K. & CHRISTOPHER, M. 2005. Leptospirosis - An Overview. *Journal of the Association of Physicians of India,* 53**,** 545 - 551.

ECDC 2013. Annual epidemiological report 2012. Reporting on 2010 surveillance data and 2011 epidemic intelligence data. *In:* CONTROL, E. C. F. D. P. A. (ed.). Stockholm.

ECDC 2014. Annual epidemiological report: Food-and waterborne diseases and zoonoses 2014. Stockholm.

ECDC 2014a. Annual epidemiological report 2014 - emerging and vector-borne diseases. *In:* CONTROL, E. C. F. D. P. A. (ed.). Stockholm.

EFSA 2007a. Scientific opinion of the panel on biological hazards on a request from EFSA on surveillance and monitoring of *Toxoplasma* in humans, foods and animals. *EFSA Journal,* 583**,** 1-64.

EFSA 2010. The Community Summary Report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. *EFSA Journal,* 8**,** 1496-1906.

EFSA. 2015. *Salmonella* [Online]. Available: http://www.efsa.europa.eu/en/topics/topic/salmonella.htm [Accessed 23/04/2015 2015].

EHRICHT, R., SLICKERS, P., GOELLNER, S., HOTZEL, H. & SACHSE, K. 2006. Optimized DNA microarray assay allowss detection and genotyping of single PCR-amplifiable target copies. *Molecular and Cellular Probes,* 20**,** 60-63.

EISEN, R. J., BORCHERT, J. N., HOLMES, J. L., AMATRE, G., VAN WYK, K., ENSCORE, R. E., BABI, N., ATIKU, L. A., WILDER, A. P., VETTER, S. M., BEARDEN, S. W., MONTENIERI, J. A. & GAGE, K. L. 2008. Early-phase Transmission of *Yersinia pestis* by Cat Fleas (*Ctenocephalides felis*) and Their Potential Role as Vectors in a Plague-endemic Region of Uganda. *The American Journal of Tropical Medicine and Hygiene,* 78**,** 949-956.

EISEN, R. J. & GAGE, K. L. 2009. Adaptive strategies of *Yersinia pestis* to persist during inter-epizootic and epizootic periods. *Veterinary Research,* 40.

ELIASSON, H. & BÄCK, E. 2007. Tularaemia in an emergent area in Sweden: An analysis of 234 cases in five years. *Scandinavian Journal of Infectious Diseases,* 39**,** 880-889.

ELIZAQUÍVEL, P., SÁNCHEZ, G. & AZNAR, R. 2012. Quantitative detection of viable foodborne *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. *Food Control,* 25**,** 704-708.

ELLIOTT, S. P. 2007. Rat Bite Fever and *Streptobacillus moniliformis*. *Clinical Microbiology Reviews,* 20**,** 13-22.

ELLIS, B. A., REGNERY, R. L., BEATI, L., BACELLAR, F., ROOD, M., GLASS, G. G., MARSTON, E., KSIAZEK, T. G., JONES, D. & CHILDS, J. E. 1999. Rats of the Genus *Rattus* are Reservoir Hosts for Pathogenic *Bartonella* Species: An Old World Origin for a New World Disease. *Journal of Infectious Diseases,* 180**,** 220-224.

ELLIS, J., OYSTON, P. C. F., GREEN, M. & TITBALL, R. W. 2002. Tularemia. *Clinical Microbiology Reviews,* 15**,** 631-646.

EPPINGER, M., GUO, Z., SEBASTIAN, Y., SONG, Y., LINDLER, L. E., YANG, R. & RAVEL, J. 2009. Draft Genome Sequences of *Yersinia*

pestis Isolates from Natural Foci of Endemic Plague in China. *Journal of Bacteriology,* 191**,** 7628-7629.

EPPINGER, M., WORSHAM, P. L., NIKOLICH, M. P., RILEY, D. R., SEBASTIAN, Y., MOU, S., ACHTMAN, M., LINDLER, L. E. & RAVEL, J. 2010. Genome Sequence of the Deep-Rooted *Yersinia pestis* Strain Angola Reveals New Insights into te Evolution and Pangenome of the Plague Bacterium. *Journal of Bacteriology,* 192**,** 1685-1699.

ERMINI, M. L., SCARANO, S., BINI, R., BANCHELLI, M., BERTI, D., MASCINI, M. & MINUNNI, M. 2011. A rational approach in probe design for nucleic acid-based biosensing. *Biosensors and Bioelectronics,* 26**,** 4785-4790.

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL. 2009. *Cowpox in Germany and France related to rodent pets* [Online]. [Accessed 03/05/2012.

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2014. Prevention measures and communication strategies for hantavirus infection in Europe. *ECDC Technical Report.* Stockholm: ECDC.

EVANDER, M., ERIKSSON, I., PETTERSSON, L., JUTO, P., AHLM, C., OLSSON, G. E., BUCHT, G. & ALLARD, A. 2007. Puumala Hantavirus Viremia Diagnosed by Real-Time Reverse Transcriptase PCR Using Samples from Patients with Hemorrhagic Fever and Renal Syndrome. *Journal of Clinical Microbiology,* 45**,** 2491-2497.

EVERETT, K. R., REES-GEORGE, J., PUSHPARAJAH, I. P. S., JANSSEN, B. J. & LUO, Z. 2010. Advantages and disadvantages of microarrays to study microbial population dynamics - a minireview. *New Zealand Plant Protection,* 63**,** 1-6.

FAINE, S. 1994. *Leptospira and Leptospirosis* Boca Raton, USA, CRC Press Inc.

FAVOROV, M. O., KOSOY, M. Y., TSAREV, S. A., CHILDS, J. E. & MARGOLIS, H. S. 2000. Prevalence of Antibody to Hepatitis E Virus among Rodents in the United States. *The Journal of Infectious Diseases,* 181**,** 449-455.

FERRY, T. & ETIENNE, J. 2007. Community acquired MRSA in Europe. *British Medical Journal,* 335**,** 947-948.

FIRTH, C., BHAT, M., FIRTH, M. A., WILLIAMS, S. H., FRYE, M. J., SIMMONDS, P., CONTE, J. M., NG, J., GARCIA, J., BHUVA, N. P., LEE, B., CHE, X., QUAN, P. L. & LIPKIN, W. I. 2014. Detection of Zoonotic Pathogens and Characterisation of Novel Viruses Carried by Commensal *Rattus norvegicus* in New York City. *American Society for MIcrobiology,* 5**,** e01933-14.

FISCHER, S. A., GRAHAM, M. B., KUEHNERT, M. J., KOTTON, C. N., SRINIVASAN, A., MARTY, F. M., COMER, J. A., GUARNER, J.,

PADDOCK, C. D., DEMEO, D. L., SHIEH, W.-J., ERICKSON, B. R., BANDY, U., DEMARIA, A., DAVIS, J. P., DELMONICO, F. L., PAVLIN, B., LIKOS, A., VINCENT, M. J., ROLLIN, P. E., PACKARD, M. M., PATEL, M., ROWLAND, C., HELFAND, R. F., NICHOL, S. T., FISHMAN, J. A., KSIAZEK, T. & ZAKI, S. R. 2006. Transmission of Lymphocytic Choriomeningitis Virus by Organ Transplantation. *New England Journal of Medicine,* 354**,** 2235-2249.

FLEGR, J., PRANDOTA, J., SOVIČKOVÁ, M. & ISRAILI, Z. H. 2014. Toxoplasmosis – A Global Threat. Correlation of Latent Toxoplasmosis with Specific Disease Burden in a Set of 88 Countries. *PLoS ONE,* 9**,** e90203.

FLEIGE, S. & PFAFFI, M. W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine,* 27**,** 126-139.

FODOR, S. P. A., READ, L.J., PIRRUNG, M.C., STRYER, L., LU, A.T., SOLAS, D. 1991. Light-Directed, Spatially Addressable Parallel Chemical Synthesis. *Science,* 251**,** 767-773.

FREDRIKKSON-AHOMAA, M. & STOLLE, A., KORKEALA, H. 2006. Molecular epidemiology of *Yersinia enterocolitica* infections. *Immunology and Medical Microbiology,* 47**,** 315-329.

FRIEDRICH, A. W., ZHANG, W., BIELASZEWSKA, M., MELLMANN, A., KÖCK, R., FRUTH, A., TSCHÄPE, H. & KARCH, H. 2007. Prevalence, Virulence Profiles and Clinical Significance of Shiga Toxin-Negative Variants of Enterohemorrhagic *Escherichia coli* O157 Infection in Humans. *Clinical Infectious Diseases,* 45**,** 39-45.

FROUSSARD, P. 1992. A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. *Nucleic Acids Research,* 20**,** 2900.

FRYE, M. J., FIRTH, C., BHAT, M., FIRTH, M. A., CHE, X., LEE, D., WILLIAMS, S. H. & LIPKIN, W. I. 2015. *Preliminary Survey of Ectoparasites and Associated Pathogens from Norway Rats in New York City*.

GAASTRA, W., BOOT, R., HO, H. T. K. & LIPMAN, L. J. A. 2009. Rat bite fever. *Veterinary Microbiology,* 133**,** 211-228.

GALIMAND, M., CARNIEL, E. & COURVALIN, P. 2006. Resistance of *Yersinia pestis* to Antimicrobial Agents. *Antimicrobial Agents and Chemotherapy,* 50**,** 3233-3236.

GANNON, V. P. J., D'SOUZA, S., GRAHAM, T., KING, R. K., RAHN, K. & READ, S. 1997. Use of the Flagellar H7 Gene as a Target in Multiplex PCR Assays and Improved Specificity in Identification of Enterohemmorrhagic *Escherichia coli* Strains. *Journal of Clinical Microbiology,* 35**,** 656-662.

GARCIA-BETANCUR, J. C., MENENDEZ, M. C., DEL PORTILLO, P. & GARCIA, M. J. 2012. Alignment of multiple complete genomes suggests that gene rearrangements may contribute towards the speciation of *Mycobacteria*. *Infection, Genetics and Evolution,* 12**,** 819-826.

GARDNER-SANTANA, L. C., NORRIS, D. E., FORNADEL, C. M., HINSON, E. R., KLEIN, S. L. & GLASS, G. E. 2009. Commensal ecology,urban landscapes, and their influence on the genetic characteristics of city-dwelling Norway rats (*Rattus norvegicus*). *Molecular Ecology,* 18**,** 2766-2778.

GARLAND, S. & WOOD, J., SKERRATT, L.F. 2011. Comparison of sensitivity between real-time detection of a Taq-Man assay for *Batrachochytrium dendrobatidis* and conventional detection. *Europe PubMed Central,* 94**,** 101-105.

GARNHAM, P. C. C. 1971. *Progress in Parasitology*, London, Athlone Press.

GARVEY, P., CONNELL, J., O'FLANAGAN, D. & MCKEOWN, P. 2014. Leptospirosis in Ireland: annual incidence and exposures associated with infection. *Epidemiology and Infection,* 142**,** 847-855.

GAVIER-WIDÉN, D., STÅHL, K., NEIMANIS, A., SEGERSTAD, C., GORTÁZAR, C., ROSSI, S. & KUIKEN, T. 2015. African swine fever in wild boar in Europe: a notable challenge. *The Veterinary record,* 176**,** 199-200.

GIKAS, A., DOUKAKIS, S., PEDIADITIS, J., KASTANAKIS, S., PSAROULAKI, A. & TSELENTIS, Y. 2002. Murine typhus in Greece: epidemiological, clinical, and therapeutic data from 83 cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 96**,** 250-253.

GLASS, G. E., GARDNER-SANTANA, L. C., HOLT, R. D., CHEN, J., SHIELDS, T. M., ROY, M., SCHACHTERLE, S. & KLEIN, S. L. 2009. Trophic Garnishes: Cat-Rat Interactions in an Urban Environment. *PLoS ONE,* 4**,** e5794.

GOMEZ, M. D., PRIOTTO, J., PROVENSAL, M. C., STEINMAN, A., CASTILLO, E. & POLOP, J. J. 2008a. A population study of house mice (*Mus musculus*) inhabiting different habitats in an Argentine urban area. *International Biodeterioration and Biodegradation,* 62**,** 270-273.

GOMEZ, M. D., PROVENSAL, M. C. & POLOP, J. J. 2008b. Effect of interspecific competition on *Mus musculus* in an urban area. *Journal of Pest Science,* 81**,** 235-240.

GORIS, M. G. A., LEEFLANG, M. M. G., LODEN, M., WAGENAAR, J. F. P., KLATSER, P. R., HARTSKEERL, R. A. & BOER, K. R. 2013. Prospective Evaluation of Three Rapid Diagnostic Tests for Diagnosis of Human Leptospirosis. *PLoS Negl Trop Dis,* 7**,** e2290.

GOSLING, J. P. 1990. A Decade of Development in Immunoassay Methodology. *Clinical Chemistry,* 36**,** 1408-1427.

GRATZ, N. G. 1999. Urbanization, Arthropod and Rodent Pests and Human Health. *Proceedings of the 3rd international conference on urban pests*.

GRAVEKAMP, C., VAN DE KEMP, F., M., CARRINGTON, D., SCHOONE, G. J., VAN EYS, G. J. J. M., EVERARD, C. O. R., HARTSKEERL, R. A. & TERPSTRA, W. J. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Journal of General Microbiology,* 139**,** 1691-1700.

GRAVES, M. H. & JANDA, M. J. 2001. Rat-Bite Fever (*Streptobacillus moniliformis*): A Potential Emerging Disease. *International Journal of Infectious Diseases,* 5**,** 151-154.

GREIN, T. W., KAMARA, K. B. O., RODIER, G., PLANT, A. J., BOVIER, P., RYAN, M. J., OHYAMA, T. & HEYMANN, D. L. 2000. Rumors of Disease in the Global Village: Outbreak Verification. *Emerging Infectious Diseases,* 6**,** 97-102.

GUBLER, D. J., REITER, P., EBI, K. L., YAP, W., NASCI, R. & PATZ, J. A. 2001. Climate Variability and Change in the United States: Potential Impacts on Vector- and Rodent-Borne Diseases. *Environmental Health Perspectives,* 109**,** 223-232.

GUERRY, P., DOIG, P., ALM, R. A., BURR, D. H., KINSELLA, N. & TRUST, T. J. 1996. Identification and characterisation of genes required for post-translational modification of *Campylobacter coli* VC167 flagellin. *Molecular Microbiology,* 19**,** 369-378.

GUMMOW, B. 2010. Challenges posed by new and re-emerging infectious diseases in livestock production, wildlife and humans. *Livestock Science,* 130**,** 41-46.

GURRALA, R., DASTJERDI, A., JOHNSON, N., NUNEZ-GARCIA, J., GRIERSON, S., STEINBACH, F. & BANKS, M. 2009. Development of a DNA microarray for simultaneous detection and genotyping of lyssaviruses. *Virus Research,* 144**,** 202-208.

HAAKE, D. & LEVETT, P. 2015. Leptospirosis in Humans. *In:* ADLER, B. (ed.) *Leptospira and Leptospirosis.* Springer Berlin Heidelberg.

HAHN, M. B., EPSTEIN, J. H., GURLEY, E. S., ISLAM, M. S., LUBY, S. P., DASZAK, P. & PATZ, J. A. 2014. Roosting behaviour and habitat selection of *Pteropus giganteus* reveal potential links to Nipah virus epidemiology. *Journal of Applied Ecology,* 51**,** 376-387.

HALLIDAY, J., DABORN, C., AUTY, H., MTEMA, Z., LEMBO, T., BRONSVOORT, B. M. D., HANDEL, I., KNOBEL, D., HAMPSON, K. & CLEAVELAND, S. 2012. Bringing together emerging and endemic

zoonoses surveillance: shared challenges and a common solution. *Philisophical Transactions of The Royal Society,* 367**,** 2872-2880.

HANDKE, W., OELSCHLEGEL, R., FRANKE, R., WIEDEMANN, L., KRUGER, D. H. & RANG, A. 2010. Generation and characterisation of genetic reassortments between Puumala and Prospect Hill hantavirus *in vitro*. *Journal of General Virology,* 91**,** 2351-2359.

HAQUE, R., ROY, S., SIDDIQUE, A., MONDAL, U., RAHMAN, S. M. M., MONDAL, D., HOUPT, E. & PETRI JR, W. A. 2007. Multiplex real-time PCR assay for detection of *Entamoeba histolytica, Giardia intestinalis* and *Cryptosporidium* spp. *The American Journal of Tropical Medicine and Hygiene,* 76**,** 713-717.

HARKER, K. S., LANE, C., GORMLEY, F.J., ADAK, G.K. 2014. National outbreaks of *Salmonella* infection in the UK, 2000-2011. *Epidemiology and Infection,* 142**,** 601-607.

HARTSKEERL, R. A., COLLARES-PEREIRA, M. & ELLIS, W. A. 2011. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infection,* 17**,** 494-501.

HESTVIK, G., WARNS-PETIT, E., SMITH, L. A., FOX, N. J., UHLHORN, H., ARTOIS, M., HANNANT, D., HUTCHINGS, M. R., MATTSSON, R., YON, L. & GAVIER-WIDEN, D. 2014. The status of tularemia in Europe in a one-health context: a review. *Epidemiology & Infection,* FirstView**,** 1-24.

HEYMAN, P., PLYUSNINA, A., BERNY, P., COCHEZ, C., ARTOIS, M., ZIZI, M., PIRNAY, J. P. & PLYUSNIN, A. 2004. Seoul hantavirus in Europe: first demonstration of the virus genome in wild *Rattus norvegicus* captured in France. *European Journal of Clinical Microbiology and Infectious Diseases,* 23**,** 711-717.

HEYMANN, D. L. & RODIER, G. R. 1998. Global Surveillance of Communicable Diseases. *Emerging Infectious Diseases,* 4**,** 362-365.

HILL, D. & DUBEY, J. P. 2002. *Toxoplasma gondii:* transmission, diagnosis and prevention. *Clinical Microbiology and Infection* 8**,** 634-640.

HIMSWORTH, C., BAI, Y., KOSOY, M. Y., WOOD, H., DIBERNARDO, A., ROBBIN, L., BIDULKA, J., TANG, P., JARDINE, C. & PATRICK, D. 2015. An Investigation of *Bartonella* spp., *Rickettsia typhi,* and Seoul Hantavirus in Rats (*Rattus* spp.) from an Inner-City Neighborhood of Vancouver, Canada: Is Pathogen Presence a Reflection of Global and Local Rat Population Structure? *Vector-borne and Zoonotic Diseases,* 15.

HIMSWORTH, C., BIDULKA, J., PARSONS, K. L., FENG, A. Y. T., TANG, P., JARDINE, C. M., KERR, T., MAK, S., ROBINSON, J. & PATRICK, D. M. 2013. Ecology of Leptospira interrogans in Norway Rats (*Rattus*

norvegicus) in an Inner-City Neighborhood of Vancouver, Canada. *PLoS Neglected Tropical Diseases,* 7**,** e2270.

HIMSWORTH, C. G., BIDULKA, J., PARSONS, K.L., FENG, A.Y.T., TANG, P., JARDINE, C.M., KERR, T., MAK, S., ROBINSON, J., PATRICK, D.M. 2013. Ecology of *Leptospira interrogan*s in Norway Rats (*Rattus norvegicus*) in an Inner-City Neighborhood of Vancouver, Canada. *PLoS Neglected Tropical Diseases,* 7**,** e2270.

HIMSWORTH, M., R.R., MONTOYA, V., HOANG, L., ROMNEY, M.G., AL-RAWAHI, G.N., KERR, T., JARDINE, C.M., PATRICK, D.M., TANG, P., WEESE, S.J. 2014. Carriage of Methicillin-Resistant *Staphylococcus aureus* by Wild Urban Norway Rats (*Rattus norvegicus*). *PLoS one,* 9**,** e87983.

HINNEBUSCH, B. J., FISCHER, E. R. & SCHWAN, T. G. 1998. Evaluation of the Role of the *Yersinia pestis* Plasminogen Activator and Other Plasmid-Encoded Factors in Temperature-Dependent Blockage of the Flea. *Journal of Infectious Diseases,* 178**,** 1406-1415.

HO, P.-L., CHOW, K.-H., LAI, E. L., PIERRA, Y., LAW, T., CHAN, P.-Y., ALEX, Y., HO, M., NG, T.-K. & YAM, W.-C. 2012. Clonality and antimicrobial susceptibility of *Staphylococcus aureus* and methicillin-resistant *S. aureus* from food and other animals. *Journal of Clinical Microbiology,* 15.

HOLMBERG, M., MCGILL, S., EHRENBORG, C., WESSLEN, L., HJELM, E., DARELID, J., BLAD, L., ENGSTRAND, L., REGNERY, R. & FRIMAN, G. 1999. Evaluation of human seroreactivity to *Bartonella* species in Sweden. *Journal of Clinical Microbiology,* 37**,** 1381-1384.

HOMAN, W. L., VERCAMMEN, M., DE BRAEKELEER, J. & VERSCHUEREN, H. 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *International Journal for Parasitology,* 30**,** 69-75.

HÖNLINGER, B., HUEMER, H. P., ROMANI, N., CZERNY, C.-P., EISENDLE, K. & HÖPFL, R. 2005. Generalised cowpox infection probably transmitted from a rat. *British Journal of Dermatology,* 153**,** 451-453.

HÖRLING, J., LUNDKVIST, A., PERSSON, K., MULLAART, M., DZAGUROVA, T., DEKONENKO, A., TKACHENKO, E. A. & NIKLASSON, B. 1995. Detection and subsequent sequencing of Puumala virus from human specimens by PCR. *Journal of Clinical Microbiology,* 33**,** 277-282.

HOWARD, C. R. 1986. *Perspectives in Medical Virology,* Amsterdam, Elsevier.

HOWE, D. K., HONORÉ, S., DEROUIN, F. & SIBLEY, D. L. 1997. Determination of Genotypes of *Toxoplasma gondii* Strains Isolated from

Patients with Toxoplasmosis. *Journal of Clinical Microbiology,* 35**,** 1411-1414.

HULETSKY, A., LEBEL, P., PICARD, F. J., BERNIER, M., GAGNON, M., BOUCHER, N. & BERGERON, M. G. 2005. Identification of Methicillin-Resistant *Staphylococcus aureus* Carriage in Less than 1 Hour during a Hospital Surveillance Program. *Clinical Infectious Diseases,* 40**,** 976-981.

HUTCHINGS, M. R. 2006. Wild Mammals and the Human Food Chain. *Animal Science,* 82**,** 765-766.

IDO, Y., HOKI, R., ITO, H. & WANI, H. 1917. The rat as a carrier of *Spirochaeta icterohaemorrhagiae,* the causative agent of Weil's disease (Spirochaetosis icterohaemorrhagica). *Journal of Experimental Medicine,* 26**,** 341-353.

INCANDELA, M. L., PERRIN, E., FONDI, M., DE JESUS LOPES RIBEIRO, A. L., MORI, G., MOIANA, A., GRAMEGNA, M., FANI, R., RICCARDI, G. & PASCA, M. R. 2013. *DprE1, a new taxonomic marker in mycobacteria*.

INGLESBY, T. V., DENNIS, D. T., HENDERSON, D. A., BARTLETT, J. G., ASCHER, M. S., EITZEN, E., FINE, A. D., FRIEDLANDER, A. M., HAUER, J., KOERNER, J. F., LAYTON, M., MCDADE, J., OSTERHOLM, M. T., O'TOOLE, T., PARKER, G., PERL, T. M., RUSSEL, P. K., SCHOCH-SPANA, M. & TONAT, K. 2000a. Plague as a Biological Weapon. *Journal of the American Medical Association,* 283**,** 2281-2290.

INGLESBY, T. V., DENNIS, D. T., HENDERSON, D. A. & ET AL. 2000b. Plague as a biological weapon: Medical and public health management. *JAMA,* 283**,** 2281-2290.

IOOS, R., KOWALSKI, T., HUSSON, C. & HOLDENRIEDER, O. 2009. Rapid in planta detection of Chalara fraxinea by a real-time PCR assay using a dual-labelled probe. *Eurpean Journal of Plant Pathology,* 125**,** 329-335.

IRIZARRY, R. A., WARREN, D., SPENCER, F., KIM, I. F., BISWAL, S., FRANK, B. C., GABRIELSON, E., GARCIA, J. G. N., GEOGHEGAN, J., GERMINO, G., GRIFFIN, C., HILMER, S. C., HOFFMAN, E., JEDLICKA, A. E., KAWASAKI, E., MARTINEZ-MURILLO, F., MORSBERGER, L., LEE, H., PETERSEN, D., QUACKENBUSH, J., SCOTT, A., WILSON, M., YANG, Y., YE, S. Q. & YU, W. 2005. Multiple-laboratory comparison of microarray platforms. *Nature Methods,* 2**,** 345-350.

JAMESON, L. J., NEWTON, A., COOLE, L., NEWMAN, E. N. C., CARROLL, M. W., BEECHING, N. J., HEWSON, R. & CHRISTLEY, R. M. 2014. Prevalence of Antibodies against Hantaviruses in Serum and Saliva of

Adults Living or Working on Farms in Yorkshire, United Kingdom. *Viruses,* 6**,** 524-534.

JAMESON, L. J., TAORI, S. K., ATKINSON, B., LEVICK, P., FEATHERSTONE, C. A., VAN DER BURGT, G., MCCARTHY, N., HART, J., OSBORNE, J. C., WALSH, A. L., BROOKS, T. J. & HEWSON, R. 2013. Pet rats as a source of hantavirus in England and Wales, 2013. *Euro Surveillance,* 18.

JANSE, I., HAMIDJAJA, R. A., BOK, J. M. & VAN ROTTERDAM, B. J. 2010. Reliable detection of *Bacillus anthracis, Francisella tularensis and Yersinia pestis* by using multiplex qPCR including internal controls for nucleic acid extraction and amplification. *BMC Microbiology,* 10.

JANSE, I., HAMIDJAJA, R. A. & REUSKEN, C. 2013. *Yersinia pestis* Plasminogen Activator Gene Homolog in Rat Tissues. *Emerging Infectious Diseases,* 19**,** 342-344.

JANSEN, A., SCHÖNEBERG, I., FRANK, C., ALPERS, K., SCHNEIDER, T. & STARK, K. 2005. Leptospirosis in Germany, 1962-2003. *Emerging Infectious Diseases,* 11**,** 1048-1054.

JANSSEN, R., KROGFELT, K. A., CAWTHRAW, S. A., VAN PELT, W., WAGENAAR, J. A. & OWEN, R. J. 2008. Host-Pathogen Interactions in *Campylobacter* Infections: the Host Perspective. *Clinical Microbiology Reviews,* 21**,** 505-518.

JARDINE, C., LINDSAY, R. L., NICHOLSON, V. M., OJKIC, D. & PRESCOTT, J. F. 2011. Longitudinal Study on the Seroprevalence of Avian Influenza, Leptospirosis, and Tularemia in an Urban population of Raccoons (Procyon lotor) in Ontario, Canada. *Vector-borne and Zoonotic Diseases,* 11**,** 37-42.

JARVINEN, A. K., HAUTANIEMI, S., EDGREN, H., AUVINEN, P., SAARELA, J., KALLIONIEMI, O. P. & MONNI, O. 2004. Are data from different gene expression microarray platforms comparable? *Genomics,* 83**,** 1164-1168.

JIRINTAI, S., TANGGIS., MULYANTO., SUPARYATMO, J. B., TAKAHASHI, M., KOBAYASHI, T., NAGASHIMA, S., NISHIZAWA, T. & OKAMOTO, H. 2014. Rat hepatitis E virus derived from wild rats (*Rattus rattus*) propagates efficiently in human hepatoma cell lines. *Virus Research,* 185**,** 92-102.

JOHNE, R., HECKEL, G., PLENGE-BÖNIG, A., KINDLER, E., MARESCH, C., REETZ, J., SCHIELKE, A. & ULRICH, R. G. 2010. Novel Hepatitis E Virus Genotype in Norway Rats, Germany. *Emerging Infectious Diseases,* 16**,** 1452-1455.

JOHNE, R., PLENGE-BÖNIG, A., HESS, M., ULRICH, R. G., REETZ, J. & SCHIELKE, A. 2010a. Detection of a novel hepatitis E-like virus in

faeces of wild rats using a nested broad-spectrum RT-PCR. *Journal of General Virology,* 91**,** 750-758.

JOHNSON, G., AYERS, M., MCCLURE, S. C. C., RICHARDSON, S. E. & TELLIER, R. 2003. Detection and Identification of *Bartonella* Species Pathogenic for Humans by PCR Amplification Targeting the Riboflavin Synthase Gene (*ribC*). *Journal of Clinical Microbiology,* 41**,** 1069-1072.

JOHNSON, N. P. A. S. & MUELLER, J. 2002. Updating the Accounts: Global Mortality of the 1918-1920 "Spanish" Influenza Pandemic. *Bulletin of the History of Medicine,* 76**,** 105-115.

JOHNSON, W. M., LIOR, H. & BEZANSON, G. S. 1983. Cytotoxic *Escherichia coli* O157:H7 associated with haemorrhagic colitis in Canada. *Lancet,* 1**,** 8314-8315.

JONES, J. L. & DUBEY, J. P. 2010. Waterborne toxoplasmosis - Recent developments. *Experimental parasitology,* 124**,** 10-25.

JONES, K. E., PATEL, N. G., LEVY, M. A., STOREYGARD, A., BALK, D., GITTLEMAN, J. L. & DASZAK, P. 2008. Global trends in emerging infectious diseases. *Nature,* 451**,** 990-993.

JONGERT, E., ROBERTS, C. W., GARGANO, N., FÖRSTER-WALDL, E. & PETERSEN, E. 2009. Vaccines against *Toxoplasma gondii*: challenges and opportunities. *Memorias do Instituto Oswaldo Cruz,* 104**,** 252-266.

JUST, F. T., GILLES, J., PRADEL, I., PFALZER, S., LENGAUER, H., HELLMANN, K. & PFISTER, K. 2008. Molecular Evidence for *Bartonella* spp. in Cat and Dog Fleas from Germany and France. *Zoonoses and Public Health,* 55**,** 514-520.

KADLEC, K., EHRICHT, R., MONECKE, S., STEINACKER, U., KASPAR, H., MANKERTZ, J. & SCHWARZ, S. 2009. Diversity of antimicrobial resistance pheno- and genotypes of methicillin resistant *Staphylococcus aureus* ST398 from diseased swine. *Journal of Antimicrobial Chemotherapy,* 64.

KALLIO, E. R., KLINGSTRÖM, J., GUSTAFSSON, E., MANNI, T., VAHERI, A., HENTTONEN, H., VAPALAHTI, O. & LUNDKVIST, Å. 2006. Prolonged survival of Puumala hantavirus outside the host: evidence for indirect transmission via the environment. *Journal of General Virology,* 87**,** 2127-2134.

KAMANI, J., AKANBI, B. O., BANETH, G., MORRICK, D. & HARRUS, S. 2013. Co-Infection with *Capillaria hepatica and Bartonella elizabethae* in a Brown rat (*Rattus norvegicus*) from Nigeria. *Israel Journal of Veterinay Medicine,* 68**,** 230-233.

KANE, M. D., JATKOE, T. A., STUMPF, C. R., LU, J., THOMAS, J. D. & MADORE, S. J. 2000. Assessment of the sensitivity and specificity of

oligonucleotide (50mer) microarrays. *Nucleic Acids Research,* 28**,** 4552-4557.

KARCH, H., BIELASZEWSKA, M., BITZAN, M. & SCHMIDT, H. 1999. Epidemiology and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections. *Diagnostic Microbiology and Infectious Disease,* 34**,** 229-243.

KARCH, H., TARR, P. I. & BIELASZEWSKA, M. 2005. Enterohaemorrhagic *Escherichia coli* in human medicine. *International Journal of Medical Microbiology,* 295**,** 405-418.

KARIWA, H., KIMURA, M., YOSHIZUMI, S., ARIKAWA, J., YOSHIMATSU, K., TAKASHIMA, I. & HASHIMOTO, N. 1996. Mode of Seoul virus infections: persistency in newborn rats and transciency in adult rats. *Archives of virology,* 141**,** 2327-2338.

KATO, C. Y., CHUNG, I. H., ROBINSON, L. K., AUSTIN, A. L., DASCH, G. A. & MASSUNG, R. F. 2013. Assessment of Real-Time PCR Assay for Detection of *Rickettsia* spp. and *Rickettsia ricketsii* in Banked Clinical Samples. *Journal of Clinical Microbiology,* 51**,** 314-317.

KAYSSER, P., VON BOMHARD, W., DOBRZYKOWSKI, L. & MEYER, H. 2010. Genetic diversity of feline cowpox virus, Germany 2000-2008. *Veterinary Microbiology,* 141**,** 282-288.

KEIM, P., JOHANSSON, A. & WAGNER, D. M. 2007. Molecular Epidemiology, Evolution, and Ecology of *Francisella*. *Annals of the New York Academy of Sciences,* 1105**,** 30-66.

KIMBALL, A. M., ARIMA, Y. & HODGES, J. R. 2005. Trade related infections: farther, faster, quieter. *Globalization and Health,* 1.

KIMURA, M., TANIKAWA, T., SUZUKI, M., KOIZUMI, N., KAMIYAMA, T., IMAOKA, K. & YAMADA, A. 2008. Detection of *Streptobacillus* spp. in feral rats by specific polymerase chain reaction. *Microbiology and Immunology,* 52**,** 9-15.

KINNUNEN, P. M., HENTTONEN, H., HOFFMAN, B., KALLIO, E. R., KORTHASE, C., LAAKKONEN, J., NIEMIMAA, J., PALVA, A., SCHLEGEL, M., ALI, H. S., SUOMINEN, P., ULRICH, R., VAHERI, A. & VAPALAHTI, O. 2011. Orthopox Virus Infections in Eurasian Wild Rodents. *Vector-Borne and Zoonotic Diseases,* 11.

KLEIN, E. Y., SUN, L., SMITH, D. L. & LAXMINARAYAN, R. 2013. The Changing Epidemiology of Methicillin-Resistant *Staphylococcus aureus* in the United States: A National Observational Study. *American Journal of Epidemiology*.

KLEIN, S. L., ZINK, M. C. & GLASS, G. E. 2004. Seoul virus infection increases aggressive behaviour in male Norway rats. *Animal Behaviour,* 67**,** 421-429.

KLEMPA, B., FICHET-CLAVET, E., LECOMPTE, E., AUSTE, B., ANISKIN, V., MEISEL, H., DENYS, C., KOIVOGUI, L., TER MEULEN, J. & KRÜGER, D. H. 2006. Hantavirus in African Wood Mouse, Guinea. *Emerging Infectious Diseases,* 12**,** 838-840.

KLUYTMANS, J. 2007. Control of meticillin-resistant *Staphylococcus aureus* (MRSA) and the value of rapid tests. *Journal of Hospital Infection,* 65**,** 100-104.

KNUST, B., STROHER, U., EDISON, L., ALBARINO, C. G., LOVEJOY, J., ARMEANU, E., HOUSE, J., CORY, D., HORTON, C., FOWLER, K. L., AUSTIN, J., POE, J., HUMBAUGH, K. E., GUERRERO, L., CAMPBELL, S., GIBBONS, A., REED, Z., CANNON, D., MANNING, C., PETERSEN, B., METCALF, D., MARSH, B., NICHOL, S. T. & ROLLIN, P. E. 2014. Lymphocytic Choriomeningitis Virus in Employees and Mice at Multipremises Feeder-Rodent Operation, United States, 2012. *Emerging Infectious Diseases,* 20**,** 240-247.

KOBAYASHI, Y. 2001. Clinical obdervation and treatment of leptospirosis. *Journal of Infection and Chemotherapy,* 7**,** 59-68.

KOEHLER, J. E. 1996. *Bartonella infections. In Advances in pediatric infectious diseases,* St. Louis, Missouri, Mosby-Year Book.

KONG, R. Y. C., LEE, S. K. Y., LAW, T. W. F., LAW, S. H. W. & WU, R. S. S. 2002. Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. *Water Research,* 36**,** 2802-2812.

KORDICK, D. L., HILYARD, E. J., HADFIELD, T. L., WILSON, K. H., STEIGERWALT, A. G. & BRENNER, D. J. 1997. *Bartonella clarridgeiae,* a newly recognised zoonotic pathogen causing inoculation papules, fever and lymphadenopathy (cat scratch disease). *Journal of Clinical Microbiology,* 35**,** 1813-1818.

KOSKINEN, M. T., HOLOPAINEN, J., PYÖRÄLÄ, S., BREDBACKA, P., PITKÄLÄ, A., BARKEMA, H. W., BEXIGA, R., ROBERSON, J., SØLVERØD, L., PICCININI, R., KELTON, D., LEHMUSTO, H., NISKALA, S. & SALMIKIVI, L. 2009. Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *Journal of Dairy Science,* 92**,** 952-959.

KOSOY, M., BAI, Y., SHEFF, K., MORWAY, C., BAGGETT, H., MALONEY, S. A., BOONMAR, S., BHENGSRI, S., DOWELL, S. F., SITDHIRASDR, A., LERDTHUSNEE, K., RICHARDSON, J. & PERUSKI, L. F. 2010. Identification of *Bartonella* Infections in Febrile Human Patients from Thailand and Their Potential Animal Reservoirs. *American Journal of Tropical Medicine and Hygiene,* 82**,** 1140-1145.

KOUNI, S., KARAKITSOS, P., CHRANIOTI, A., THEODORIDOU, M., CHROUSOS, G. & MICHOS, A. 2013. Evaluation of viral co-infections

in hospitalized and non-hospitalized children with respiratory infections using microarrays. *Clinical Microbiology and Infection,* 19**,** 772-777.

KRAMSKI, M., MEISEL, H., KLEMPA, B., KRÜGER, D. H., PAULI, G. & NITSCHE, A. 2007. Detection and Typing of Human Pathogenic Hantaviruses by Real-Time Reverse Transcription-PCR and Pyrosequencing. *Clinical Chemistry,* 53**,** 1899-1905.

KREIL, D. P., RUSSELL, R. R. & RUSSELL, S. 2006. Microarray Oligonucleotide Probes. *Methods in Enzymology,* 410**,** 73-98.

KRUGER, D. H., ULRICH, R. & HOFMANN, J. 2013. Hantaviruses as Zoonotic Pathogens in Germany. *Deutsches Ärzteblatt International,* 110**,** 461-467.

KUEHN, A., SCHULZE, C., KUTZER, P., PROBST, C., HLINAK, A., OCHS, A. & GRUNOW, R. 2013. Tularaemia seroprevalence of captured and wild animals in Germany: the fox (*Vulpes vulpes*) as a biological indicator. *Epidemiology & Infection,* 141**,** 833-840.

KUGELER, K. J., STAPLES, J. E., HINCKLEY, A. F., GAGE, K. L. & MEAD, P. S. 2015. Epidemiology of Human Plague in the United States, 1900–2012. *Emerging Infectious Diseases,* 21**,** 16-22.

KUHN, K. G., FALKENHORST, G., CEPER, T. H., DALBY, T., ETHELBERG, S., MØLBAK, K. & KROGFELT, K. A. 2012. Detecting non-typhoid *Salmonella* in humans by ELISAs: a literature review. *Journal of Medical Microbiology,* 61**,** 1-7.

KUMAR, R. M. 2009. The Widely Used Diagnostics "DNA Microarray" - A Review. *American Journal of Infectious Diseases,* 5**,** 207-218.

LAFFERTY, K. D. 2009. The ecology of climate change and infectious diseases. *Ecology,* 90**,** 888-890.

LALIBERTÉ, J. & CARRUTHERS, V. B. 2008. Host cell manipulation by the human pathogen *Toxoplasma gondii*. *Cellular and Molecular Life Sciences,* 65**,** 1900-1915.

LAMBERT, M. S., QUY, R. J., SMITH, R. H. & COWAN, D. P. 2008. The effect of habitat management on home-range size and survival of rural Norway rat populations. *Journal of Applied Ecology,* 45**,** 1753-1761.

LARSSON, P., OYSTON, P. C. F., CHAIN, P., CHU, M. C., DUFFIELD, M., FUXELIUS, H.-H., GARCIA, E., HALLTORP, G., JOHANSSON, D., ISHERWOOD, K. E., KARP, P. D., LARSSON, E., LIU, Y., MICHELL, S., PRIOR, J., PRIOR, R., MALFATTI, S., SJOSTEDT, A., SVENSSON, K., THOMPSON, N., VERGEZ, L., WAGG, J. K., WREN, B. W., LINDLER, L. E., ANDERSSON, S. G. E., FORSMAN, M. & TITBALL, R. W. 2005. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nat Genet,* 37**,** 153-159.

LAU, C. L., SMYTHE, L. D., CRAIG, S. B. & WEINSTEIN, P. 2010. Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 104**,** 631-638.

LAUDISOIT, A., FALAY, D., AMUNDALA, N., AKAIBE, D., DE BELLOCQ, J. G., VAN HOUTTE, N., BRENO, M., VERHEYEN, E., WILSCHUT, L., PAROLA, P., RAOULT, D. & SOCOLOVSCHI, C. 2014. High Prevalence of Rickettsia typhi and Bartonella Species in Rats and Fleas, Kisangani, Democratic Republic of the Congo. *The American Journal of Tropical Medicine and Hygiene,* 90**,** 463-468.

LAUKKENEN, R., MARTÍNEZ, P. O., SIEKKINEN, K. M., RANTA, J., MAIJALA, R. & KORKEALA, H. 2008. Transmission of Yersinia pseudotuberculosis in the Pork Production Chain from Farm to Slaughterhouse. *Applied and Environmental Microbiology,* 74**,** 5444-5450.

LE MOINE, V., VANNIER, P. & JESTIN, A. 1987. Microbiological Studies of Wild Rodents in Farms as Carriers of Pig Infectious Agents. *Preventive Veterinary Medicine,* 4**,** 399-406.

LEAL-BALBINO, T. C., LEAL, N. C., LOPES, C. V. & DE ALMEIDA, A. M. P. 2004. Differences in the Stability of the Plasmids of *Yersinia pestis* Cultures in Vitro: Impact on Virulence. *Memorias do Instituto Oswaldo Cruz,* 99**,** 727-732.

LEEK, J. T., SCHARPF, R. B., BRAVO, H. C., SIMCHA, D., LANGMEAD, B., JOHNSON, W. E., GEMAN, D., BAGGERLY, K. & IRIZARRY, R. A. 2010. Tackling the widespread and critical impact of batch effects in high-throughout data. *Nature Reviews Genetics,* 11**,** 733-739.

LEEK, J. T., SCHARPF, R.B., BRAVO, H.C., SIMCHA, D., LANGMEAD, B., JOHNSON, W.E., GEMAN, D., BAGGERLY, K., IRIZARRY, R.A. 2010. Tackling the widespread and critical impact of batch effects in high-throughout data. *Nature Reviews Genetics,* 11**,** 733-739.

LEMOINE, S., COMBES, F. & LE CROM, S. 2009. An evaluation of custom microarray applications: the oligonucleotide design challenge. *Nucleic Acids Research,* 37**,** 1726-1739.

LEQUIN, R. M. 2005. Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clinical Chemistry,* 51**,** 2415-2418.

LEVADITI, C., NICOLAU, S. & POINCLOUX, P. 1925. Sur le role etiologique de *Streptobacillus moniliformis* (nov. spec.) dans l'erytheme polymorphe aigu septicemique. *Comptes rendus,* 180.

LEVANTESI, C., BONADONNA, L., BRIANCESCO, R., GROHMANN, E., TOZE, S. & TANDOI, V. 2012. Salmonella in surface and drinking water:

Occurrence and water-mediated transmission. *Food Research International,* 45**,** 587-602.

LEVETT, P. N. 2001. Leptospirosis. *Clinical Microbiology Reviews,* 14**,** 296-326.

LEVETT, P. N. 2004. Leptospirosis: A forgotten zoonosis? *Clinical and Applied Immunology Reviews,* 4**,** 435-448.

LEVIEUGE, A., ABOUBAKER, M. H., TERRIER, O., DRANCOURT, M. & DAVOUST, B. 2010. Real-time PCR detection of Leptospira sp. in rodents from Toulon harbour (France). *Revue de Médecine Vétérinaire,* 161**,** 264-266.

LI, T. C., AMI, Y., SUZAKI, Y., YASUDA, S. P., YOSHIMATSU, K., TAKEDA, N. & TAKAJI, W. 2013a. Characterisation of full genome of rat hepatitis e virus strain from Vietnam. *Emerging Infectious Diseases,* 19**,** 118.

LI, W., GUAN, D., SU, J., TAKEDA, N., WAKITA, T., LI, T.-C. & KE, C. W. 2013b. High prevalence of rat hepatitis E virus in wild rats in China. *Veterinary Microbiology*.

LI, W. & RUAN, K. 2009. MicroRNA detection by microarray. *Analytical and Bioanalytical Chemistry,* 394**,** 1117-1124.

LIM, Y., -H., HIROSE, K., IZUMIYA, H., ARAKAWA, E., TAKAHASHI, H., TERAJIMA, J., ITOH, K., -I., TAMURA, K., KIM, S., -I. & WATANABE, H. 2003. Multiplex Polymerase Chain Reaction Assay for Selective Detection of *Salmonella enterica* Serovar Typhimurium. *Japanese Journal of Infectious Diseases,* 56**,** 151-155.

LIN, M.-H., CHEN, T.-C., KUO, T.-T., TSENG, C.-C. & TSENG, C.-P. 2000. Real-Time PCR for Quantitative Detection of *Toxoplasma gondii*. *Journal of Clinical Microbiology,* 38**,** 4121-4125.

LINTON, D., LAWSON, A. J., OWEN, R. J. & STANLEY, J. 1997. PCR detection, identification to species level, and fingerprinting of campylobacter jejuni and campylobacter coli direct from diarrheic samples. *Journal of Clinical Microbiology,* 35**,** 2568-2572.

LITRUP, E., TORPDAHL, M., MALORNY, B., HUEHN, S., HELMS, M., CHRISTENSEN, H. & NIELSEN, E. M. 2010. DNA microarray analysis of *Salmonella* serotype Typhimurium strains causing different symptoms of disease. *BMC Microbiology,* 10.

LLEDÓ, L., GEGÚNDEZ, M. I., SAZ, J. V., BAHAMONTES, N. & BELTRÁN, M. 2003. *Lymphocytic Choriomeningitis Virus* Infection in a Province of Spain: Analysis of Sera From the General Population and Wild Rodents. *Journal of Medical Virology,* 70**,** 273-275.

LOÏEZ, C., HERWEGH, S., WALLET, F., ARMAND, S., GUINET, F. & COURCOL, R. J. 2003. Detection of *Yersinia pestis* in Sputum by Real-Time PCR. *Journal of Clinical Microbiology,* 41**,** 4873-4875.

LONG, C., JONES, T. F., VUGIA, D. J., SCHEFTEL, J., STROCKBINE, N., RYAN, P., SHIFERAW, B., TAUXE, R. V. & GOULD, L. H. 2010. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* Infections, FoodNet 1996-2007. *Emerging Infectious Diseases,* 16**,** 566-567.

LONG, W. H., XIAO, H. S., GU, X. M., ZHANG, Q. H., YANG, H. J., ZHAO, G. P. & LIU, J. H. 2004. A universal microarray for detection of SARS coronavirus. *Journal of Virological Methods,* 121**,** 57-63.

LOY, A. & BODROSSY, L. 2006. Highly parallel microbial diagnostics using oligonucleotide microarrays. *Science Direct,* 363**,** 106-119.

LUCIANI, F., CIONE, E., CORSONELLO, A., GUIDO, F., DE SANTIS, S., CANNATARO, R., PERRI, M., CAROLEO, M. C. & CANNATARO, A. M. 2014. sun. *International Journal of Infectious Diseases,* 27**,** 10-12.

LUDWIG, K., SARKIM, V., BITZAN, M., KARMALI, M. A., BOBROWSKI, C., RUDER, H., LAUFS, R., SOBOTTKA, I., PETRIC, M., KARCH, H. & MÜLLER-WIEFEL, D. E. 2002. Shiga Toxin-Producing *Escherichia coli* Infection and Antibodies against Stx2 and Stx1 in Household Contacts of Children with Enteropathic Hemolytic-Uremic Syndrome. *Journal of Clinical Microbiology,* 40**,** 1773-1782.

MACÉ, G., FEYEUX, C., MOLLARD, N., CHANTEGRET, C., AUDIA, S., REBIBOU, J. M., SPAGNOLO, G., BOUR, J. B., DENOYEL, G. A., SAGOT, P. & REYNES, J. M. 2013. Severe Seoul hantavirus infection in a pregnant woman, France, October 2012. *Euro Surveillance,* 18.

MADHUBASHINI, M., GEORGE, S. & CHANDRASEKARAN, S. 2013. Streptobacillus moniliformis endocarditis: Case report and review of literature. *Indian Heart Journal,* 65**,** 442-446.

MAJOWICZ, S. E., SCALLAN, E., JONES-BITTON, A., SARGEANT, J. M., STAPLETON, J., ANGULO, F. J., YEUNG, D. H. & MARTYN, K. D. 2014. Global Incidence of Human Shiga-Toxin-Producing Escherichia coli Infections and Deaths: A Systematic Review and Knowledge Synthesis. *Foodborne Pathogens and Disease,* 11**,** 447-455.

MAKSYUTOV, R. A., GAVRILOVA, E. V., MEYER, H. & SHCHELKUNOV, S. N. 2015. Real-time PCR assay for specific detection of cowpox virus. *Journal of Virological Methods,* 211**,** 8-11.

MANEERAT, Y., CLAYSON, E. T., MYINT, K. S. A., YOUNG, D. G. & INNIS, B. L. 1996. Experimental infection of the laboratory rat with the hepatitis E virus. *Journal of Medical Virology,* 48**,** 121-128.

MANIGOLD, T. & VIAL, P. 2014. Human hantavirus infections: epidemiology, clinical features, pathogenesis and immunology. *Swiss Medical Weekly*.

MANITZ, J., KNEIB, T., SCHLATHER, M., HELBING, D. & BROCKMANN, D. 2014. Origin Detection During Food-borne Disease Outbreaks - A Case Study of the 2011 EHEC/HUS Outbreak in Germany. *PLoS Currents,* 6**,** ecurrents.outbreaks.f3fdeb08c5b9de7c09ed9cbcef5f01f2.

MARANO, N., ARGUIN, P. M. & PAPPAIONANOU, M. 2007. Impact of Globalization and Animal Trade on Infectious Disease Ecology. *Emerging Infectious Diseases,* 13**,** 1807-1809.

MARENNIKOVA, S. S. 1979. Field and experimental studies of poxvirus infections in rodents. *Bulletin of the World Health Organisation,* 57**,** 461-464.

MARICIC, T., WHITTEN, M. & PÄÄBO, S. 2010. Multiplexed DNA Sequence Capture of Mitochondrial Genomes Using PCR Products. *PLoS one,* 5.

MARTINEZ-OLONDRIS, P., RIGOL, M. & TORRES, A. 2010. What lessons have been learnt from animal models of MRSA in the lung? *European Respiratory Journal,* 35**,** 198-201.

MARTÍNEZ, M. A., RÍO., S.-D., M.D.L.D., G., R.M., CHIU, C. Y., GRENINGER, A. L., CONTRERAS, J. F., LÓPEZ, S., ARIAS, C. F. & ISA, P. 2014. DNA microarray for detection of gastrointestinal viruses. *Journal of Clinical Microbiology,* 52.

MARUYAMA, S., MITSUZANE, A., NAKAGAWA, K., KABEYA, H., YOKOYAMA, E. & KATSUBE, Y. 2000. Prevalence of *Salmonella* species in house rats from the buildings in Yokohama city. *Journal of Veterinary Epidemiology,* 4**,** 27-32.

MASI, E., PINO, F. A., SANTOS, M. D. G. S., GENEHR, L., ALBUQUERQUE, J. O. M., BANCHER, A. M. & ALVES, J. C. M. 2010. Socioeconomic and environmental risk factors for urban rodent infestation in Sao Paolo, Brazil. *Journal of Pest Science,* 83**,** 231-241.

MATHEWS, F., MACDONALD, D. W., TAYLOR, G. M., GELLING, M., NORMAN, R. A., HONESS, P. E., FOSTER, R., GOWER, C. M., VARLEY, S., HARRIS, A., PALMER, S., HEWINSON, G. & WEBSTER, J. P. 2006. Bovine tuberculosis (*Mycobacterium bovis*) in British farmland wildlife: the importance to agriculture. *Proceedings of the Royal Society B: Biological Sciences,* 273**,** 357-365.

MAURIN, M., RAOULT, D. 2012. *Rickettsia typhi (Murine tyhpus)* [Online]. Available: http://www.antimicrobe.org/new/r06.asp [Accessed 20/05/2014 2014].

MCCAUSLAND, M. M. & CROTTY, S. 2007. Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo. *Journal of Virological Methods,* 147**,** 167-176.

MCCLUSKEY, B. J. 2003. *Use of sentinel herds in monitoring and surveillance systems,* Iowa, Iowa State Press.

MCEVOY, M. B., NOAH, N. D. & PILSWORTH, R. 1987. Outbreak of fever caused by *Streptobacillus moniliformis*. *Lancet,* 330**,** 1361-1363.

MCGILL, S., WESSLEN, L., HJELM, E., HOLMBERG, M., ROLF, C. & FRIMAN, G. 2001. Serological and epidemiological analysis of the prevalence of *Bartonella* spp. antibodies in Swedish elite orienteers 1992-1993. *Scandinavian Journal of Infectious Diseases,* 33**,** 423-428.

MCKEE, G. & PEWARCHUK, J. 2013. Rat-bite fever. *CMAJ,* 185**,** 1346.

MCNABB, S. J. N. 2010. Comprehensive effective and efficient global public health surveillance. *BMC Public Health,* 10.

MEERBURG, B. G., JACOBS-REITSMA, W. F., WAGENAAR, J. A. & KILJSTRA, A. 2006. Presence of *Salmonella* and *Campylobacter* spp. in Wild Small Mammals on Organic Farms. *Applied and Environmental Microbiology,* 72**,** 960-962.

MEERBURG, B. G., SINGLETON, G. R. & KIJLSTRA, A. 2009. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology,* 35**,** 221-270.

MERHEJ, V. & RAOULT, D. 2011. Rickettsial evolution in the light of comparative genomics. *Biological Reviews,* 86**,** 379-405.

MERTENS, M., HOFMANN, J., PETRAITYTE-BURNEIKIENE, R., ZILLER, M., SASNAUSKAS, K., FRIEDRICH, R., NIEDERSTRASSER, O., KRÜGER, D. H., GROSCHUP, M. H., PETRI, E., WERDERMANN, S. & ULRICH, R. G. 2011. Seroprevalence study in forestry workers of a non-endemic region in eastern Germany reveals infections by Tula and Dobrava-Belgrade hantaviruses. *Medical Microbiology and Immunology,* 200**,** 263-268.

MEYER, B., DROSTEN, C. & MÜLLER, M. A. 2014. Serological assays for emerging coronaviruses: Challenges and pitfalls. *Virus Research,* 194**,** 175-183.

MEYER, H., PFEFFER, M. & RZIHA, H.-J. 1994. Sequence alterations within and downstream of the A-type inclusion protein allow differentiation of *Orthopoxvirus* species by polymerase chain reaction. *Journal of General Virology,* 75**,** 1975-1981.

MICHELET, L., BONNET, S., MADANI, N. & MOUTAILLER, S. 2013. Discriminating *Francisella tularensis* and *Francisella*-like endosymbionts in Dermacentor reticulatus ticks: Evaluation of current molecular techniques. *Veterinary Microbiology,* 163**,** 399-403.

MIKHAILOVICH, V., GRYADUNOV, D., KOLCHINSKY, A., MAKAROV, A.A., ZASEDATELEV, A. 2008. DNA microarrays in the clinic: infectious diseases. *BioEssays,* 30**,** 673-682.

MILDENBERG, Z., WESTCOTT, D., BELLAICHE, M., DASTJERDI, A., STEINBACH, F. & DREW, T. 2009. Equine Encephalosis Virus in Israel. *Transboundary and Emerging Diseases,* 56**,** 291-291.

MILLER, M. B. 2009. *Solid and liquid phase array technologies,* Washingtom, American Society of Microbiology Press.

MILLS, J. N. & CHILDS, J. E. 1998. Ecologic Studies of Rodent Reservoirs: Their relevance for Human Health. *Emerging Infectious Diseases,* 4**,** 529-537.

MILLS, J. N., GAGE, K.L., KHAN, A.S 2010. Potential Influence of Climate Change on Vector-Borne and Zoonotic Disease. *Environmental Health Perspectives*.

MILLS, J. N., KSIAZEK, T. G., PETERS, C. J. & CHILDS, J. E. 1999. Long-term studies of hantavirus reservoir populations in the southwestern United States: a synthesis. *Emerging Infectious Diseases,* 5**,** 135-142.

MOGOLLON-PASAPERA, E., OTVOS JR, L., GIORDANO, A. & CASSONE, M. 2009. Bartonella: emerging pathogen or emerging awareness? *International Journal of Infectious Diseases,* 13**,** 3-8.

MOHAMED, M. R., RAHMAN, M. M., RICE, A., MOYER, R. W., WERDEN, S. J. & MCFADDEN, G. 2009. Cowpox Virus Expresses a Novel Ankyrin Repeat NF-κB Inhibitor That Controls Inflammatory Cell Influx into Virus-Infected Tissues and Is Critical for Virus Pathogenesis. *Journal of Virology,* 83**,** 9223-9236.

MORSE, S. S. 1995. Factors in the Emergence of Infectious Diseases. *Emerging Infectious Diseases,* 1**,** 7-15.

MOSTOWY, S., INWALD, J., GORDON, S., MARTIN, C., WARREN, R., KREMER, K., COUSINS, D. & BEHR, M. A. 2005. Revisiting the Evolution of *Mycobacterium bovis*. *Journal of Bacteriology,* 187**,** 6386-6395.

MÜLLER, R., DITZEN, A., HILLE, K., STICHLING, M., EHRICHT, R., ILLMER, T., EHNINGER, G. & ROHAYEM, J. 2009. Detection of herpesvirus and adenovirus co-infections with diagnostic DNA-microarrays. *Journal of Virological methods,* 155**,** 161-166.

MURPHY, F. 2008. Emerging zoonoses: The challenge for public health and biodefence. *Preventive Veterinary Medicine,* 86**,** 216-223.

MYINT, K. S. A., ENDY, T. P., GIBBONS, R. V., LARAS, K., MAMMEN, M. P. J., SEDYANINGSIH, E. R., SERIWATANA, J., GLASS, J. S., NARUPITI, S. & CORWIN, A. L. 2006. Evaluation of Diagnostic Assays for Hepatitis E Virus in Outbreak Settings. *Journal of Clinical Microbiology,* 44**,** 1581-1583.

NAKAJIMA, H., INOUE, M., MORI, T., ITOH, K., ARAKAWA, E. & WATANABE, H. 1992. Detection and identification of *Yersinia pseudotuberculosi*s and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *Journal of Clinical Microbiology,* 30**,** 2484-2486.

NARDONNE, A., CAPEK, I., BARANTON, G., CAMPÈSE, C., POSTIC, D., VAILLANT, V., LIÉNARD, M. & DESENCLOS, J. C. 2004. Risk Factors for Leptospirosis in Metropolitan France: Results of a National Case-Control Study 1999-2000. *Clinical Infectious Diseases,* 39**,** 751-753.

NIELSEN, E. M., SKOV, M. N., MADSEN, J. J., LODAL, J., JESPERSEN, J. B. & BAGGESEN, D. L. 2004. Verocytotoxin-Producing *Escherichia coli in* Wild Birds and Rodents in Close Proximity to Farms. *Applied and Environmental Microbiology,* 70**,** 6944-6947.

NOGAREDA, F., LE STRAT, Y., VILLENA, I., DE VALK, H. & GOULET, V. 2014. Incidence and prevalence of *Toxoplasma gondii* infection in women in France, 1980–2020: model-based estimation. *Epidemiology & Infection,* 142**,** 1661-1670.

ODA, S., KABEYA, H., SATO, S., SHIMONAGNE, A., INOUE, K., HYASHIDANI, H., TAKADA, N., FUJITA, H., KAWABATA, H. & MARUYAMA, S. 2015. Isolation of Pathogenic *Yersinia enterocolitica* 1B/O:8 from *Apodemus* mice in Japan. *Journal of Wildlife Diseases,* 51**,** 260-264.

OLIVEIRA, D. C., TOMASZ, A. & DE LENCASTRE, H. 2002. Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Lancet Infectious Diseases,* 2**,** 180-189.

OOTEMAN, M. C., VAGO, A. R. & KOURY, M. C. 2006. Evaluation of MAT, IgM ELISA and PCR methods for the disgnosis of human leptospirosis. *Journal of Microbiological Methods,* 65**,** 247-257.

ORTIZ-RIAÑO, E., CHENG, B. Y. H., DE LA TORRE, J. C. & MARTÍNEZ-SOBRIDO, L. 2011. The C-Terminal Region of Lymphocytic Choriomeningitis Virus Nucleoprotein COntains Distinct and Segregable Functional Domains Involved in NP-Z Interaction and Counteraction of the Type I Interferon Response. *Journal of Virology,* 85**,** 13038-13048.

OSTFELD, R. S. & HOLT, R. D. 2004. Are predators good for your health? Evaluating evidence for top-down regulation of zoonotic disease reservoirs. *Frontiers in Ecology and the Environment,* 2**,** 13-20.

OWEN, M. R. & TREES, A. J. 1998. Vertical transmission of *Toxoplasma gondii* from chronically infected house (*Mus musculus*) and field (*Apodemus sylvaticus*) mice determined by polymerase chain reaction. *Parasitology,* 116**,** 299-304.

PAHLITZSCH, R., HAMMARIN, A.-L. & WIDELL, A. 2006. A Case of Facial Cellulitis and Necrotizing Lymphadenitis due to Cowpox Virus Infection. *Clinical Infectious Diseases,* 43**,** 737-742.

PALACIOS, G., QUAN, P. L., JABADO, O. J., CONLAN, S., HIRSCHBERG, D. L., LIU, Y., ZHAI, J., RENWICK, N., HUI, J., HEGYI, H., GROLLA, A., STRONG, J. E., TOWNER, J. S., GEISBERT, T. W., JAHRLING, P. B., BÜCHEN-OSMOND, C., ELLERBROK, H., SANCHEZ-SECO, M. P., LUSSIER, Y., FORMENTY, P., NICHOL, S. T., FELDMANN, H., BRIESE, T. & LIPKIN, W. I. 2007. Panmicrobial Oligonucleotide Array for Diagnosis of Infectious Diseases. *Emerging Infectious Diseases,* 13**,** 73-81.

PANTI-MAY, J. A., HERNANDEZ-BETANCOURT, S., RUIZ-PINA, H. & MEDINA-PERALTA, S. 2012. Abundance and population parameters of commensal rodents present in rural households in Yucatan, Mexico. *International Biodeterioration & Biodegradation,* 66**,** 77-81.

PAWESKA, J. T., VAN VUREN, P. J., KEMP, A., BUSS, P., BENGIS, R. G., GAKUYA, F., BREIMAN, R. F., NJENGA, M. K. & SWANEPOEL, R. 2008. Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo. *Veterinary Microbiology,* 127**,** 21-28.

PELLUDAT, C., DUFFY, B. & FREY, J. E. 2009. Design and development of a DNA microarray for rapid identification of multiple European quarantine phytopathogenic bacteria. *European Journal of Plant Pathology,* 125**,** 413-423.

PERALTA, B., MATEU, E., CASAS, M., DE DEUS, N., MARTÍN, M. & PINS, S. 2009. Genetic characterization of the complete coding regions of genotype 3 hepatitis E virus isolated from Spanish swine herds. *Virus Research,* 139**,** 111-116.

PERLMAN, S. J., HUNTER, M. S. & ZCHORI-FEIN, E. 2006. The emerging diversity of *Rickettsia*. *Proceedings of the Royal Society,* 273**,** 2097-2106.

PETERS, C. J. 2006. Lymphocytic Choriomeningitis Virus - An Old Enemy up to New Tricks. *New England Journal of Medicine,* 354**,** 2208-2211.

PHILIPS, C. B. & PARKER, R. R. 1938. The Persistence of the Viruses of Endemic (Murine) Typhus, Rocky Mountain Spotted Fever, and Boutonneuse Fever in Tissues of Experimental Animals. *Public Health Reports,* 53**,** 1246-1251.

PLACE, E. H. & SUTTON, L. E. 1934. Erythema arthriticum epidemicum (Haverhill fever). *Archives of Internal Medicine,* 54**,** 659-684.

PLYUSNINA, A., IBRAHIM, I., WINOTO, I., PORTER, K.R., GOTAMA, I.B.I., LUNDKVIST, A., VAHERI, A., PLYUSNIN, A. 2004.

Identification of Seoul Hantavirus in *Rattus norvegicus* in Indonesia. *Scandinavian Journal of Infectious Diseases,* 36**,** 356-359.

POCOCK, M. J. O., SEARLE, J. B. & WHITE, P. C. L. 2004. Adaptations of animals to commensal habitats: population dynamics of house mice *Mus musculus domesticus* on farms. *Journal of Animal Ecology,* 73**,** 878-888.

POLAND, J. D. & BARNES, A. M. 1979. *Plague,* Boca Raton, FL, CRC Press.

POLLEY, L. 2005. Navigating parasite webs and parasite flow: Emerging and re-emerging parasitic zoonoses of wildlife origin. *International Journal for Parasitology,* 35**,** 1279-1294.

PROMED-MAIL 2014. Plague-China (02): (Gansu), Fatality. *Pro-MED-mail* 27.

PSAROULAKI, A., ANTONIOU, M., TOUMAZOS, P., MAZERIS, A., IOANNOU, I., CHOCHLAKIS, D., CHRISTOPHI, N., LOUKAIDES, P., PATSIAS, A., MOSCHANDREA, I. & TSELENTIS, Y. 2010. Rats as indicators of the presence and dispersal of six zoonotic microbial agents in Cyprus, an island ecosystem: a seroepidemiological study. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 104**,** 733-739.

PUI, C. F., WONG, W. C., CHAI, L. C., LEE, H. Y., NOORLIS, A., ZAINAZOR, T. C. T., TANG, J. Y. H., GHAZALI, F. M., CHEAH, Y. K., NAKAGUCHI, Y., NISHIBUCHI, M. & RADU, S. 2011. Multiplex PCR for the concurrent detection and differentiation of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium. *Tropical Medicine and Health,* 39**,** 9-15.

PUPPE, W., WEIGL, J., GRÖNDHAL, B., KNUF, M., ROCKAHR, S., VON BISMARCK, P., ARON, G., NIESTERS, H. G. M., OSTERHAUS, A. D. M. E. & SCHMITT, H.-J. 2013. Validation of a multiplex reverse transcriptase PCR ELISA for the detection of 19 respiratory tract pathogens. *Infection,* 41**,** 77-91.

PURCELL, R. H. & EMERSON, S. U. 2010. Hidden Danger: The Raw Facts about Hepatitis E Virus. *The Journal of Infectious Diseases,* 202**,** 819-821.

PURCELL, R. H., ENGLE, R. E., ROOD, M. P., KABRANE-LAZIZI, Y., NGUYEN, H. T., GOVINDARAJAN, S., ST.CLAIRE, M. & EMERSON, S. U. 2011. Hepatitis E Virus in Rats, Los Angeles, California USA. *Emerging Infectious Diseases,* 17**,** 2216-2222.

RAMACHANDRAN, S., SINGHAL, M., MCKENZIE, K. G., OSBORN, J. L., ARJYAL, A., DONGOL, S., BAKER, S. G., BASNYAT, B., FARRAR, J., DOLECEK, C., DOMINGO, G. J., YAGER, P. & LUTZ, B. 2013. A Rapid, Multiplexed, High-Throughput Flow-Through Membrane Immunoassay: A Convenient Alternative to ELISA. *Diagnostics,* 3**,** 244-260.

RAMSDEN, C., MELO, F. L., FIGUEIREDO, L. M., HOLMES, E. C. & ZANOTTO, P. M. A. 2008. High Rates of Molecular Evolution in Hantaviruses. *Molecular Biology and Evolution,* 25**,** 1488-1492.

RAOULT, D., ABOUDHARAM, G., CRUBÉZY, E., LARROUY, G., LUDES, B. & DRANCOURT, M. 2000. Molecular identification by "suicide PCR" of *Yersinia pestis* as the agent of Medieval Black Death. *PNAS,* 97**,** 12800-12803.

RAOULT, D. & ROUX, V. 1997. Rickettsioses as Paradigms of New or Emerging Infectious Diseases. *Clinical Microbiology Reviews,* 10**,** 694-719.

RASKO, D. A., ROSOVITZ, M. J., MYERS, G. S. A., MONGODIN, E. F., FRICKLE, W. F., GAJER, P., CRABTREE, J., SEBAIHIA, M., THOMSON, N. R., CHAUDHURI, R., HENDERSON, I. R., SPERANDIO, V. & RAVEL, J. 2008. The Pangenome Structure of *Escherichia coli*: Comparative Genomic Analysis of E. coli Commensal and Pathogenic Isolates. *Journal of Bacteriology,* 190**,** 6881-6893.

RASTAWICKI, W., SZYCH, J., ROKOSZ, N., ZACHARCZUK, K. & GIERCCZYŃSKI, R. 2013. Seasonality of *Yersinia enterocolitica* bioserotype 1B/O:8 infections in Poland. *Epidemiology and Infection,* 141**,** 2039-2042.

RELOGIO, A., SCHWAGER, C., RICHTER, A., ANSORGE, W. & VALCARCEL, J. 2002. Optimisation of oligonucleotide-based DNA microarrays. *Nucleic Acids Research,* 30.

REUSKEN, C. & HEYMAN, P. 2013. Factors driving hantavirus emergence in Europe. *Current Opinion in Virology,* 3**,** 92-99.

REUTER, G., FODOR, D., FORGÁCH, P., KÁTAI, A. & SZŰCS, G. 2009. Characterisation and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. *Journal of Clinical Virology,* 44**,** 277-281.

REUTER, G., FODOR, D., KÁTAI, A. & SZŰCS, G. 2006. Identification of a novel variant of human hepatitis E virus in Hungary. *Journal of Clinical Virology,* 36**,** 100-102.

RICHARD, V., RIEHM, J. M., PERLINOT, H., RAHELINIRINA, S., MAHERISOA, R., FANJASOA, R., SAMUEL, A., SCHOLZ, H. C. & MINOARISOA, R. 2015. Pneumonic Plague Outbreak, Northen Madagascar, 2011. *Emerging Infectious Diseases,* 21**,** 8-15.

RIEHM, J. M., RAHALISON, L., SCHOLZ, H. C., THOMA, B., PFEFFER, M., RAZANAKOTO, L. M., DAHOUK, S. A., NEUBAUER, H. & TOMASO, H. 2011. Detection of *Yersinia pestis* using real-time PCR in patients with suspected bubonic plague. *Molecular and Cellular Probes,* 25**,** 8-12.

RILEY, L. W., REMIS, R. S., HELGERSON, S. D., MCGEE, H. B., WELLS, J. G., DAVIS, B. R., HEBERT, R. J., OLCOTT, E. S., JONHSON, L. M., HARGRETT, N. T., BLAKE, P. A. & COHEN, M. L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype *New England Journal of Medicine,* 308**,** 681-685.

RIVIERE, Y., AHMED, R., SOUTHERN, P. J., BUCHMEIER, M. J. & OLDSTONE, M. B. A. 1985. Genetic Mapping of Lymphocytic Choriomeningitis Virus Pathogenicity: Virulence in Guinea Pigs Is Associated with the L RNA Segment. *Journal of Virology,* 55**,** 704-709.

ROPP, S. L., JANICE, Q. J., KNIGHT, C., MASSUNG, R. F. & ESPOSITO, J. J. 1995. PCR Strategy for Identification and Differentiation of Smallpox and Other Orthopoxviruses. *Journal of Clinical Microbiology,* 33**,** 2069-2076.

ROSEF, O., GONDROSEN, B., KAPPERUD, G., UNDERAL, B. 1983. Isolation and characterisation of *Campylobacter jejuni* and *Campylobacter coli* from domestic and wild animals in Norway. *Applied and Environmental Microbiology,* 46**,** 855-859.

ROSENBERG, R. 2014. Detecting the emergence of novel, zoonotic viruses pathogenic to humans. *Cellular and Molecular Life Sciences***,** 1-11.

ROSNER, B. M., STARK, K. & WERBER, D. 2010. Epidemiology of reported *Yersinia enterocolitica* infections in Germany 2001-2008. *BMC Public Health,* 10.

ROUNDS, J. M., BOXRUD, D. J., JAWAHIR, S. L. & SMITH, K. E. 2011. Dynamics of *Escherichia coli* O157:H7 outbreak detection and investigation, Minnesota 2000-2008. *Epidemiology and Infection*.

ROZYNEK, E., DZIERZANOWSKA-FANGRAT, K., JOZWIAK, P., POPOWSKI, J., KORSAK, D. & DZIERZANOWSKA, D. 2005. Prevalence of potential virulence markers in Polish *Campylobacter jejuni* and *Campylobacter coli* isolates obtained from hospitalised children and from chicken carcasses. *Journal of Medical Microbiology,* 54**,** 615-619.

RUSSELL, L. B. 1986. *Is Prevention better than Cure?,* Washington D.C., The Brookings Institution.

RUSSELL, S., MEADOWS, K. A. & RUSSEL, R. R. 2009. *Microarray Technology in Practice,* Cambridge, Academic Press.

SACHSE, K., HOTZEL, H., SLICKERS, P., ELLINGER, T. & EHRICHT, R. 2005. DNA microarray-based detection and identification of *Chlamydia* and *Chlamydophila* spp. *Molecular and Cellular Probes,* 19**,** 41-50.

SAFIULLAH, S. A., SALEH, A. A. & MUNWAR, S. 2009. Laboratory Methods for Diagnosing Leptospirosis: A Review. *Bangladesh Journal of Medical Microbiology,* 3**,** 39-43.

SALVATO, M. S. & SHIMOMAYE, E. M. 1989. The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. *Virology,* 173**,** 1-10.

SAMUEL, W. M., PYBUS, M. J. & KOCAN, A. A. 2001. *Parasitic Diseases of Wild Animals,* Iowa, Iowa State University Press.

SAMUEL, W. M., PYBUS, M.J., KOCAN, A.A. 2001. *Parasitic Diseases of Wild Mammals,* Iowa, Iowa State University Press.

SÁNCHEZ-VARGAS, F. M., ABU-EL-HAIJA, M. A. & GÓMEZ-DUARTE, O. G. 2011. *Salmonella* infections: An update on epidemiology, management, and prevention. *Travel Medicine and Infectious Disease,* 9**,** 263-277.

SÁNCHEZ-VIZCAÍNO, J. M., MUR, L. & MARTÍNEZ-LÓPEZ, B. 2013. African swine fever (ASF): Five years around Europe. *Veterinary Microbiology,* 165**,** 45-50.

SANNÖ, A., ASPÁN, A., HESTVIK, G. & JACOBSON, M. 2014. Presence of *Salmonella* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Escherichia coli* O157:H7 in wild boars. *Epidemiology and Infection,* 142**,** 2542-2547.

SARKAR, U., NASCIMENTO, S. F., BARBOSA, R., MARTINS, R., NUEVO, H., KALOFONOS, I., GRUNSTEIN, I., FLANNERY, B., DIAS, J., RILEY, L. W., REIS, G. M. & KO, A. I. 2002. Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. *American Journal of Tropical Medicine and Hygiene,* 66**,** 605-610.

SAULEDA, S., ONG, E., BES, M., JANSSEN, A., CORY, R., BABIZKI, M., SHIN, T., LINDQUIST, A., HOANG, A., VANG, L., PIRON, M., CASAMITJANA, N., KOPPELMAN, M., DANZIG, L. & LINNEN, J. M. 2014. Seroprevalence of hepatitis E virus (HEV) and detection of HEV RNA with a transcription-mediated amplification assay in blood donors from Catalonia (Spain). *Transfusion***,** n/a-n/a.

SCALLAN, E., GRIFFIN, P. M., ANGULO, F. J., TAUXE, R. V. & HOEKSTRA, R. M. 2011. Foodborne Illness Acquired in the United States—Unspecified Agents. *Emerging Infectious Diseases,* 17**,** 16-22.

SCHAFER, I. J., MILLER, R., STROHER, U., KNUST, B., NICHOL, S. T. & ROLLIN, P. E. 2014. Notes from the Field: A Cluster of Lymphocytic Choriomeningitis Virus Infections Transmitted Through Organ Transplantation - Iowa, 2013. *Morbidity and Mortality Weekly Report* 63**,** 249-249.

SCHENA, M. 2003. *Microarray Analysis,* Hoboken, New Jersey, John Wiley & Sons.

SCHIELKE, A., ROSNER, M. B. & STARK, K. 2014. Epidemiology of campylobacterisosis in Germany - insights from 10 years of surveillance. *BMC Infectious Diseases,* 14.

SCHMITT, P., SPLETTSTÖSSER, W., PORSCH-OZCÜRÜMEZ, M., FINKE, E. J. & GRUNOW, R. 2005. A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. *Epidemiology and Infection,* 133**,** 759-766.

SCHUENEMANN, V. J., BOS, K., DEWITTE, S., SCHMEDES, S., JAMIESON, J., MITTNIK, A., FORREST, S., COOMBES, B. K., WOOD, J. W., EARN, D. J. D., WHITE, W., KRAUSE, J. & POINAR, H. N. 2011. Targeted enrichment of ancient pathogens yielding the pPCP1 plasmid of *Yersinia pestis* from victims of the Black Death. *PNAS,* 108**,** E746-E752.

SCOTT, G. M., THOMSON, R., MALONE-LEE, J. & RIDGWAY, G. L. 1988. Cross-infection between animals and man: possible feline transmission of *Staphylococcus aureus* infection in humans? *Journal of Hospital Infection,* 12**,** 29-34.

SEBBANE, F., JARRETT, C. O., GARDNER, D., LONG, D. & HINNEBUSCH, B. J. 2006. Role of the *Yersinia pestis* plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague. *PNAS,* 103**,** 5526-5530.

SEIDEL, M. & NIESSNER, R. 2008. Automated analytical microarrays: a critical review. *Analytical and Bioanalytical Chemistry,* 391**,** 1521-1544.

SEVERGNINI, M., CREMONESI, P., CONSOLANDI, C., DE BELLIS, G., CASTIGLIONI, B. 2011. Advances in DNA Microarray Technology for the Detection of Foodborne Pathogens. *Food and Bioprocess Technology,* 4**,** 936-953.

SHARMA, N., HOTTA, A., YAMAMOTO, Y., FUJITA, O., UDA, A., MORIKAWA, S., YAMADA, A. & TANABAYASHI, K. 2013. Detection of *Francisella tularensis*-Specific Antibodies in Patients with Tularemia by a Novel Competitive Enzyme-Linked Immunosorbent Assay. *Clinical and Vaccine Immunology,* 20**,** 9-16.

SHCHELKUNOV, S. N., GAVRILOVA, E. V. & BABKIN, I. V. 2005. Multiplex PCR detection and species differentiation of orthopoxviruses pathogenic to humans. *Molecular and Cellular Probes,* 19**,** 1-8.

SIBOLD, C., MEISEL, H., LUNDKVIST, Å., SCHULZ, A., CIFIRE, F., ULRICH, R., KOZUCH, O., LABUDA, M. & KRÜGER, D. H. 1999. Short report: simultaneous occurrence of Dobrava, Puumala, and Tula Hantaviruses in Slovakia. *American Journal of Tropical Medicine and Hygiene,* 61**,** 409-411.

SILVA, J., LEITE, D., FERNANDES, M., MENA, C., GIBBS, P. A. & TEIXEIRA, P. 2011. *Campylobacter* spp. as a foodborne pathogen: a review. *Frontiers in Microbiology,* 2.

SINGH, D. V., ISAC, S. R. & COLWELL, R. R. 2002. Development of a hexaplex PCR assay for rapid detection of virulence and regulatory genes in *Vibrio cholerae* and *Vibrio mimicus*. *Journal of Clinical Microbiology,* 40**,** 4321-4324.

SINT, D., RASO, L. & TRAUGOTT, M. 2012. Advances in multiplex PCR: balancing primer effectiveness and improving detection success. *Methods in Ecology and Evolution,* 3**,** 898-905.

SKOTTMAN, T., PIIPARINEN, H., HYYTIÄINEN, H., MYLLYS, V., SKURNIK, M. & NIKKARI, S. 2006. Simultaneous real-time PCR detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. *European Journal of Clinical Microbiology and Infectious Diseases,* 26**,** 207-211.

SKOV, M. N., MADSEN, J. J., RAHBEK, C., LODAL, J., JESPERSEN, J. B., JØRGENSEN, J. C., DIETZ, H. H., CHRIÉL, M. & BAGGESEN, D. L. 2008. Transmission of *Salmonella* between wildlife and meat-production animals in Denmark. *Journal of Applied Microbiology,* 105**,** 1558-1568.

SLACK, A. T., SYMONDS, M. L., DOHNT, M. F. & SMYTHE, L. D. 2006. Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. *BMC Microbiology,* 6.

SLIFKA, M. K., MATLOUBIAN, M. & AHMED, R. 1995. Bone marrow is a major site of long-term antibody production after acute viral infection. *Journal of Virology,* 69**,** 1895-1902.

SMYTH, G. K., MICHAUD, J. & SCOTT, H. S. 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics,* 21**,** 2067-2075.

SMYTHE, L. D., SMITH, I. L., SMITH, G. A., DOHNT, M. F., SYMONDS, M. L., BARNETT, L. J. & MCKAY, D. B. 2002. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira spp*. *BMC Infectious Diseases,* 2.

SPICHLER, A. S., VILAÇA, P. J., ATHANAZIO, D. A., ALBUQUERQUE, J. O. M., BUZZAR, M., CASTRO, B., SEGURO, A. & VINETZ, J. M. 2008. Predictors of Lethality in Severe Leptospirosis in Urban Brazil. *American Journal of Tropical Medicine and Hygiene,* 79**,** 911-914.

STAMM, I., HAILER, M., DEPNER, B., KOPP, P. A. & RAU, J. 2013. *Yersinia enterocolitica* in Diagnostic Fecal Samples from European Dogs and Cats: Identification by Fourier Transform Infrared Spectroscopy and Matrix-

Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *Journal of Clinical Microbiology,* 51**,** 887-893.

STEPHENSON, J. R. & WARNES, A. 1998. *Methods in Molecular Medicine,* New Jersey, Humana Press.

STRYJEWSKI, M. E. & COREY, G. R. 2014. Methicillin-Resistant *Staphylococcus aureus*: An Evolving Pathogen. *Clinical Infectious Diseases,* 58**,** S10-S19.

STUART, A. M., PRESCOTT, C. V., MACINTYRE, S., SETHAR, A., NEUMAN, B. W., MCCARTHY, N. D., WIMALARATHNA, H. & MAIDEN, M. C. J. 2011. The role of rodents as carriers of disease on UK farms: a preliminary investigation. *8th European Vertebrate Pest Management Conference,* 432 Julius-Kuhn Archiv**,** 198-199.

SU, C., SHWAB, E. K., ZHOU, P., ZHU, X. Q. & DUBEY, J. P. 2010. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitology,* 137**,** 1-11.

SUN, Z., PENG, Y., ZHANG, M., WANG, K., BAI, J., LI, X., NING, B. & GAO, Z. 2014. Simultaneous and highly sensitive detection of six different foodborne pathogens by high-throughput suspension assay technology. *Food Control,* 40**,** 300-309.

TAGLIAPIETRA, V., ROSÁ, R., HAUFFE, H. C., LAAKKONEN, J., VOUTILAINEN, L., VAPALAHTI, O., VAHERI, A., HENTTONEN, H. & RIZZOLI, A. 2009. Spatial and Temporal Dynamics of Lymphocytic Choriomeningitis Virus in Wild Rodents, Northern Italy. *Emerging Infectious Diseases,* 15**,** 1019-1025.

TATINENI, S., SAGARAM, U. S., GOWDA, S., ROBERTSON, C. J., DAWSON, W. O., IWANAMI, T. & WANG, N. 2008. In Planta Distribution of '*Candidatus Liberibacter asiaticus*' as revealed by Polymerase Chain Reaction (PCR) and Real-Time PCR. *Phytopathology,* 98**,** 592-599.

TAUXE, R. V. 2002. Emerging foodborne pathogens. *International Journal of Food Microbiology,* 78**,** 31-41.

TAYLOR, L. H., LATHAM, S. M. & WOOLHOUSE, M. E. J. 2001. Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences. *The Royal Society,* 356**,** 983-989.

TENTER, A. M. 2009. *Toxoplasma gondii* in animals used for human consumption. *Memorias do Instituto Oswaldo Cruz,* 104**,** 364-369.

TENTER, A. M., HECKEROTH, A. R. & WEISS, L. M. 2000. Toxoplasma gondii: from animals to humans. *International Journal for Parasitology,* 31**,** 1217-1258.

THOERNER, P., BIN KINGOMBE, C. I., BOGLI-STUBER, K., BISSIG-CHOISAT, B., WASSENAAR, T. M., FREY, J. & JEMMI, T. 2003. PCR Detection of Virulence Genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and Investigation of Virulence Gene Distribution. *Applied and Environmental Microbiology,* 69**,** 1810-1816.

THOMPSON, A. R. C., KUTZ, S. J. & SMITH, A. 2009. Parasite zoonoses and Wildife: Emerging Issues. *Environmental Research and Public Health,* 6**,** 678-693.

THRELFALL, E. J. 2002. Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. *FEMS Microbiology Reviews,* 26**,** 141-148.

TIQUIA, S. M., WU, L., CHONG, S. C., PASSOVETS, S., XU, D., XU, Y. & ZHOU, J. 2004. Evaluation of 50-mer oligonucleotide arrays for detecting microbial populations in environmental samples. *BioTechniques,* 36**,** 664-675.

TOMASO, H., REISINGER, E. C., AL DAHOUK, S., FRANGOULIDIS, D., RAKIN, A., LANDT, O. & NEUBAUER, H. 2003. Rapid detection of *Yersinia pestis* with multiplex real-time PCR assays using fluorescent hybridisation probes. *FEMS Immunology and Medical Microbiology,* 38**,** 117-126.

TORTOLI, E. 2003. Impact of Genotypic Studies on Mycobacterial Taxonomy: the New Mycobacteria of the 1990s. *Clinical Microbiology Reviews,* 16**,** 319-354.

TORTOLI, E., URBANO, P., MARCELLI, F., SIMONETTI, T. M. & CIRILLO, D. M. 2012. Is Real-Time PCR Better than Conventional PCR for *Mycobacterium tuberculosis* Complex Detection in Clinical Samples? *Journal of Clinical Microbiology,* 50**,** 2810-2813.

TRAJMAN, A., STEFFEN, R. E. & MENZIES, D. 2013. Interferon-Gamma Release Assays versus Tuberculin Skin Testing for the Diagnosis of Latent Tuberculosis Infection: An Overview of the Evidence. *Pulmonary Medicine,* 2013**,** 11.

TRAN, P. H., PEIFFER, D. A., SHIN, Y., MEEK, L. M., BRODY, J. P. & CHO, K. W. Y. 2002. Microarray optimisations: increasing spot accuracy and automated identification of true microarray signals. *Nucleic Acids Research,* 30**,** e54.

TRAUB, R., WISSEMAN, C. L. & FARHANG-AZAD, A. 1978. The ecology of murine typhus: a critical review. *Tropical diseases bulletin,* 75**,** 237-317.

TRAWEGER, D. & SLOTTA-BACHMAYR, L. 2005. Introducing GIS-modelling into the management of a bron rat (*Rattus norvegicus*) (Mamm. Rodentia Muridae) population in an urban habitat. *Journal of Pest Science,* 78**,** 17-24.

TRAWEGER, D., TRAVNITZKY, R., MOSER, C., WALZER, C. & BERNATZKY, G. 2006. Habitat preferences and distribution of the brown rat (*Rattus norvegicus*) in the city of Salzburg (Austria): implications for an urban rat management. *Journal of Pest Science,* 79**,** 113-125.

TSANGARAS, K., SIRACUSA, M. C., NIKOLAIDIS, N., ISHIDA, Y., CUI, P., VIELGRADER, H., HELGEN, K. M., ROCA, A. L. & GREENWOOD, A. D. 2014. Hybridisation Capture Reveals Evolution and Conservation across the Entire Koala Retrovirus Genome. *PloS One,* 9**,** e95633.

TSIEN, C. L., LIBERMANN, T., GU, X. & KOHANE, I. S. On reporting fold differences. Pacific Symposium on Biocomputing, 2001. 250.

UTTAMCHANDANI, M., NEO, J. L., ONG, B. N. Z. & MOOCHHALA, S. 2008. Applications of microarrays in pathogen detection and biodefence. *Trends in Biotechnology,* 27**,** 53-61.

UYTTENDAELE, M., VANWILDEMEERSCH, K. & DEBEVERE, J. 2003. Evaluation of real-time PCR vs automated ELISA and a conventional culture methof using a semi-solid medium for detection of *Salmonella*. *Letters in Applied Microbiology,* 37**,** 386-291.

VALLAT, B. 2007. Protecting the World from Emerging Diseases Linked to Globalisation. *Editorials from the Director General.* Paris, France: World Organisation for Animal Health (OIE).

VAN DE GIESSEN, A. W., VAN SANTEN-VERHEUVEL, M. G., HENGEVELD, P. D., BOSCH, T., BROENS, E. M. & REUSKEN, C. B. E. M. 2009. Occurrence of methicillin-resistant *Staphylococcus aureus* in rats living on pig farms. *Preventive Veterinary Medicine,* 91**,** 270-273.

VAN DUIJKEREN, E., BOX, A. T., HECK, M. E., WANNET, W. J. & FLUIT, A. C. 2004. Methicillin-resistant staphylococci isolated from animals. *Veterinary Microbiology,* 103**,** 91-97.

VAN LOO, I., HUIJSDENS, X., TIEMERSMA, E., DE NEELING, A., VAN DE SANDE-BRUINSMA, N. & BEAUJEAN, D. 2007. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. *Emerging Infectious Diseases,* 13**,** 1834-1839.

VANANTWERPEN, G., VAN DAMME, I., DE ZUTTER, L. & HOUF, K. 2014. Within-batch prevalence and quantification of human pathogenic *Yersinia enterocolitica* and *Y. pseudotuberculosis* in tonsils of pigs at slaughter. *Veterinary Microbiology,* 169**,** 223-227.

VANDAMME, P., DEWHIRST, F. E., PASTER, B. J. & ON, S. L. W. 2005. *Genus I. Campylobacter,* New York, USA., Springer.

VAYSSIER-TAUSSAT, M., MOUTAILLER, S., MICHELET, L., DEVILLERS, E., BONNET, S., CHEVAL, J., HÉBERT, C. & ELOIT, M. 2013. Next

Generation Sequencing Uncovers Unexpected Bacterial Pathogens in Ticks in Western Europe. *PLoS one,* 8**,** e81439.

VOGEL, G. 2014. Are Bats Spreading Ebola Across Sub-Saharan Africa? *Science,* 344**,** 140.

VOLOKHOV, D., RASOOLY, A., CHUMAKOV, K. & CHIZHIKOV, V. 2002. Identifiaction of Listeria species by microarray-based assay. *Journal of Clinical Microbiology,* 40**,** 4720-4728.

VORA, G. J., MEADOR, C. E., STENGER, D. A. & ANDREADIS, J. D. 2004. Mucleic Acid Amplification Strategies for DNA Microarray-Based Pathogen Detection. *Applied and Environmental Microbiology,* 70**,** 3047-3054.

VOROU, R. M., PAPAVASSILIOU, V. G. & PIERROUTSAKOS, I. N. 2008. Cowpox virus infection: an emerging health threat. *Current Opinions in Infectious Diseases,* 21**,** 153-156.

VYAS, A., KIM, S. K., GIACOMINI, N., BOOTHROYD, J. C. & SAPOLSKY, R. M. 2007. Behavioural changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. *PNAS,* 104**,** 6442-6447.

WALTER, G., BOTELHO-NEVERS, E., SOCOLOVSCHI, C., RAOULT, D. & PAROLA, P. 2012. Murine Typhus in Returned Travelers: A Report of Thirty-Two Cases. *American Journal of Tropical Medicine and Hygiene,* 86**,** 1049-1053.

WANG, C., HE, H. 2013. Leptospira spp. In Commensal Rodents, Beijing, China. *Journal of Wildlife Diseases,* 49**,** 461-463.

WANG, D., COSCOY, L., ZYLBERBERG, M., AVILA, P. C., BOUSHEY, H. A., GANEM, D. & DERISI, J. L. 2002a. Microarray-based detection and genotyping of viral pathogens. *PNAS,* 99**,** 15687-15692.

WANG, D., URISMAN, A., LIU, Y., -T., SPRINGER, M., KSIAZEK, T. G., ERDMAN, D. D., MARDIS, E. R., HICKENBOTHAM, M., MAGRINI, V., ELDRED, J., LATREILLE, J. P., WILSON, R. K., GANEM, D. & DERISI, J. L. 2003a. Viral Discovery and Sequence Recovery Using DNA Microarrays. *PLoS Biology,* 1**,** e2.

WANG, D., URISMAN, A., LIU, Y. T., SPRINGER, M., KSIAZEK, T. G., ERDMAN, D. D., MARDIS, E. R., HICKENBOTHAM, M., MAGRINI, V., ELDRED, J., LATREILLE, J. P., WILSON, R. K., GANEM, D. & DERISI, J. L. 2003b. Viral Discovery and Sequence Recovery Using DNA Microarrays. *Public Library of Science Biology,* 1**,** 257-260.

WANG, G., CLARK, C. G., TAYLOR, T. M., PUCKNELL, C., BARTON, C., PRICE, L., WOODWARD, D. L. & RODGERS, F. G. 2002b. Colony Multiplex PCR Assay for Identification and Differentiation of

Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, and *C. fetus* subcp. *fetus*. *Journal of Clinical Microbiology,* 40**,** 4744-4747.

WANG, H. Y., MALEK, R. L., KWITEK, A. E., GREENE, A. S., LUU, T. V., BEHBAHANI, B., FRANK, B., QUACKENBUSH, J. & LEE, N. H. 2003a. Assessing unmodified 70-mer oligonucleotide probe performance on glass-slide microarrays. *Genome Biology,* 4**,** R5.

WANG, L. C., PAN, C. H., SEVERINGHAUS, L. L., LIU, L. Y., CHEN, C. T., PU, C. E., HUANG, D., LIR, J. T., CHIN, S. C., CHENG, M. C., LEE, S. H. & WANG, C. H. 2008. Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. *Veterinary Microbiology,* 127**,** 217-226.

WANG, T. T. & PALESE, P. 2009. Unraveling the Mystery of Swine Influenza Virus. *Cell,* 137**,** 983-985.

WARD, M. P. 2002. Seasonality of canine leptospirosis in the United States and Canada and its association with rainfall. *Preventive Veterinary Medicine,* 56**,** 203-213.

WATSON, M., DUKES, J., ABU-MEDIAN, A.-B., KING, D. P. & BRITTON, P. 2007. DetectiV: visualisation, normalization and significance testing for pathogen-detection mircoarray data. *Genome Biology,* 8**,** 1901-1912.

WEBSTER, J. P. 2001. Rats, cats, people and parasites: the impact of latent toxoplasmosis on behavior. *Microbes and Infection,* 33**,** 1037-1045.

WEBSTER, J. P. & MACDONALD, D. W. 1995. Parasites of wild brown rats (Rattus norvegicus) on UK farms. *Parasitology,* 111**,** 247-255.

WEESE, J. S., ARCHAMBAULT, M., WILLEY, B. M., HEARN, P., KREISWIRTH, B. N., SAID-SALIM, B., MCGEER, A., LIKHOSHVAY, Y., PRESCOTT, J. F. & LOW, D. E. 2005. Methicillin-resistant *Staphyloccus aureus* in horses and horse personnel. *Emerging Infectious Diseases,* 20**,** 601-613.

WERNERSSON, R., JUNCKER, A. S. & NIELSEN, H. B. 2007. Probe selection for DNA microarrays using OligoWiz. *Nature Protocols,* 2**,** 2677-2691.

WERNERSSON, R. & NIELSEN, H. B. 2005. OligoWiz 2.0 - integrating sequence feature annotation into the design of microarray probes. *Nucleic Acids Research,* 33**,** 611-615.

WHO 2000. The Increasing Incidence of Human Campylobacteriosis. . *In:* RESPONSE, D. O. C. D. S. A. (ed.) *Report and Proceedings of a WHO Consultation of Experts.*

WHO 2012. The Global View of Campylobacteriosis. *In:* ORGANISATION, W. H. (ed.). Geneva: WHO Press.

WHO 2014. World health statistics 2014. *In:* ORGANISATION, W. H. (ed.). Geneva: WHO Press.

WHO. 2014b. *The top 10 causes of death* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs310/en/ [Accessed 27/03/2015 2015].

WHO 2014c. Global tuberculosis report. *In:* ORGANISATION, W. H. (ed.). France: WHO Press.

WIECHMANN, I. & GRUPE, G. 2004. Detection of *Yersinia pestis* DNA in two early medieval skeletal finds from Aschheim (Upper Bavaria, 6th century A.D.). *American Journal of Physical Anthropology,* 126**,** 48-55.

WILCOX, B. A. & GUBLER, D. J. 2005. Disease Ecology and the Global Emergence of Zoonotic Pathogens. *Environmental Health and Preventive Medicine,* 10**,** 263-272.

WILLIAMS, E. S. & BARKER, I. K. 2001. *Infectious Diseases of Wild Mammals,* Iowa State University Press.

WILLIAMS, E. S., YUILL, T., ARTOIS, M., FISCHER, J. & HAIGH, S. A. 2002. Emerging infectious diseases in wildlife. *Revue scientifique et technique de l'Office international des Epizooties* 21**,** 139-157.

WILLIAMS, R. H., MORLEY, E. K., HUGHES, J. M., DUNCANSON, P., TERRY, R. S., SMITH, J. E. & HIDE, G. 2005. High levels of congenital transmission of *Toxoplasma gondii* in longitudinal and cross-sectional studies on sheep farms provides evidence of vertical transmission in ovine hosts. *Parasitology,* 130**,** 301-307.

WOJCIECHOWSKA, B., MIKULSKA-SKUPIEŃ, E., PLATT-SAMORAJ, A., SZCZERBA-TUREK, A. & SZWEDA, W. 2010. Occurrence of *Yersinia enterocolitica* in canine excrements contaminating urban lawns. *Bulletin of the Veterinary Institute in Pulawy,* 54**,** 153-159.

WOLFF, J. O. & SHERMAN, P. W. 2007. *Rodent Societies: An Ecological & Evolutionary Perspective,* Chicago, The University of Chicago Press.

WOODFORD, M. H. 2009. Veterinary aspects of ecological monitoring: the natural history of emerging infectious diseases of humans, domestic animals and wildlife. *Tropical Animal Health Production,* 41**,** 1023-1033.

WOOLHOUSE, M. E. J. 2002. Population biology of emerging and re-emerging pathogens. *Trends in Microbiology,* 10**,** 3-7.

WORLD HEALTH ORGANISATION. 2003. *Human Leptospirosis: guidance for diagnosis, surveillance and control* [Online]. Malta. [Accessed 17/04/2012 2012].

WORLD HEALTH ORGANISATION. 2005. *Drug-resistant Salmonella* [Online]. [Accessed 03/10/2012.

WÖSTEN, M. S. M. M., WAGENAAR, J. A. & VAN PUTTEN, J. P. M. 2004. The FlgS/FlgR Two-component Signal Transduction System Regulates the *fla* Regulon in *Campylobacter jejuni*. *The Journal of Biological Chemistry,* 279**,** 16214-16222.

WRIGHT, R., JOHNSON, D. J., NEUMANN, M., KSIAZEK, G., ROLLIN, P., KEECH, R. V., BONTHIUS, D. J., HITCHON, P., GROSE, C. F., BELL, W. E. & BALE, J. F. 1997. Congenital Lymphocytic Choriomeningitis Virus Syndrome: A Disease That Mimics Congenital Toxoplasmosis or Cytomegalovirus Infection. *Pediatrics,* 100.

WU, W., ZHANG, S., QU, J., ZHANG, Q., LI, C., LI, J., JIN, C., LIANG, M. & LI, D. 2014. Simultaneous detection of IgG antibodies associated with viral hemorrhagic fever by a multiplexed Luminex-based immunoassay. *Virus Research,* 187**,** 84-90.

XU, W., BAK, S., DECKER, A., PAQUETTE, S. M., FEYEREISEN, R. & GALBRAITH, D. W. 2001. Microarray-based analysis of gene expression in very large families: the cytochrome P450 gene superfamily of *Arabidopsis thaliana*. *Gene,* 272**,** 61-74.

YIM, L., BETANCOR, L., MARTINEZ, A., GIOSSA, G., BRYANT, C., MASKELL, D. & CHABALGOITY, J. A. 2010. Differential Phenotypic Diversity among Epidemic-Spanning *Salmonella enterica* Serovar Enteritidis Isolates from Humans or Animals. *Applied and Environmental Microbiology,* 76**,** 6812-6820.

YOKOYAMA, E., MARUYAMA, S., KABEYA, H., HARA, S., SATA, S., KUROKI, T. & YAMAMOTO, T. 2007. Prevalence and Genetic Properties of *Salmonella enterica* Serovar Typhimurium Definitive Phage Type 104 Isolated from *Rattus norvegicus* and *Rattus rattus* House Rats in Yokohama City, Japan. *Applied and Environmental Microbiology,* 73**,** 2624-2630.

ZEIER, M., HANDERMANN, M., BAHR, U., RENSCH, B., MÜLLER, S., KEHM, R., MURANYI, W. & DARAI, G. 2005. New Ecological Aspects of Hantavirus Infection: A Change of A Paradigm and a Challenge of Prevention - A Review. *Virus Genes,* 30**,** 157-180.

ZELL, R., KRUMBHOLZ, A. & WUTZLER, P. 2008. Impact of global warming on viral diseases: what is the evidence? *Current Opinion in Biotechnology,* 19**,** 652-660.

ZHAN, L., HAN, Y., YANG, L., GENG, J., LI, Y., GAO, H., GUO, Z., FAN, W., LI, G., ZHANG, L., QIN, C., ZHOI, D. & F., Y. 2008. The Cyclic AMP Receptor Protein, CRP, Is Required for Both Virulence and Expression of the Minimal CRP Regulon in *Yersinia pestis* Biovar microtus. *Infection and Immunity,* 76**,** 5028-5037.

ZHOU, D. & YANG, R. 2009. Molecular Darwinian Evolution of Virulence in *Yersinia pestis*. *Infection and Immunity,* 77**,** 2242-2250.

ZIWA, M. H., MATEE, M. I., KILONZO, B. S. & B.M., H. O. 2013. Evidence of *Yersinia pestis* DNA in rodents in plague outbreak foci in Mbulu and Karatu Districts, northern Tanzania. *AJOL,* 15**,** 1-8.

APPENDIX

9.1. SOP





SOP for sample handling and shipment

March 2011

Authors:
Abu-Bakr A. K. Abu-Median
Paul Barrow
Charalambos Billinis
Dolores Gavier-Widén
Duncan Hannant
Richard Lea
Kitty Maassen
Eszter Papp
Liljana Petrovska
Lisa Yon

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Purpose and scope

This SOP was prepared in the framework of the WildTech EU FP7 project (contract number 222633).
The purpose of this SOP is to describe the shipment process of samples of tissues (including coagulated blood), liquid blood (+anticoagulant), serum, faeces, urine, saliva, vectors, cerebro-spinal fluid (CSF). The samples will be processed for nucleic acid (NA) or serology array.

Background

The Associate and Collaborative Partners and the WildTech consortium will cooperate in this process based on the bilateral agreements signed by the Associate and Collaborative Partners and the Co-ordinator of the WildTech project (University of Nottingham, School of Veterinary Medicine and Science).

Responsibilities[1]

Source Laboratory (Associate Partner (AP) / Collaborative Partner (CP) / Partner)

1. Enters details of each sample into the sample information sheet and sends a copy to the National Veterinary Institute in Sweden (SVA) to wildtechsamples@sva.se.
2. Awaits authorisation and sample barcodes from SVA (non-EU) or CERETETH (EU) on the input information.
3. Awaits packaging material and instructions from SVA (WP5).
4. Awaits packaging material and instructions from CERETETH (WP4).
5. Prepares the samples for shipment based on the instructions in 4.2.
6. Prepares necessary documentation including permits, dispatch and shipping documents based on the material and documents received from CERETETH/SVA and if necessary, from the Receiver.

SVA, Sweden (WP5 leader)

1. Asks for the samples from the relevant Source Labs of WP5.
2. Checks and authorises the sample information sheet sent by the Source Lab (both EU and non-EU).
3. Sends the barcode to the Source Labs of WP5 and to CERETETH for the samples of WP4.

[1] Based on WHO/CDS/EPR/2007.2 and requirements of WildTech.

4. Makes advance arrangements with the receiver of the WP5 samples including investigating the need for an import permit and CITES permit.
5. Makes arrangements with the carrier to ensure that the shipment (for the Source Labs of WP5):
   a. is paid for;
   b. will be accepted for appropriate transport;
   c. is undertaken by the most direct routing, avoiding arrival at weekends.
6. Sends appropriate instructions and packaging material to the Source Labs of WP5, including the Shipment Air Waybill filled in with all the details (payer's account number of SVA, receiver's details, etc.).
7. Notifies the receiver of transportation arrangements once these have been made, well in advance of expected arrival time.

Contact details: **Dolores Gavier-Widén**, DVM, MS, PhD, Associate Prof.
Head of R&D Division
Department of Pathology and Wildlife Diseases
National Veterinary Institute (SVA)
Sweden
Phone: +46 18 674215
Fax: +46 18 309162
E-mail: wildtechsamples@sva.se

CERETETH, Greece (WP4 leader)

1. Asks for the samples from the relevant Source Labs of WP4.
2. Sends the barcode to the Source Labs of WP4.
3. Makes advance arrangements with the receiver of the WP4 samples including investigating the need for an import permit and CITES permit.
4. Makes arrangements with the carrier to ensure that the shipment (for the Source Labs of WP4):
   a. is paid for;
   b. will be accepted for appropriate transport;
   c. is undertaken by the most direct routing, avoiding arrival at weekends.

(Only transportation directly arranged by CERETETH will be paid for.)

5. Sends appropriate instructions and packaging material to the Source Labs of WP4, including the Shipment Air Waybill filled in with all the details (payer's account number of CERETETH, receiver's details, etc.).
6. Notifies the receiver of transportation arrangements once these have been made, well in advance of expected arrival time.

Contact details: **Prof. Charalambos Billinis**
Dean
Faculty of Veterinary Medicine
University of Thessaly
Trikalon 224, 43100, Karditsa
GREECE

Phone: +302441066011, +302441066000
Fax: +302441066092

E-mail: billinis@vet.uth.gr, wildtech@cereteth.gr

Receiver (UNOTT, VLA, IAH, CVI, FLI, CERETETH)

1. Obtains the necessary authorisation(s) from national authorities for the importation of the material.
2. Provides the sender with the required import permit(s), letter(s) of authorisation, or other document(s) required by the national authorities.
3. Arranges for the most timely and efficient collection on arrival.
4. Immediately acknowledges receipt to the Source Lab and CERETETH/SVA.

Contact details:

UNOTT: Prof. Paul Barrow
School of Veterinary Medicine and Science
University of Nottingham, Sutton Bonington Campus
UK
Phone: +44 115 951 6428
Fax: +44 115 951 6440
E-mail: paul.barrow@nottingham.ac.uk

VLA: Prof. Martin Woodward
c/o Dr. Liljana Petrovska
Veterinary Laboratories Agency, Weybridge
UK
Phone: +44 1932 341111
Fax: +44 1932 347046
E-mail: m.j.woodward@vla.defra.gsi.gov.uk
l.petrovska@vla.defra.gsi.gov.uk

IAH: Prof. Peter Mertens
Institute for Animal Health, Ash Road, Pirbright, Woking, UK
Phone: +44 1483 231189
Fax: +44 1483 323448
E-mail: peter.mertens@bbsrc.ac.uk

CVI: Dr. C.B.M. (Kitty) Maassen
Central Veterinary Institute of Wageningen UR
Edelhertweg 15, 8219 PH Lelystad
The Netherlands
Phone: +31 320 23 80 84
Fax: +31 320 23 89 61
E-mail: kitty.maassen@wur.nl

FLI: Dr. Konrad Sachse
Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Institute of Molecular Pathogenesis,
Naumburger Str. 96a, 07743 Jena, Germany
Phone: +49-3641-804 2334

Fax: +49-3641-804 2228
E-mail konrad.sachse@fli.bund.de

CERETETH: Prof. Charalambos Billinis
Dean
Faculty of Veterinary Medicine
University of Thessaly
Trikalon 224, 43100, Karditsa
GREECE

Phone: +302441066011, +302441066000
Fax: +302441066092
E-mail: billinis@vet.uth.gr, wildtech@cereteth.gr

Shipping process

Preparatory activities

Identification of sample need

- The WildTech Co-ordinator's Management Group (CMG) identifies the need for the samples from the Partner, Associate or Collaborative Partner based on information from WP2 and 3 Partners and CERETETH (WP6 PhD projects) about the need for samples from certain species or with certain pathogens.
- The leader of the Wildlife Team, Lisa Yon, informs the relevant WP leader (WP4 Leader CERETETH or WP5 leader SVA) to request the detailed sample information from the Source Lab.
- The Receiver is identified on a case by case basis depending on the pathogen(s), species and the infectivity of the sample.

Sample request

- The WP4 and 5 leaders request the sample from the Source Lab and send them the sample information sheet to be filled in for each sample.
- The Source Lab sends the filled-in sample information sheet to SVA, Sweden to the following email address: wildtechsamples@sva.se.
- SVA checks the data and authorises the shipment or asks for corrections. In case of authorisation it also obtains the barcodes for the shipment.
- SVA informs CERETETH of the authorised shipment from WP4 countries and the barcodes progress via the following email address: wildtech@cereteth.gr and sends the barcodes by mail.

Shipment preparation

- CERETETH (for WP4 samples) and SVA (for WP5 samples) organise the shipment with the carrier, including timing and payment.
- CERETETH and SVA send to the Source Lab the instructions and the packaging material and documents, including the Shipment Air Waybill filled in with all the details.

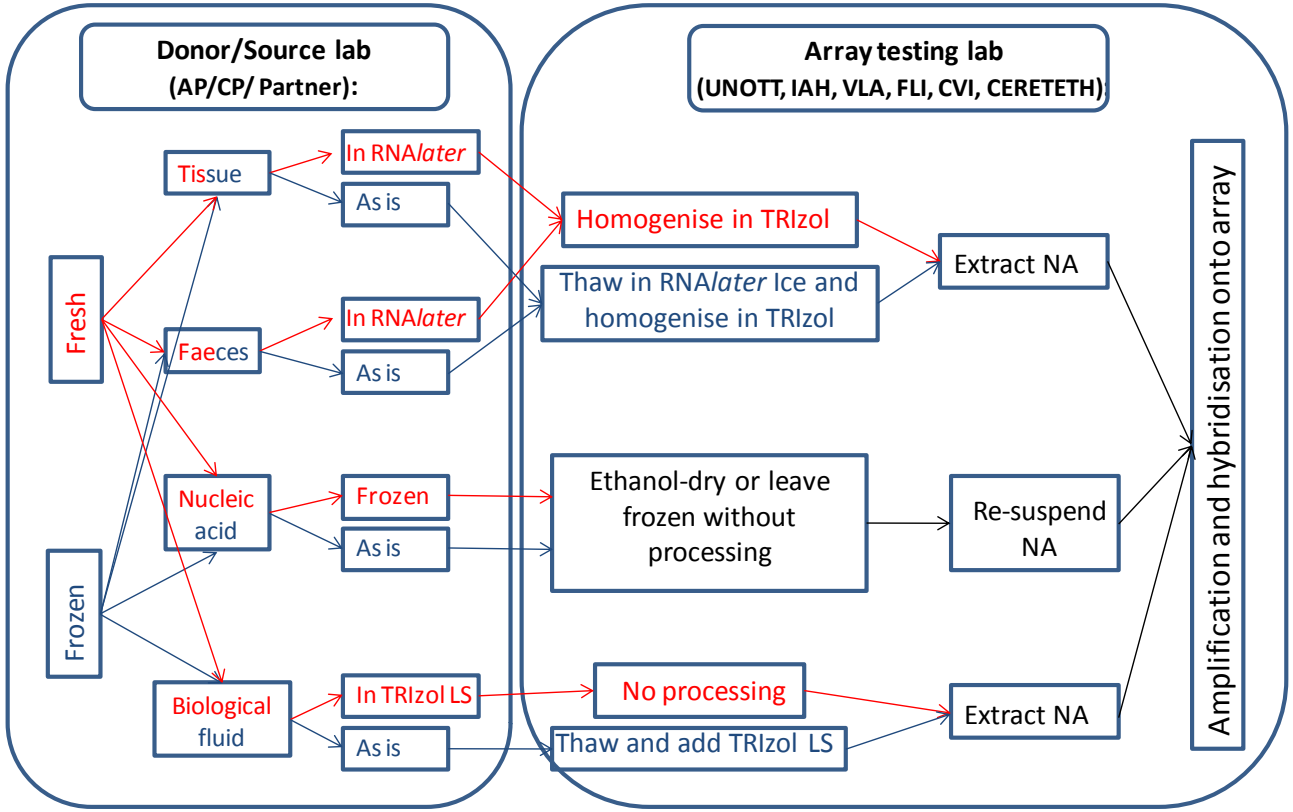
Sample preparation

Samples need to be taken and processed for transport in the Source Laboratory.

After obtaining the sample to send to WildTech, the rest of the material should be identified with the barcode stickers and saved again in the freezer bank of the Source Lab.

Samples for NA analysis

Handling and processing of samples for NA array testing



1. Samples may be taken from frozen archived material or from opportunistic (fresh) samples that were collected for other purposes (i.e. not collected for the purpose of the WildTech project).
2. Samples should be taken using the appropriate SOP developed for use in each laboratory.
    - Where possible samples should be handled in a safety cabinet (Class 1, Class 2 or Class 3, as appropriate) and in biohazard level room depending on the infectious agents.
    - Samples have to be cut or picked with new disposable scalpel blades or sterile instruments/pipettes to avoid cross-contamination between samples.

Sample weights and volumes:

At the Source Laboratory:

- When a fresh tissue is collected in the field or following experimental infection:

- Aliquot a suitable volume of RNA*later®* Tissue Collection solution in sterile screw-capped containers.
- Dissect and slice the tissue less than 0.5 cm in any one dimension.
- Submerge the sliced tissue in approximately 5 volumes of RNA*later* (e.g., a 0.5 g sample requires about 2.5 ml of RNA*later*) already in the containers at room temperature.

- A fresh faecal sample (0.2 g) should be suspended in 1 ml RNA*later* pre-aliquoted in sterile screw-capped containers.

- Biological Fluids (including liquid blood, urine, CSF, saliva) when fresh, a volume of 0.25 ml should be mixed with 0.75ml TRIzol LS reagent in a screw top microcentrifuge tube. Whole blood may be diluted 1:1 with water prior to mixing with TRIzol LS. For homogenisation, pass the suspension several times through a pipette.

- ***Note:*** *Fresh tissue, faeces or biological fluids (above) should be shipped at 2-8˚C. All frozen/archived samples should be shipped as they are on dry ice or with cold packs (subject to assessment).*

- Pure nucleic acid (50 µl minimum at 50 ng/µl minimum) should be aliquoted in sterile screw-capped microcentrifuge tubes and shipped on dry ice.

At the Receiver (Array Testing) Lab:

- A fragment of the fresh tissue sample weighing 50-100 mg should be cut out from the sample in RNA*later* and transferred into a small plastic homogenisation/safelock tube containing 1 ml TRIzol and homogenised immediately using an appropriate homogeniser. Any remaining material in RNA*later* must be stored at -20˚C. A frozen tissue sample should be thawed in RNA*later* ice and then processed as a fresh sample.

- A fresh faecal sample received in RNA*later* should be centrifuged and the pellet re-suspended in TRIzol and homogenised. A frozen faecal sample is treated similar to a frozen tissue sample.

- Fresh biological fluids should remain stored in TRIzol LS as received from source without further processing. Frozen samples should be thawed and thoroughly mixed (vortexed briefly), then mixed with TRIzol LS and homogenised as described for a fresh biological fluid sample.

- NA should be extracted from homogenates above using appropriate protocols.

Samples for serology analysis at VLA

1. Samples should be taken using the appropriate SOP developed for use in each laboratory/centre.
    - Where possible samples should be handled in a safety cabinet (Class 1, Class 2 or Class 3, as appropriate), and in biohazard level room depending on the infectious agents.

2. Samples are received as serum or whole blood collected without anticoagulant

    - Serum samples should be sent in the tubes provided with the packaging materials. The remaining serum should be identified with the barcode sticker and saved in the serum bank in the freezer.
    - Where possible, separate serum from coagulated blood before storing.
    - Collected samples should be kept frozen.

3. It is preferable the samples were taken from live animals. Where not possible, post-mortem blood and body fluids from dead animals may also be used.

4. Samples may be taken from frozen archived material or from opportunistic (fresh) samples that were collected for other purposes (i.e. not collected for the purpose of the WildTech project).

5. Sample volumes:
    - Whole blood samples: minimum volume of 0.2 ml
    - Serum and other liquid samples (including urine, CSF, saliva): a volume of 0.2 ml in a screw top Eppendorf tube.
    - These are recommended volumes but lower volumes may also be tested

Sample packaging

CERETETH and SVA will send detailed packaging instructions and the necessary documentation for each shipment (see annex 6).

Shipment type and category *(category 2/3; SAPO; UN packaging categories) will be specified on a case by case basis in collaboration with the Source Laboratory and the*

Receiver, depending on the infectivity of the material and the pathogen(s). Unless otherwise stated, shipments will usually belong to UN packaging Category B.

No more than 50 filled Eppendorf tubes should be packed together in one box (but several boxes can be sent together in one shipment). The tubes should have absorbent material wrapped around each, enough to absorb the whole volume of liquid in case all of it leaks, and placed in a clear impermeable plastic bag which is tied at the neck. This should then be placed within a second plastic bag so that the tubes are "double-bagged".
The bag should be placed in a hard screw-cap plastic transport container which must meet UN class 6.2 specifications and packaging instruction (PI)602. For examples see Annex.

The hard transport container should be surrounded by frozen cold packs (i.e. not dry ice [frozen carbon dioxide]) in a polystyrene container normally used for cooled chemical transport.

The outer shipping package must be marked infectious and bear the UN Packaging Specification Marking.

- Category A: An infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals.
    - UN 2814: Infectious substances which cause disease in humans or both in humans and animals
    - UN 2900: Infectious substances which cause disease only in animals
- Category B: An infectious substance which does not meet the criteria for inclusion in Category A.
    - UN 3373: all samples

Note: UN-approved packaging supplier listings may be obtained from carriers or from the appropriate national ministry or department, e.g. the Ministry of Transport, etc.

All packages must be accompanied by appropriate documentation that is fixed to the outer package. These are:

- The shippers' Declaration of Dangerous Goods.
- A packing list/proforma invoice which includes the receiver's address, the number of packages, detail of contents, weight, value (note: state that there is "no commercial value" as the items are supplied free of charge).
- An import and/or export permit and/or declaration if required.

See Annex for further detail.

All infectious goods must have a specific hazard label(s) and an International Infectious Substance label affixed to the outside of each package.

Address labels will be pre-filled by CERETETH/SVA and must have:

- the receiver's name, address and telephone number;
- the shipper's name, address and telephone number;

- if applicable: the UN shipping name (Infectious Substances Affecting Humans or Animals as the case may be) followed by the scientific name of the substance;
- if applicable: appropriate UN Number (see above);
- storage and shipment temperature requirements
    - serum storage prior to shipment: -20°C
    - sample in TRIzol storage prior to shipment: 2-8°C
    - all samples during shipment: variable/as instructed
    - serum samples after arrival: freeze at -80°C
    - samples in TRIzol after arrival: 2-8°C

References

http://www.unpackaging.com/un_cert.htm#plastic
WHO/CDS/EPR/2007.2.
http://www.coolmybeer.co.uk/communities/6/004/006/150/936/images/4517753315.jpg
http://www.caa.co.uk/docs/33/CAP675.PDF
WHO/EMC/97.3.
http://www.cites.org/eng/app/index.shtml

Annex

Pre-shipment checklist for the Source Laboratories

1. Sample information sheet is completed by the Source Lab.
2. Sample information sheet is emailed to SVA to: wildtechsamples@sva.se.
3. Approval to send the samples is obtained from SVA.
4. Bar codes stickers to identify the samples are received from SVA/CERETETH.
5. Sample-preparation and packaging materials are received from SVA/CERETETH.
6. Import permits and other transportation documents are received.

List of sample packaging material

1. Eppendorf Tubes
2. Absorbent material
3. Plastic bag (a watertight, leak-proof and impermeable receptacle)
4. Plastic bag (a second watertight, leak-proof plastic bag)
5. Hard screw-cap plastic transport container
6. Outer packaging
7. TRIzol
8. RNA*later*
9. Scalpel blades
10. Labels

Packaging, marking and labelling infectious substances for transport

Because of the differences in the hazards posed by category A and category B substances, there are variations in the packaging, labelling and documentation requirements for the two categories. Packaging requirements are determined by the UN and are contained in ICAO and IATA regulations in the form of Packaging Instruction (PI) *P602* (*620*) and *P650*. Currently category A substances may only be transported in packaging that meets the United Nations class *6.2* specifications and complies with Packing Instruction *P620* (PI602 for the air mode) whilst category B substances should be shipped in *P650* packaging as a minimum requirement. Both of these require a triple packaging system.

Basic Triple Packaging System

This system shall be used for all infectious substances. It consists of three layers as follows.

i. Primary receptacle. A primary watertight, leak-proof receptacle containing the specimen. The receptacle is packaged with enough absorbent material to absorb all fluid in case of breakage.
ii. Secondary packaging. A second durable, watertight, leak-proof packaging to enclose and protect the primary receptacle(s). Several cushioned primary receptacles may be placed in one secondary packaging, but sufficient additional absorbent material shall be used to absorb all fluid in case of breakage.
iii. Outer packaging. Secondary packagings are placed in outer shipping packagings with suitable cushioning material. Outer packagings protect their contents from outside influences, such as physical damage, while in transit. The smallest overall external dimension shall be 10x10 cm.

NOTE 1: Cold packs must be placed outside the secondary receptacle.

NOTE 2: The correct process of the samples can be achieved by removing 2mm in diameter from the samples and transfer it to a screw-cap1ml Eppendorf tube containing 500uL Trizol. Each package should not contain more than 50 filled Eppendorf tubes.

Each completed package is normally required to be correctly marked, labeled and accompanied with appropriate shipping documents (as applicable). The requirements for these aspects are described below.

Category A, packaging

Infectious substances must be shipped using the UN 602 (620) Packaging Instructions (Fig 1). This involves the use of the basic triple packaging with additional labelling and documentation requirements. The outer shipping package must bear the UN Packaging Specification Marking (Fig 2). UN-approved packaging supplier listings may be obtained from carriers or from an appropriate national ministry, e.g. the Ministry of Transport.

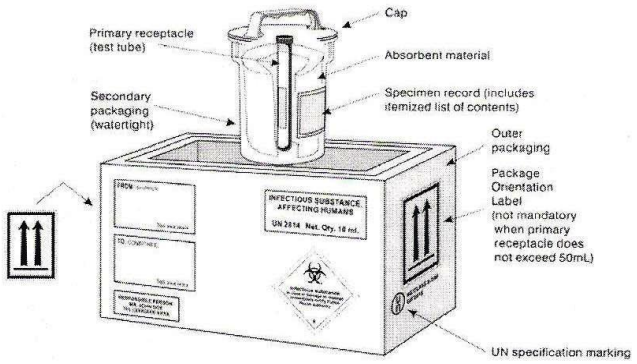
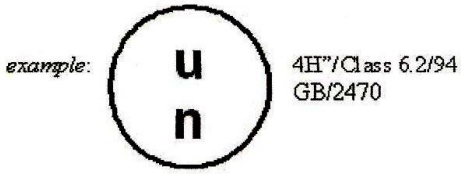


Figure 1. Example of triple packaging system for the packaging and labelling of Category A infectious substances (Figure kindly provided by IATA, Montreal, Canada)

Figure 2

Packaging Specification Marking



This example shows one bid test tube instead of 50 small, but it is a similar triple packaging system.

The marking consists of:

1. the United Nations packaging symbol.
2. the type of packing (4H)
3. the United Nations class specification (Class 6.2)
4. the last two digits of the year of manufacture (1994)
5. State authority (GB, Great Britain)
6. the manufacturer's code (2470)

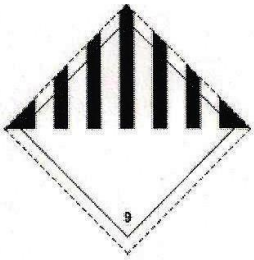
Category A, labeling



Label name:	Infectious substance
Minimum dimensions:	100 × 100 mm
(for small packages:	50 × 50 mm)
No. of labels per package:	1
Colour:	Black and white

The words "INFECTIOUS SUBSTANCE" shall be shown. The statement "In case of damage or leakage immediately notify a Public Health Authority" is required in some countries.

Figure 3. Hazard label for Category A infectious substances and for genetically modified microorganisms and organisms that meet the definition of an infectious substance, Category A



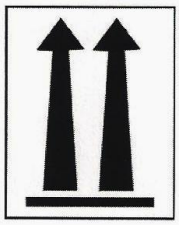
Label name:	Miscellaneous dangerous substances
Minimum dimensions:	100 × 100 mm
(for small packages:	50 × 50 mm)
No. of labels per package:	1
Colour:	Black and white

Figure 4. Hazard label for certain noninfectious genetically modified microorganisms and organisms (UN3245) and for carbon dioxide, solid (dry ice) (UN1845).



Label name:	Non flammable, non-toxic gas
Minimum dimensions:	100 × 100 mm
(for small packages:	50 × 50 mm)
No. of labels per package:	1
Colour:	Green and white or green and black

Figure 5. Hazard label for liquid nitrogen; substances packed using liquid nitrogen (see section on Refrigerants) shall bear this label in addition to the primary risk label (e.g. the label shown in Figure 3



Label name:	Orientation label
Minimum dimensions:	Standard A7: 74 × 105 mm
No. per package:	2 on opposite sides
Colour:	Black and white or red and white

The words "THIS SIDE UP" or "THIS END UP" may also be displayed on the top cover of the package.

Figure 6. Orientation label to indicate position of closures on the primary receptacles.

Category B, packaging

Packaging Instruction P650 as well as the basic triple packaging system is required for the preparation and transportation of Category B.

For Liquids:

- Either the primary or the secondary receptacle must be capable of withstanding, without leakage an internal pressure producing a pressure differential of not less that 95kPa.
- Primary and secondary container must be leak proof.
- Primary container must not contain more than 1L.
- Outer packaging must not contain more than 4 liters.
- Absorbent material sufficient to absorb entire contents to be contained in the secondary packaging
- If multiple receptacles are placed in a single secondary packaging, they must be either individually wrapped or separated to prevent contact between them.

For Solids:

- Primary and secondary packaging must be sift proof.
- Primary container must not exceed the outer packaging weight limit.
- Outer packaging must not contain more than 4kg.

- Cushioning material sufficient to protect primary container to be contained in the secondary packaging and sufficient absorbent material must be used.
- If multiple primary receptacles are placed in a single secondary packaging, they must be either individually wrapped or separated to prevent contact between them.

For Liquids and Solids:

- An itemized list of contents must be enclosed between the secondary packaging and the outer packaging.
- At least one surface of the outer packaging must have a minimum dimension of 100mm x 100mm.

Category B, labelling

For transport the UN3373 mark must be displayed on the external surface of the outer packaging and must be clearly visible and legible (Fig 7).

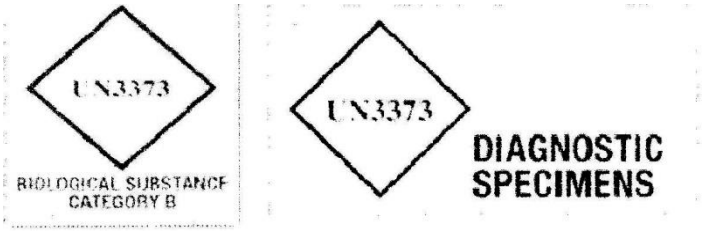


Figure 7.

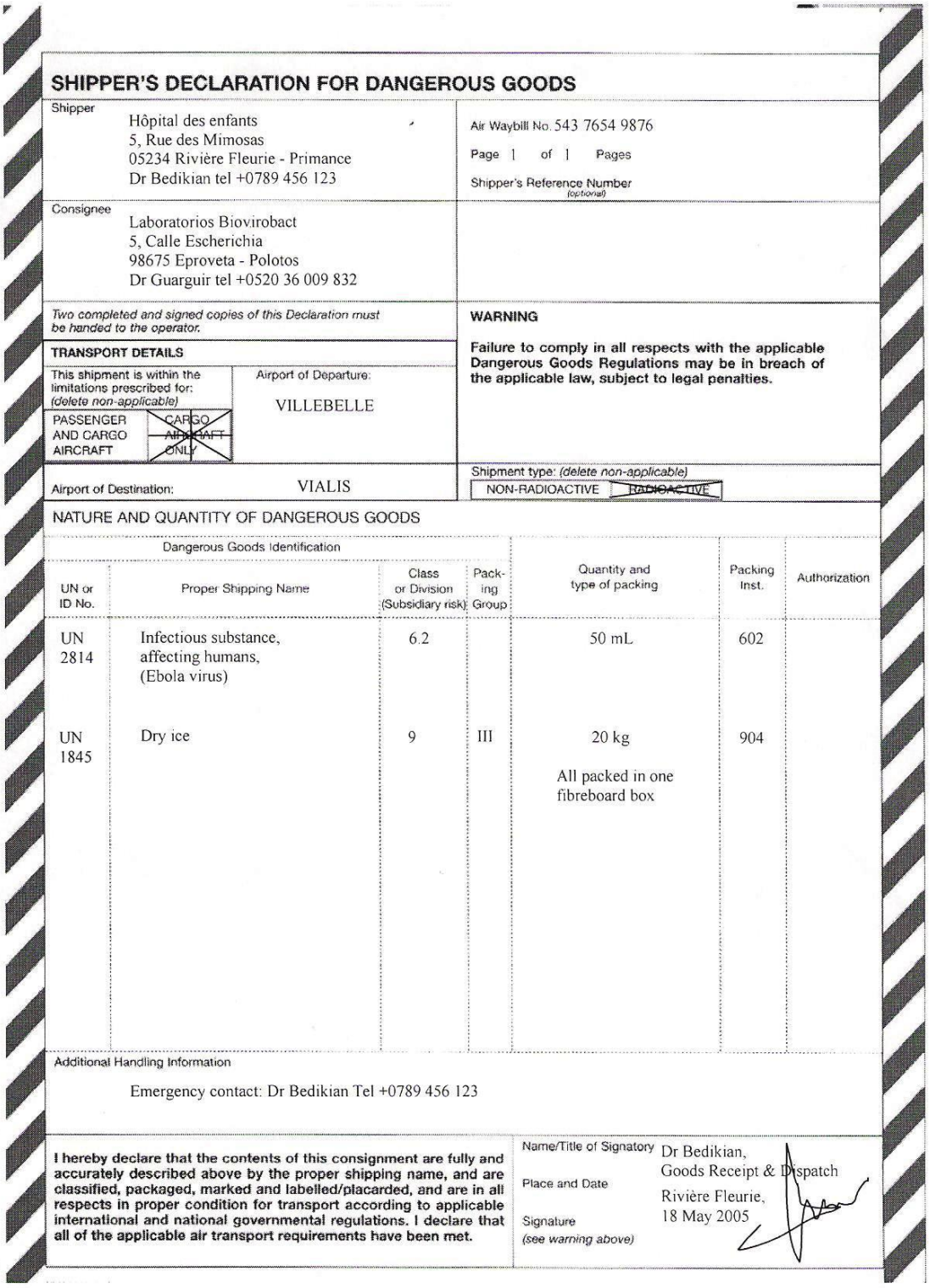
Marking *(Categories A and B)*

Each package shall display the following information on the outer packaging or the overpack.

- The shipper's (consignor's) name and address.
- The receiver's (consignee's) name and address.
- The United Nations number followed by the proper shipping name (UN 2814 "INFECTIOUS SUBSTANCES AFFECTING HUMANS" or UN 2900 "INFECTIOUS SUBSTANCES AFFECTING ANIMALS" or UN 3373 "BIOLOGICAL SUBSTANCE CATEGORY B", as appropriate).
- Temperature storage requirements (optional).

Documentation *(Categories A and B)*

- For air the shipper's Declaration for Dangerous Goods (Fig 8).The shipper's Declaration for Dangerous Goods is required only for category A.
- A packing list/proforma invoice that includes the receiver's address, the number of packages, detail of contents, weight, value,
- An import and/or export permit and/or declaration if required.
- An air waybill for air transport or equivalent documents for road, rail and sea journeys.



SHIPPER'S DECLARATION FOR DANGEROUS GOODS

Shipper
Hôpital des enfants
5, Rue des Mimosas
05234 Rivière Fleurie - Primance
Dr Bedikian tel +0789 456 123

Air Waybill No. 543 7654 9876
Page 1 of 1 Pages
Shipper's Reference Number (optional)

Consignee
Laboratorios Biovirobact
5, Calle Escherichia
98675 Eproveta - Polotos
Dr Guarguir tel +0520 36 009 832

Two completed and signed copies of this Declaration must be handed to the operator.

WARNING

Failure to comply in all respects with the applicable Dangerous Goods Regulations may be in breach of the applicable law, subject to legal penalties.

TRANSPORT DETAILS

This shipment is within the limitations prescribed for: *(delete non-applicable)*
PASSENGER AND CARGO AIRCRAFT ~~CARGO AIRCRAFT ONLY~~

Airport of Departure: VILLEBELLE

Airport of Destination: VIALIS

Shipment type: *(delete non-applicable)*
NON-RADIOACTIVE ~~RADIOACTIVE~~

NATURE AND QUANTITY OF DANGEROUS GOODS

Dangerous Goods Identification						
UN or ID No.	Proper Shipping Name	Class or Division (Subsidiary risk)	Packing Group	Quantity and type of packing	Packing Inst.	Authorization
UN 2814	Infectious substance, affecting humans, (Ebola virus)	6.2		50 mL	602	
UN 1845	Dry ice	9	III	20 kg All packed in one fibreboard box	904	

Additional Handling Information

Emergency contact: Dr Bedikian Tel +0789 456 123

I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name, and are classified, packaged, marked and labelled/placarded, and are in all respects in proper condition for transport according to applicable international and national governmental regulations. I declare that all of the applicable air transport requirements have been met.

Name/Title of Signatory: Dr Bedikian, Goods Receipt & Dispatch
Place and Date: Rivière Fleurie, 18 May 2005
Signature *(see warning above)*

Figure 8. Declaration for Dangerous Goods

Emergency response procedures (category A and B)

The following guidance is primarily of use by operators in the event that a package containing infectious substances (of either Category A or B) is involved in an incident resulting in spillage.

Mitigation procedures:

1. Isolate spill or leak area immediately in all directions.
2. Keep unauthorised personnel away.
3. Obtain identity of substance involved if possible and report the spill to the appropriate authorities.
4. Do not touch or walk through spilled material.
5. Do not touch damaged containers or spilled material unless wearing appropriate protective clothing.
6. Be particularly careful to avoid contact with broken glass or sharp objects that may cause cuts or abrasions that could significantly increase the risk of exposure.
7. Damaged packages containing solid CO2 as a refrigerant may produce water or frost from condensation of air. Do not touch this liquid as it could be contaminated by the contents of the package.
8. Liquid nitrogen may be present and can cause severe burns.
9. Absorb spilled materials with earth, sand or other non-combustible material while avoiding direct contact.
10. Cover damaged package or spilled material with damp towel or rag and keep wet with liquid bleach or other disinfectant. Liquid bleach will generally effectively inactivate the released substance.

CITES permits

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists endangered animals species into 3 categories (appendix I, II and III) according to the level or type of protection from over-exploitation needed for the species. It is not anticipated that Wild Tech will work on material from CITES-listed species. However, should such material become available and of interest for the project, the necessary CITES permits will be obtained.

References:

Guidance on regulations for the Transport of Infectious Substances 2009–2010 Applicable (WHO)
IATA Guidance Document: Infectious Substances

Table 9.1.Oligonucleotide probe sequence information.
1: Yersinia_01 ArrayTube
2: Toxoplasma_01 ArrayTube
3: Hanta_HEV_01 ArrayTube
4: WT_Rodent_Chip_02 ArrayStrip
5: WT_Rodent_Chip_03 ArrayStrip

Probe ID	Probe Sequence	Array	Family	Genus	Species	Derived from
BartonellaUPS_455	AATGCGACACGTCCGCAGTTTAGCCGTTGTTCTCAGCAGTCTATGGCCGCATCAGTAAGT	4,5	Bartonellaceae	Bartonella	*B.quintana*	ftsZ
BartonellaUPS_456	TGTTTATGTTCCACGTCGTTCTGGTGAGTTGCATCCCCATGTAGCACAAGATCAACGTAC	4,5	Bartonellaceae	Bartonella	*B.quintana*	ftsZ
BartonellaUPS_458	GGAAATGCAACAGATGCGGGCTCGTTCAGCACCGATGCGTATGCCTGAGTTAAAGGATTT	4,5	Bartonellaceae	Bartonella	*B.quintana*	ftsZ
BartonellaUPS_459	TATTCGTGTTTCTGTGGTTGCAACAGGAATCGATCGTGAGGTTAATGATGTGGTTCAGCC	4,5	Bartonellaceae	Bartonella	*B.quintana*	ftsZ
BartonellaUPS_460	AGAACTGAACATTCAAGATGATCCGCTTCTTGATATCGCTATCGCACTTGAAAACATTGC	4,5	Bartonellaceae	Bartonella	*B.quintana*	gltA
BartonellaUPS_461	TGATCCTTTCCGTCTTATGGGTTTTGGTCACCGAGTTTATAAAAATTATGATCCACGTGC	4,5	Bartonellaceae	Bartonella	Multiple	gltA
BartonellaUPS_462	TTTATTCTGGCATTACATTAAAAGCTCTAGGTTTTCCGACCGAAATGTTTACTGTTCTTT	4,5	Bartonellaceae	Bartonella	Multiple	gltA
BartonellaUPS_464	ATCATGCAACAAACCTGCCATGAAGTTTTAAAAGAACTGAACATTCAAGATGATCCGCTT	4,5	Bartonellaceae	Bartonella	Multiple	gltA
BartonellaUPS_465	TTCTTCAGATGATGATCCCAAGCCTTCTGGCGATCTAGACAAAACAAGTCCACCGTGGGC	4,5	Bartonellaceae	Bartonella	Multiple	16S-23S intergenic spacer
BartonellaUPS_466	AGCTGGGAGAGCACCTGCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGTCCGGCTCCA	4,5	Bartonellaceae	Bartonella	Multiple	16S-23S intergenic spacer
BartonellaUPS_467	GTAGGGGACCTGTGGCTGGATCACCTCCTTTCTAAGGATGATCAAGAATTGGAACTCCCT	4,5	Bartonellaceae	Bartonella	Multiple	16S-23S intergenic spacer
BartonellaUPS_468	AAGAGGCCCCTACCTCTGAAAGAGTGAAGGCGCCCTTTAAGGTGCAGTGCTCATGTGTTT	4,5	Bartonellaceae	Bartonella	*B. hensalae*	16S-23S intergenic spacer
BartonellaUPS_469	GCGTTTTATAAGAGGATGCCGGGGAAGGTTTTCCGGTTTATCCCGGAGGGCTTGTAGCTC	4,5	Bartonellaceae	Bartonella	Multiple	16S-23S intergenic spacer

BartonellaUPS_470	GGTGAAGACGCTGAAATTACGACATATCTTGAAAAAGCGATGACATCTGAATTACAGGGA	4,5	Bartonellaceae	Bartonella	Multiple	NADH
BartonellaUPS_471	ACGGTGTATTCACTGTACACGTTGTATCCGTTTTACAACAGAAGTTGCAGGTATTTCAGA	4,5	Bartonellaceae	Bartonella	Multiple	NADH
Campylo_Owiz_472	AATATTCCAATACCAACATTAGTGTGCGATGGAAGGACGCTTAGGGCTAAGGGGGCTA	4	Campylobacteraceae	Campylobacter	*Campylobacter*	23srRNA
Campylo_Owiz_473	CTAAGTTTAGCTAATGTTGCCCGTACCGTAAACCGACACAGGTGGGTGGGATGAGTATTC	4	Campylobacteraceae	Campylobacter	*Campylobacter*	23srRNA
Campylo_Owiz_474	GTAAACCGACACAGGTGGGTGGGATGAGTATTCTAAGGCGCGTGGAAGAACTCTCTTTA	4	Campylobacteraceae	Campylobacter	*Campylobacter*	23srRNA
Campylo_Owiz_475	TTTGAAAAATTTGATAGTGATTATGTTTTTGGATGGCACAATATGCCTTTTGGTAGCGAT	4,5	Campylobacteraceae	Campylobacter	*Campylobacter*	23srRNA
Campylo_Owiz_476	TTTTGGATGGCACAATATGCCTTTTGGTAGCGATAAGAAATTTTATCTTAAAAAAGGTGC	4, 5	Campylobacteraceae	Campylobacter	*C. jejuni*	23srRNA
Campylo_Owiz_477	AACCAATACTTGTCTTAATGCTTATGGTAATGGAATTGTCCATTATCCTTGTGATGCAAG	4	Campylobacteraceae	Campylobacter	*C. jejuni*	cdtA
Campylo_Owiz_478	CGCACAAATGTGGAAACTTATCCCTATGAGCAATACAGCGGTTCAAATTAAAAATTTA	4	Campylobacteraceae	Campylobacter	*C. jejuni*	cdtA
Campylo_Owiz_479	TCAAGTGGTTCAACACTTTCTCAGTTTGCAACTATGAAAACAACTGCTTTTGGAGTAA	4	Campylobacteraceae	Campylobacter	*C. jejuni*	flaA
Campylo_Owiz_480	ATATTTTAATCAGCGGTAGCAATCTTTCTTCTGCAGGTTTTGGTGCAACTCAATTTAT	4, 5	Campylobacteraceae	Campylobacter	*C. jejuni*	hipO
Campylo_Owiz_481	AGAAGAGTGTGAAAGTGCCATTAACTCTTATAATTTCACTTCTCAAATAGACTTTGAAA	4, 5	Campylobacteraceae	Campylobacter	*C. jejuni*	cdtA
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Campylo_Owiz_483	GAAATTTTACCACTCGCCAAAGTGCCCCAAAATGCTATGATATGAACGCTAGTATTTA	4, 5	Campylobacteraceae	Campylobacter	*C. jejuni*	cdtA
Campylo_Owiz_484	TAATTCCAATCTCATCACCGCAGTTCCTGCTAGACGTAATCCTTATTTTAACCTCGTAGA	4, 5	Campylobacteraceae	Campylobacter	*C. jejuni*	cdtA
Campylo_Owiz_485	CGGAATTTACTTCCATTATGATGGTATCAGAGCTTCCTATCGGGGAATTAAAAGAAGT	4, 5	Campylobacteraceae	Campylobacter	*C. jejuni*	tonB1
Campylo_Owiz_486	ATCAGAGCTTCCTATCGGGGAATTAAAAGAAGTTTCGATTGATCAAAAAAAGAGTAATTC	4	Campylobacteraceae	Campylobacter	*C. jejuni*	tonB1
Campylo_Owiz_487	AGAAGAGTGTGAAAGTGCCATTAACTCTTATAATTTCACTTCTCAAATAGACTTTGAAA	5	Campylobacteraceae	Campylobacter	*C. jejuni*	FlgS
Campylo_Owiz_488	GGGTAGTGTAGCGGCTCATTTGGCACATGAGATAAGAAATCCTATTGGATCTATATCT	5	Campylobacteraceae	Campylobacter	*C. jejuni*	FlgS
Campylo_Owiz_489	TAAGCATATAGTTCTTGAAATCCAAAAGGCTATTGCTAGAGTGGAGCGTATCGTAAAT	5	Campylobacteraceae	Campylobacter	*C. jejuni*	FlgS

Cowpox_495	CAGGTCGGTGTCGGAGATTCGAATCTCTTTGAGACGACTTATGTCATGACTGCATCTTTC	4	Poxviridae	Orthopoxvirus	CPXV	unknown
Cowpox_Owiz_496	TGTTCAAACTTTGTTGTTACATTAGTAATCTTTTTTTCCAAATTAGTTAGTCGTTGTTTG	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_497	GCCGTTACACACTTACCTTCAAGAGCATACCAAACATAGTCCTCGTGTGGTGTATGCTCT	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_498	GACTCCTCTTCATGCCGCCTTCCAGAATTGTAACAACAAGGTTGCCTCATACCTCGTATA	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_499	GATGCAGTCATGACATAAGTCGTCTCAAAGAGATTCGAATCTCCGACACCGACCTGTATA	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_500	ATAGCCTACTGCGTATTAAGGGACGAGTCATTCGCGGAACTGCATCGTAAATTCTGTTTA	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_811	ATACCTACGGCGTACAAGACGCCTGATGAGGATTTTCTAGGAACAGATGGAGGCAGATTT	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_812	GATGTGGCTGAACAGATACCTGCTACGGGCATTGTCATCTTTAAAACTCTCCACTTTCCA	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_813	TTACAACGGCAAGTCCAGCATTCGCTCTGGGATATACAGTAGAAGGTTCCTTCTTCCAAA	4	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_814	TCTTCGAAGAAACCGGGTGGAGGATAATCATTTGCAAACGATGCAACAAATAATGATGCG	4	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_815	AAAGCGAGCTGAATCAGTATCCGTATCTGAATGCGGTTCAGCAGTCCCACCTATAAGGTA	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_816	TAGTGCATCCATGGCTTCATCTTCTGATTTTGTGTCGAGGTCGTCGTTTTCAAATATGGA	4, 5	Poxviridae	Orthopoxvirus	CPXV	unknown
CowpoxUPS_821	ATGAGTGCTTGGTATAAGGAGCCCAATTCCATTATTCTCTTGGCTGCTAAAAGTGATGTC	4, 5	Poxviridae	Orthopoxvirus	CPXV	HA gene
CowpoxUPS_822	ACCTGATTATATAGATAATTCTAATTGCTCGTCGGTATTCGAAATCGCGACTCCGGGACC	4, 5	Poxviridae	Orthopoxvirus	CPXV	HA gene
CowpoxUPS_823	TCCACAACAGACGAGACTCCGGAATCAATTACTGATAATGAAGAAGATCACGCTGTCTCA	4, 5	Poxviridae	Orthopoxvirus	CPXV	HA gene
CowpoxUPS_824	CATTGACTTCTGAAGATGCCGGTACTTATGTATGTGCATTCTTTATGACAAATGACACCA	5, 5	Poxviridae	Orthopoxvirus	Cowpox	HA gene
CowpoxUPS_825	ATCGACTATAGACGTAATACTATCTGGATCTACACATTCACCGGAAACTAGTTCTGAGAA	5	Poxviridae	Orthopoxvirus	Cowpox	HA gene
Ctrl_097	GGTGCGTGTGAGCGTGTTGCTTTTGTCTCTAAGGACATGAGCTTGATCATATTGCCGGTC	3	Reoviridae	Orbivirus	Bluetongue virus	Bluetongue virus 16 isolate IND2010/cattle/16 segment 8, complete sequence
Ctrl_098	GTTGCAAAGCAGATTAAATTGAAGGATGAACGGTTTATGAGTCTGTCAAGCGCTATGCCT	3	Reoviridae	Orbivirus	Bluetongue virus	Bluetongue virus strain USA2010 segment 8, complete sequence

Ctrl_099	AGCGCTATGCCTCAAGCGAGTGGTGGATTTGATCGTATGATTGTTACGAAGAAGCTTAAG	3	Reoviridae	Orbivirus	Bluetongue virus	Bluetongue virus 10 isolate IND2004/01 segment 8 NS2 gene, complete cds
Ctrl_100	TGTTTTGATGAGTCATCGAAAAGGTATGAGTTACAGTGTGTGGGTGCGTGTGAGCGTGTT	3	Reoviridae	Orbivirus	Bluetongue virus	Bluetongue virus 2 isolate sheep/08/Ind/ABT/Hisar segment 8, complete sequence
Ctrl_101	CAAGGAGTACATTGAAAAAGTTGCAAAGCAGATTAAATTGAAGGATGAACGGTTTATGAG	3	Reoviridae	Orbivirus	Bluetongue virus	Bluetongue virus 13 isolate FL99 22364-8 NS2 protein (S8) gene, complete cds
E.coliUPS_510	TTGGCTAGTTACGGAGTTTCTGGACATTATTCTCACGGCGTGGTATATCCAGCTCCATCT	4	Enterobacteriaceae	Escherichia	*E.coli*	plasmidO157
E.coliUPS_511	TCATCATTGTTACACAACGCGCTCCTTGGTCTGGCAAATGTTTATAATGGCTTACGGGAA	4, 5	Enterobacteriaceae	Escherichia	*E.coli*	efa1/lifA partial
E.coliUPS_512	TCATGCCATATGGGTTGCGGGTTCCCCACCTGAAAGCATCTCGGACTATATTAAGACTTT	4, 5	Enterobacteriaceae	Escherichia	*E.coli*	efa1/lifA partial
E.coliUPS_513	CAGGCCAATCAAAACAGCATCAGAAGCCCGACTACGCTTCTTTGATAAAATGGTTTCGAA	4, 5	Enterobacteriaceae	Escherichia	*E.coli*	efa1/lifA partial
E.coliUPS_514	ACATCCTGAACATGCAACAATACCATATGACAACGACAATGAAAGTAATAAATTGCTAAA	4, 5	Enterobacteriaceae	Escherichia	*E.coli*	efa1/lifA partial
E.coliUPS_515	CTCTCTGGAAGATGTTGTTTCTTTAGGTGAAATGATTCAGAAGGAAATTTATGGGCATGA	4, 5	Enterobacteriaceae	Escherichia	*E.coli*	efa1/lifA partial
E.coliUPS_517	ACATAGAGATGGCTATAGCAGATATAAAAAGGGAGTACTATTCGCATACAGTTGATATTG	4, 5	Enterobacteriaceae	Escherichia	*E.coli*	efa1/lifA partial
E.coliUPS_518	GCAAGTGCGCTGGCGACGCCTGATTGTGTAACTGGAAAGGTGGAGTATACAAAATATAAT	4	Enterobacteriaceae	Escherichia	*E. coli 0157*	stx1
E.coliUPS_519	ATGTCGCATAGTGGAACCTCACTGACGCAGTCTGTGGCAAGAGCGATGTTACGGTTTGTT	4, 5	Enterobacteriaceae	Escherichia	*E. coli 0157*	stx1
E.coliUPS_520	GGGGAAGGTTGAGTAGTGTCCTGCCTGATTATCATGGACAAGACTCTGTTCGTGTAGGAA	4, 5	Enterobacteriaceae	Escherichia	*E. coli 0157*	stx1
E.coliUPS_523	GATTCATCCACTCTGGGGGCAATTCTGATGCGCAGAACTATTAGCAGTTGAGGGGGTAAA	4, 5	Enterobacteriaceae	Escherichia	*E. coli 0157*	stx1
E.coliUPS_524	AAGTGTCAAAGAAAGTCGACGAGGTGCTAGCCATCGAGCTACGTCTGCTCTCCATGGTAT	4	Enterobacteriaceae	Escherichia	*E.coli*	tir2
E.coliUPS_525	TGTCTTTACCTTCAGGATCAATGGACTGCAAGCGAGCGTACTCTTGATCACTTAAAACAA	4	Enterobacteriaceae	Escherichia	*E.coli*	tir2
E.coliUPS_526	CCCGCACTTGGATTTCCTAATAACCGTGCGCCGTTATCAGTAGTATCCCGGGGAGGATGT	4	Enterobacteriaceae	Escherichia	*E.coli*	tir2
E.coliUPS_527	AGTTGCACTTGCAGCTGCATCAGGGTCGGTCGTGGTTGGGCTATCCGGCTCCGGCGTCAA	4	Enterobacteriaceae	Escherichia	*E.coli*	tir2
E.coli_828	ACTGTGACAGCTGAAGCTTTACGTTTTCGGCAAATACAGAGGGGATTTCGTACAACACTG	5	Enterobacteriaceae	Escherichia	*E. coli 0157*	stx1

E.coli_829	GATGATCTCAGTGGGCGTTCTTATGTAATGACTGCTGAAGATGTTGATCTTACATTGAAC	5	Enterobacteriaceae	Escherichia	*E. coli 0157*	stx1
FTM_FSC147_01	CACCAGTCATTTTTTCTTATCTCACTCAAAACAGAAAATCTATACTCTTCATACGATAA	5	Fransicellaceae	Francisella	*sub sp mediasiatica*	F_tularensis_mediasiatica_FSC147_RD1 gi|32469339|gb|AF469616.1| Francisella tularensis subsp. mediasiatica strain FSC147 region of difference 1 (RD1), complete sequence_474-532
FTM_FSC147_02	AAATTATAGTTCCCATCATGATATACGGCTTATATATCCAATTAAAGACTGAACCTAAAATTTTT	5	Fransicellaceae	Francisella	*sub sp mediasiatica*	F_tularensis_mediasiatica_FSC147_RD1 gi|32469339|gb|AF469616.1| Francisella tularensis subsp. mediasiatica strain FSC147 region of difference 1 (RD1), complete sequence_655-719
FTM_FSC147_03	TTTCTTCTATTATCTGGCAATTAGGTTCTAATTCAGGCGTGCCATACGAGATATT	5	Fransicellaceae	Francisella	*sub sp mediasiatica*	F_tularensis_mediasiatica_FSC147_RD1 gi|32469339|gb|AF469616.1| Francisella tularensis subsp. mediasiatica strain FSC147 region of difference 1 (RD1), complete sequence_718-772
FTM_FSC147_04	TACTTTCAACAACTTGAATTCCTTTATTAGAATTGCCATAATTATATTCATTTGACTTATTTTTG	5	Fransicellaceae	Francisella	*sub sp mediasiatica*	F_tularensis_mediasiatica_FSC147_RD1 gi|32469339|gb|AF469616.1| Francisella tularensis subsp. mediasiatica strain FSC147 region of difference 1 (RD1), complete sequence_799-863
FTH_FSC155_01	TGCTGTTACTCCAATATATAGAGTACCATTTTTCTTACTCGCCAAAATATACACAAAA	5	Fransicellaceae	Francisella	*sub sp holarctica*	F_tularensis_holarctica_FSC155_RD1 gi|32469343|gb|AF469617.1| Francisella tularensis subsp. holarctica strain FSC155 region of difference 1 (RD1), complete sequence_678-735
FTH_FSC155_02	GTTTTTCAAATAGCATTCAACATCTCTCTTATAGCTACCTGCATTATGCAACTTT	5	Fransicellaceae	Francisella	*sub sp holarctica*	F_tularensis_holarctica_FSC155_RD1 gi|32469343|gb|AF469617.1| Francisella tularensis subsp. holarctica strain FSC155 region of difference 1 (RD1), complete sequence_203-257
FTH_FSC155_04	AGGATCCACCCCAAAGACTCGAATATACTTCTCTCTAAGCTCTACTAACTTTTCT	5	Fransicellaceae	Francisella	*sub sp holarctica*	F_tularensis_holarctica_FSC155_RD1 gi|32469343|gb|AF469617.1| Francisella tularensis subsp. holarctica strain FSC155 region of difference 1 (RD1), complete sequence_829-883
FTH_FSC155_05	CATTCTCCACCTAGTCTAGAATGCTCTCATATAAGTCTAACCAATCAGGATTAAACT	5	Fransicellaceae	Francisella	*sub sp holarctica*	F_tularensis_holarctica_FSC155_RD1 gi|32469343|gb|AF469617.1| Francisella tularensis subsp. holarctica strain FSC155

						region of difference 1 (RD1), complete sequence_446-502
FTN_FSC040_01	TTTCAATTTTAGTATGTAATGGTTTACAGGTAGCACAAAGCATATTTGCAAGAGTA	5	Fransicellaceae	Francisella	*sub sp novicida*	F_tularensis_novicida_FSC040_RD1 gi|32469349|gb|AF469619.1| Francisella tularensis subsp. novicida strain FSC040 region of difference 1 (RD1), complete sequence_3064-3119
FTN_FSC040_02	GTATCTAGAGTTTTCTCTCTTAGATTCTTAGGTACAAAACTAACTTGTCCGCCATCA	5	Fransicellaceae	Francisella	*sub sp novicida*	F_tularensis_novicida_FSC040_RD1 gi|32469349|gb|AF469619.1| Francisella tularensis subsp. novicida strain FSC040 region of difference 1 (RD1), complete sequence_3117-3173
FTN_FSC040_03	ATATGGATTATCTCCACAAATCATCAGTGCAAAGTGCTCTAAAAAATAGTTTCTA	5	Fransicellaceae	Francisella	*sub sp novicida*	F_tularensis_novicida_FSC040_RD1 gi|32469349|gb|AF469619.1| Francisella tularensis subsp. novicida strain FSC040 region of difference 1 (RD1), complete sequence_2769-2823
FTN_FSC040_04	AAAACACCATTCTGTATTTCTTCAAGTTGATTATTGCTTAAACCCTTTGCTAATA	5	Fransicellaceae	Francisella	*sub sp novicida*	F_tularensis_novicida_FSC040_RD1 gi|32469349|gb|AF469619.1| Francisella tularensis subsp. novicida strain FSC040 region of difference 1 (RD1), complete sequence_1631-1685
FTN_FSC040_05	ACTTTTCTTTGGCTGCCTTTAATAGATTTTCACAATACTTGTCATTTATATTAACAACTTC	5	Fransicellaceae	Francisella	*sub sp novicida*	F_tularensis_novicida_FSC040_RD1 gi|32469349|gb|AF469619.1| Francisella tularensis subsp. novicida strain FSC040 region of difference 1 (RD1), complete sequence_2351-2411
FTT_SCHU S4_7026	CTGGCGGTAATGATAAGCTTACGCGTAAGATGATCGATGAGTATACCAAGTTTGTTGGTA	5	Fransicellaceae	Francisella	*sub sp tularensis*	aspS
FTT_SCHU S4_19222	AGCGTAGAATTATGATTGGTAACTATGTCCTAGCATCGAGTCAGTATGATTCTTACTACA	5	Fransicellaceae	Francisella	*sub sp tularensis*	gatA
FTT_SCHU S4_66023	TTTACTGGTAACCCTGGTAAGTTCGTATCACTTAAGGATACTGTAGCAAGCTTCAAAGCT	5	Fransicellaceae	Francisella	*sub sp tularensis*	atpD
FTT_SCHU S4_92642	AGAATCTTCTTAAGGCTGGAACAATTACACCAGAAGAAGCCGCTAAATACATTGTAGTAA	5	Fransicellaceae	Francisella	*sub sp tularensis*	pilT
FTT_SCHU S4_113928	TTGCAAATAAACTTGATCTGCGGCAATATAGGCGCCTGTTGCAATGACTATGATAGTTAT	5	Fransicellaceae	Francisella	*sub sp tularensis*	dsbB
FTT_SCHU S4_158585	GATGAAGCTTTTTGATGGTCGTACTGGTAAGCCTTTTGATAGACATGTAACTGTTGGTTA	5	Fransicellaceae	Francisella	*sub sp tularensis*	rpoB

FTT_SCHU S4_159779	TACCTGTATTACCACCGGATTTAAGACCATTGGTACCAATCGAAGGTGGTAGATTTGCAA	5	Fransicellaceae	Francisella	*sub sp tularensis*	rpoC
FTT_SCHU S4_262183	TCAATTATGGCTGCTAGAACTTTAGAAACACGCAAAGATCGTCTGATGACTTTGATGATG	5	Fransicellaceae	Francisella	*sub sp tularensis*	feoB
FTT_SCHU S4_383305	ATGACCATCTCTTTTTTCTACTTGAGCAATTACGCTATCAATGTACTTCTGAGCATTCAT	5	Fransicellaceae	Francisella	*sub sp tularensis*	gdh
FTT_SCHU S4_396790	TGCGATGCGTTATACAGAGTCGCGTTTATCTAAATATGCGGTATTACTTCTAGATGAATT	5	Fransicellaceae	Francisella	*sub sp tularensis*	parC
FTT_SCHU S4_484220	AATAGCGTCAGGAAATGTCCAGGTGCTACAATGTGATCAGGAATTGTATGGTAATAATGC	5	Fransicellaceae	Francisella	*sub sp tularensis*	ostA1
FTT_SCHU S4_553718	TTCATTAAATACCGTCTTAGCAAGACATAATCCTAAACCACGACGCGTAGTAGGGTCTGC	5	Fransicellaceae	Francisella	*sub sp tularensis*	nrdB
FTT_SCHU S4_654359	CATTTTACACAAGTACTAGTGGTAGTGTTGATCGAGCTGAGACATTATTAAAACAATTCT	5	Fransicellaceae	Francisella	*sub sp tularensis*	hflC
FTT_SCHU S4_732507	CTTTTTTCTCATCATAGATCATTACAGGAATGATAGGGTGCTCACCTGGAATTAAATCAA	5	Fransicellaceae	Francisella	*sub sp tularensis*	kbl
FTT_SCHU S4_748642	TATTTCCCATTAGAAACTTACTTATACCATAGGCAATACCTAGACCAGCTCCAATTAATC	5	Fransicellaceae	Francisella	*sub sp tularensis*	glpT
FTT_SCHU S4_786574	ATCTCTGACAGCTTTATAACCGGTGAATCTACTACAGCTCAACAGCGAGAGCAATCTTTC	5	Fransicellaceae	Francisella	*sub sp tularensis*	deoD
FTT_SCHU S4_870249	AGAACTCAAGAAATTCAGCGCCTGATAGGTAGAGCATTGCGTGCTAGTGTTGATCTTACA	5	Fransicellaceae	Francisella	*sub sp tularensis*	rph
FTT_SCHU S4_889471	GGAAAAAAACCACCATCGCTAACTATATAGGTTCCACCTAGTACGCCACCAGTATTATTT	5	Fransicellaceae	Francisella	*sub sp tularensis*	FTT0881c
FTT_SCHU S4_923392	TAGCTTTAAATATAAACTCTCCGGCAAATAGTTTGGTATTCTCGCTGTAGCAACCATTAC	5	Fransicellaceae	Francisella	*sub sp tularensis*	ileS
FTT_SCHU S4_927117	AGATGTGACATATAATGGTCGTGATTACCGTATTTCTCAGGGGAATAACGCCTTTATATT	5	Fransicellaceae	Francisella	*sub sp tularensis*	maeA
FTT_SCHU S4_1039158	ACTATTGCTCTTGCTGAATATATCGGCGGAACTACACAAGCCTTTACTGATTTAATGAAT	5	Fransicellaceae	Francisella	*sub sp tularensis*	dacD
FTT_SCHU S4_1068478	TATCTCTGATACCTCTAGTGTTCGGTTAGTCTCATAACCTGGGATTTTCATAAGCTGTAG	5	Fransicellaceae	Francisella	*sub sp tularensis*	dnaB
FTT_SCHU S4_1198552	TAAATCCACCACCAGCGAGATTACCAGCTCTTGGTAGCGTGACAGCTAAAGCAAAACCAA	5	Fransicellaceae	Francisella	*sub sp tularensis*	ggt
FTT_SCHU S4_1201862	TAGCTTCTAATATCATTTACAAGCTCGTACATCTGTGCGGCACTATAATTTACAACTGCA	5	Fransicellaceae	Francisella	*sub sp tularensis*	FTT1185c
FTT_SCHU S4_1268912	TTTGCAAACTATTGGTGAAGACCCTATACAGCGTCTAGTATTACCAAGAATAACTGCTTT	5	Fransicellaceae	Francisella	*sub sp tularensis*	FTT1247
FTT_SCHU S4_1276241	AGAGAAATTGAGTTATGCATACTTAAGTTAGACTATGTAAGAGAGGTAGCTGTCACTGGA	5	Fransicellaceae	Francisella	*sub sp tularensis*	fadD1

FTT_SCHU S4_1337808	ACATCACACCAACAAGACCAGCGCCTATCTGACTTGCTAAACGAATACGCTGAGCCTCAC	5	Fransicellaceae	Francisella	*sub sp tularensis*	uvrA
FTT_SCHU S4_1527194	ATAGCTGCTGCAATTAGAAGTACCTTTCTACGTCCATAGCGCTTTGATAAAAAACCACTA	5	Fransicellaceae	Francisella	*sub sp tularensis*	galP1
FTT_SCHU S4_1624392	CCTGCTATGGATAACGATACTCTACGTAGGGGCAAACCAACTTGTCATATCAAATTTAAT	5	Fransicellaceae	Francisella	*sub sp tularensis*	ispA
FTT_SCHU S4_1843502	GTTATTGGTTGATGGACCGCTTCAGTATGATGCTGCGATGATTGAAAGTGTCGCTAAGAG	5	Fransicellaceae	Francisella	*sub sp tularensis*	pta
FTT_SCHU S4_1856019	GTTGCCAAGTATTATTTGCGCCACCTGTAGATGCAATAACCAAGCCATCTTTTGCATGAA	5	Fransicellaceae	Francisella	*sub sp tularensis*	FTT1768c
HANTA_UPS_S56	ATGGCATTAGACGACCCAAGCACCTTTATGTATCCATGCCGACAGCACAGTCTACCATGA	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_UPS_S57	ATGGGATCCGGAAGCCAAAACATCTATTTTTGTCAATGCCTAATGCCCAGTCTAGCATGA	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_UPS_S58	ATGGTGGACCACTTCCATCTTGGTGATGACATGGATCCTGAACTTAGGGGTCTAGCTCAG	3, 5	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_UPS_S59	CACCCGGTCGTTTCCGGACTATTGTCTGTGGCTTGTTTCCTGCTCAAATCATGCATAGAA	3, 5	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_UPS_S60	TCTCCACAAGCACCATGGGTTTTTGCCTGTGCGCCTGATCGATGCCCTCCCACTTGTATT	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_UPS_M61	GGATGGGGCTGTAATCCTCCTGACTGCCCAGGGGTAGGTACAGGGTGTACAGCCTGTGGT	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	G1 and G2 glycoproteins
HANTA_UPS_M62	CTGTGGGCTGCTAGTGCATCAGAAATACCCTTAAGCCCTACGTGGAATGATAATGCTCAT	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	G1 and G2 glycoproteins
HANTA_M65	TGATGATGGGGCACCAGAATGTGGTATGTCATGTTGGTTTAAAAAATCAGGTGAATGGAT	3, 5	Bunyaviridae	Hantavirus	Multiple *Hantaviruses*	G1 and G2 glycoproteins
HANTA_M64	CTGACCTGGAATTAGACTTCTCCCTACCATCCTCTGCAAGTTACACATATAGGAGGCAGT	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	G1 and G2 glycoproteins
HANTA_L66	GGCATGACATTGTTGATCAGATGATAAAGCATGACTGGTCAGATAACAAGGATTCAGAGG	3, 4	Bunyaviridae	Hantavirus	Multiple hantaviruses	RNA dependent RNA polymerase
HANTA_L67	ATTGCATTTGAAAAGGCATTAATTGCTACAGCCACATGGTTTCAATACTATACAGAGGAT	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	RNA dependent RNA polymerase
HANTA_L68	ATTCAGGTAAACAATATGATGCCTATTGTGTTCAGACATATAACGAAGAAACAAAGCTCT	3, 4	Bunyaviridae	Hantavirus	Multiple hantaviruses	RNA dependent RNA polymerase
HANTA_L69	GAAAACATTGATGTGGAAGCTGATATTGAACATTTCTTACAAGATGAAAGTTCATATACA	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	RNA dependent RNA polymerase
HANTA_L70	AAGGTTTATGTATTGAGAAGGTATATAGCCCAACCAATATTATATTGATGACTAGATATC	3, 5	Bunyaviridae	Hantavirus	Multiple hantaviruses	RNA dependent RNA polymerase

HANTA_L71	GAGTACATCTCCTATGGTGGTGAAAGGAAAATTTTATGTATTCAATCAGCTTTAGAAAAA	3, 4, 5	Bunyaviridae	Hantavirus	Multiple hantaviruses	RNA dependent RNA polymerase
HANTA_S72	AAGGATGCGGAAAAGCAGTATGAAAAGGACCCTGATGACCTTAACAAAAGGGCATTGAGT	3, 5	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_S73	AACTAGAGGGAGACAGACTACTAAGGACAACAAGGGAATGAGGATTCGCTTCAAGGATGA	3, 5	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_S74	GGTGCCCCCCTACCTGCCTGTTTATTGCAGGCATGGCAGAGTTAGGTGCATTCTTTTCTG	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTA_S75	ATCATTCTACCAGTCTTACCTTCGAAGGACACAATCAATGGGAATCCAGCTGGACCAGCG	3	Bunyaviridae	Hantavirus	Multiple hantaviruses	N protein
HANTAUPS_560	ACTTCTCGGATTGACTGTTGACCAATCTACAGTTGGTGCAAGCGGTATCTATCCATCCTT	4, 5	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_561	AAATCACGGGTTGTCATTGTCCAGGGGAATGTGGAAGGAACTGCAGAGGCAATCTGTGCA	4, 5	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_562	AGATAGCCCACGGTTTCTTTTAGCACATGCTTTCCATACTATCCGTGATGTTCGCTATCA	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_563	TTAGGAGCATCACCTCAAATCTCACAACTTGCAGTCTCACTTAGTACGAGTAAGGTTGAG	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_564	TTCTCTGTATCCAGGCGGCCCTTGAAAAAGCTCTCCGCTGGGCATCTGGAGAGAGCTTTA	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_565	CAATGGCCCAGTGCAAGGAATGATGGTGTCGTTCAATACATGAGATTGGTACAGGCAGAA	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_566	AAACCTCATCCCAGAAACCCCTGCATCTATTCTTGCACATGATATGGCACACAGATACAT	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_567	GAAGACCACAGCATGGCATATCGGGCATTTAGTTAGGGATATTACTGAGAGCTTGATTGC	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_568	CTGACCTACCAGGTCTTGGCTATTTCGATGATCTTGCTGCTGCCCAGACGAGGTGCGTCA	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_569	AGACGGGCATCATGGAGAGGTACGAGGCAATTGGTTGCAGGGTAACTTGAACAAGTGTTC	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_570	GATGGAGGAGGTTTCGAAAGAGCTAGTTAGGTGTGGGATGCGATTTAATACTGAGCAAGT	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTAUPS_571	CTCCAATTCCACTGGGAGGAAGTGGTGCAATGTCAATAATGGAACTTTCAACAGCCGGTA	4	Bunyaviridae	Hantavirus	Hantavirus	RNAdependent-RNApolymerase
HANTA_L830	CATGACATTGTTGACCAGATGATAAAACATGAATGGTCAGACAATAAAGACAGCGAGGAG	5	Bunyaviridae	Hantavirus	Multiple *Hantaviruses*	L segment
HANTA_L831	GAATTTGCTCACCACTCAGATGATGCACTTTTTATATATGGATACTTGGAACCAGCTGAT	5	Bunyaviridae	Hantavirus	Multiple *Hantaviruses*	L segment

Hanta_L832	TGTCAAGAAAATTGAGGCGGTATATCGATTCTATGGAGACTCTGGAGCCCCATGTAAAGG	5	Bunyaviridae	Hantavirus	Multiple *Hantaviruses*	L segment
Hanta_L833	CCGGATGGTCATCATGGTGAAGTTAGGGGTAATTGGTTGCAAGGTAACTTAAATAAATGC	5	Bunyaviridae	Hantavirus	Multiple *Hantaviruses*	L segment
Hanta_L834	TTTGAATTTGCTCATCATTCAGATGATGCTTTGTTCATTTACGGTTATCTAGAGCCAGTT	5	Bunyaviridae	Hantavirus	Multiple *Hantaviruses*	L segment
HEV_UPS_76	CAGGCTATAACCATGACGTCTCTATACTCCGAGCGTGGATCCGAACGACTAAAATAGTTG	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_77	CTTGACCTATGTAATCGTGATGTCTCGCGCATCACATTCTTCCAGAAAGACTGTAACAAG	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_78	GCGCTCACGATAACGGAAGACACAGCCCGCACGGCCAACCTAGCACTAGAGATCGACGCT	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_79	TCGCATTAGAGCTGGAATTTAGGAACTTGACACCTGGAAACACCAACACTCGTGTATCCC	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 2 Capsid protein
HEV_UPS_80	CCTTACTACCATCCAGCAGTATTCTAAGACGTTCTACGTCCTTCCGCTCCGYGGAAAGCT	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF2 Capsid protein
HEV_UPS_81	TCGGGAATAAGACTTTCCGGACGACGGTGACCGATGGTGCCCATCTTGAAGCGAACGGTC	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_82	AGTGCAGGTTACAACCATGACGTCTCTATACTTCGTGCGTGGATTCGTACGACTAAAATA	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_83	CCGATGTATGTGAGCTCATACGTGGGGCTTATCCTAAAATCCAGACCTCGAGTCGCGTGC	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_84	GAATACCGTTTGGAATATGGCGATCATAGCACACTGCTATGAGTTTCGCGATTTTCGGGT	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_UPS_85	TGCAATCGTGATGTCTCGCGCATCACATTTTTCCAAAAGGATTGCAACAAGTTTACAACT	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_86	CCGGCAGTTGGTTTTCCGTCCTGAAGTGCTTTGGAATCATCCTATCCAGAGGGTTATACA	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_87	GAAGATGCCCTAACTGCAGTGATTACCGCTGCCTATTTGACCATCTGCCATCAGCGCTAC	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_88	CCTAGAGTGCTTGTGTTCGATGAGTCAGTGCCCTGCCGCTGTAGGACTTTCCTAAAGAAG	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_89	GTATATACCAGGTCCCCGTTGGTCTCAGTTTTGATGCCTGGGAGCGTAACCACCGCCCCG	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_90	TTGTGGGGAGGTATGGCCGTAAGACGAAGTTGTACGAGGCAGCCCACTCTGATGTTCGTG	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_91	GTATCATCTGGTCCGGTCAGCCTGGATTCTGCAGGCGCCGAAGGAGTCTCTTAAAGGTTT	3, 5	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 NSP polypeptide
HEV_92	ATTAAACCTGTGCTTGACCTTACAAACTCTATCATACAGCGGGTAGAATGAATAACATGT	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 1 and 3

HEV_93	TTACCGCCCGCTGGTGCCGAATGCTGTTGGTGGTTATGCCATTTCCATTTCTTTTTGGCC	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF 2 Capsid protein
HEV_94	TCGGCGGCCTGCCGACAGAATTGATTTCGTCGGCTGGGGGTCAGCTGTTTTACTCCCGCC	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF2 Capsid protein
HEV_95	GGTTGCGCTTTCCAGTCTACTATCGCTGAGCTTCAGCGCCTTAAAATGAAGGTAGGTAAA	3	Hepeviridae	Hepevirus	Hepatitis E Virus	ORF2 Capsid protein
HEVUPS_572	TTAAGCTGTATACATCTGTAGAGAATGCTCAGCAGGATAAGGGTATTGCAATCCCGCATG	4	Hepeviridae	Hepevirus	HEV	Capsid protein gene ORF2
HEVUPS_573	GGCATAGCCTCCGAGCATGTTATCCCAAGTGAGCGCCTACACTATCGTAACCAAGGTTGG	4, 5	Hepeviridae	Hepevirus	HEV	Capsid protein gene ORF2
HEVUPS_574	TCAGTCTACTGTCGCTGAGCTTCAGCGCCTTAAGATGAAGGTGGGTAAAACTCGGGAGTT	4, 5	Hepeviridae	Hepevirus	HEV	Capsid protein gene ORF2
HEVUPS_575	CCAGGACGGCACCAATACTCATATAATGGCTACAGAAGCTTCTAATTATGCCCAGTACCG	4	Hepeviridae	Hepevirus	HEV	Capsid protein gene ORF2
HEVUPS_576	TTTACTAGTACTAATGGTGTCGGTGAGATCGGCCGCGGGATAGCGCTTACCCTGTTTAAC	4	Hepeviridae	Hepevirus	HEV	Capsid protein gene ORF2
HEVUPS_577	CGCGGTAAGCTCTCCTTTTGGGAGGCAGGTACTACTAAAGCCGGGTACCCTTATAATTAT	4	Hepeviridae	Hepevirus	HEV	Capsid protein gene ORF2
HEVUPS_578	GTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATCCCCTATATTCATCCAA	4	Hepeviridae	Hepevirus	HEV	ORF3
HEVUPS_579	CTGGACCTCGTGTTCGCCAACCTGCCCGACCACTCGGCTCCGCTTGGCGTGACCAGGCCC	4, 5	Hepeviridae	Hepevirus	HEV	ORF3
HEVUPS_580	GCTGTTCCTCATGTTTTTGCCTATGCTGCCCGCGCCACCGCCCGGTCAGCCGTCTGGCCG	4, 5	Hepeviridae	Hepevirus	HEV	ORF3
HEVUPS_581	TGAATAACATGTCTTTTGCTGCGCCCATGGGTTCGCGACCATGCGCCCTCGGCCTATTTT	4	Hepeviridae	Hepevirus	HEV	ORF3
HEVUPS_582	TGTCTTGCACCGTTGCTTCCTTAAACCTGTTGGTCGCGACGTTCAACGGTGGTATACCGC	4	Hepeviridae	Hepevirus	HEV	ORF1
HEVUPS_583	GTCCTGTGGAATCACCCAATCCAGCGCGTGATCCACAATGAGCTTGAGCAGTATTGCCGA	4, 5	Hepeviridae	Hepevirus	HEV	ORF1
HEVUPS_584	CTTTCCCGGCTACAGACAGAGATTCTTATAAACCTGATGCAGCCCCGGCAGCTCGTTTTT	4, 5	Hepeviridae	Hepevirus	HEV	ORF1
HEVUPS_585	ACCCGTGGCCCTGCAGCGAACTGCCGCCGGTCCGCCCTTCGTGGGCTTCCACCTGCTGAT	4, 5	Hepeviridae	Hepevirus	HEV	ORF1
HEVUPS_586	TGTCTTGCACCGTTGCTTCCTTAAACCTGTTGGTCGCGACGTTCAACGGTGGTATACCGC	4	Arenaviridae	Arenavirus	HEV	ORF1
LCMVUPS_595	ACTGGGTGCTTGTCTTTCAGCCTTTCAAGATCATTAAGATTTGGATACTTGACTGTGTA	4, 5	Arenaviridae	Arenavirus	LCMV	GP-C and NP
LCMVUPS_597	TTCTATCCAGTAAAAGGATGGGTCAGATTGTGACAATGTTTGAGGCTTTGCCTCACATCA	4	Arenaviridae	Arenavirus	LCMV	glycoprotein C

LCMVUPS_598	TGTACAATTTCGCCACCTGTGGGATATTAGCACTGGTCAGCTTCCTTTTTCTGGCTGGTA	4	Arenaviridae	Arenavirus	LCMV	glycoprotein C
LCMVUPS_601	ACCCCAGTGTGCATTTTGCATAGCCAGCCATATTTGTCCCACACTTTATCTTCATATTCT	4, 5	Arenaviridae	Arenavirus	LCMV	S segment
LCMVUPS_603	CCGTGTGAATACTTGGAGTCCTGCTTGAATTGCTTCTGGTCCGTAGGTTCCCTGTAAAAA	4, 5	Arenaviridae	Arenavirus	LCMV	S segment
LCMVUPS_604	CCCTCAATGTCAATCCATGTGGGAGCATTGGGATCAATCCCTCCCATCAAGTCTTTCAAC	5	Arenaviridae	Arenavirus	LCMV	S segment
LCMVUPS_605	CTAGATGAATTGGCCACAAGTTTTTCATTTGTGAGATCAATTGTTGTGTTCTCCCATGCT	5	Arenaviridae	Arenavirus	LCMV	S segment
LCMVUPS_606	AGATCTGGGAGCCTTGCTTTGGAGGCGCTTTCAAAAATGATGCAGTCCATGAGTGCAC	4,5	Arenaviridae	Arenavirus	LCMV	GP-C and NP
LCMVUPS_607	CACATACTGGTCATCACCTAGTTTGAGATTTTGTAGAAACATTAAGAACAAAAATGGGCA	4	Arenaviridae	Arenavirus	LCMV	L segment
LCMVUPS_609	CAAATCTCCAATATAGAGTTCTCTATTTCCCCCAACCTGCTCTTTATAAGATAGTGCAAA	4	Arenaviridae	Arenavirus	LCMV	L segment
LCMVUPS_611	TTATCGTGATCACGGGTATCAAGGCTGTCTACAATTTTGCCACCTGTGGGATATTCGCAT	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LCMVUPS_612	GACCGGCGAAACTAGTGTCCCCAAGTGCTGGCTTGTCACCAATGGTTCTTACTTAAATGA	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LCMVUPS_613	TCTTGCTGCAGAGCTTAAGTGTTTCGGGAACACAGCAGTTGCGAAATGCAATGTAAATCA	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LCMVUPS_614	GTCCAGGATTCTCCTTTCCCAAGAGAAGACTAAGTTCCTCACTAGGAGACTAGCGGGCAC	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LCMVUPS_615	TTCCTACTTCTGGCTGGCAGGTCCTGTGGCATGTACGGTCTTAAGGGACCCGACATTTAC	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LCMVUPS_616	CAGGAAGCCGATAACATGATTACAGAGATGTTGAGGAAGGATTACATAAAGAGGCAGGGG	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LCMVUPS_617	ACTTTGTCAGACTCTTCAGGGGTGGAGAATCCAGGTGGTTATTGCCTGACCAAATGGATG	4,5	Arenaviridae	Arenavirus	LCMV	Nucleoprotein Segment S
LeptospiraUPS_618	GCCACATTGGTTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCA	4	Leptospiraceae	Leptospira	*Leptospira interrogans*	Lip32
LeptospiraUPS_619	CTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGG	4	Leptospiraceae	Leptospira	*Leptospira interrogans*	Lip32
LeptospiraUPS_620	GATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAA	4	Leptospiraceae	Leptospira	*Leptospira interrogans*	Lip32
LeptospiraUPS_623	TTCTCCAGAATTAAAGCAGTCAGGTTCACGAGCTTGTAGCTGAGTTCCGATAACATGAAA	4	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1027
LeptospiraUPS_624	CGATGATAACTCCTCCGTTAGCCATTGGAGAAGTTGTATAAGCTCCAAGAGTTGCATTCC	4	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1027

LeptospiraUPS_625	TCTGGAGAGCGTGGATTTGAAGAGTCGGAATCCGAAGGGGCGTTGTCGTTGGCATTTGCG	4	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1027
LeptospiraUPS_629	CATGTCATCGGAACCCACGCTCAGTCTCAGGATCAGAACTGTTCAAATCTAGGAATACCA	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1029
LeptospiraUPS_630	CGGATCACTATCCGATATACGGTTTTGTATACGCGGATCCTTCTACTCCTACAAAGTCCG	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1029
LeptospiraUPS_631	TTGGGACGCAAGCTTAGGCAATTTTAGATCTTATTCGCTCGTTAACGGAGGAGTTGTAAT	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1029
LeptospiraUPS_633	TTCTGGGGGTTAAATTTCAATTCACTTATCCAGGAGAGAATGCTGTAACGATTCGTCCTC	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	FlaA
LeptospiraUPS_634	CTTCAATTTGGATCTCTCGACTTTATTGGATGGAGACCATTGACCGTTTACATCCCTC	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	FlaA
LeptospiraUPS_636	AGCTACTCTGGATTATTATTATCTTACGATTTGGGATGGTGGTAATTGGGATGGATATT	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SphH
LeptospiraUPS_637	TAATAAACATGGTCGCAAATATCATATCATTGGAACTGACGTTCAAAATACTTCTTGTT	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SphH
LeptospiraUPS_639	TTTCCTCAGACGATTATTCAATGGTTATCTAATAGTAGTCAAGAATGGGCTGGATGGGCA	5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SecY
LeptospiraUPS_640	TTAAATAGAATCACTCTTCCCGGAGCTATGTTTCTTGCAGGTTTGGCATTAGCACCTTA	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SecY
LeptospiraUPS_641	TTGATCTTCGCTGGAATTATCGGAAGACTGCCTGAGTCTATGGTTCAACTTTTCTCTAC	4,5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SecY
LeptospiraUPS_642	TTTGGTTTATACATTCGGTGGAACTTCTCTTCTAATTATGGTAGGGGTTGCGCTCGAG	5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SecY
LeptospiraUPS_643	AAAAATGGTTCAGGCCAAGAGTCAATCTATTCCTTTCAAAGTAAACGGCGCGAACGTG	5	Leptospiraceae	Leptospira	*Leptospira interrogans*	SecY
Leptospira_826	TTCAATCAGCTGCTAATGGTAAATACATTCAAGCAGATCCTAATAGAAAAAACGGATGGT	5	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1029
Leptospira_827	ATTTAAATCCTTCCTGCATAAAAAATGGTCTCGTTAGAATTGAATCTTCCCGTTTTTTAA	5	Leptospiraceae	Leptospira	*Leptospira interrogans*	LA1029
MRSA_Owiz_644	AAAAGACTATAAGGTTGCTGATAAAAGTGGTCAAGCAATAACATATGCTTCTAGAAAT	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	blaZ
MRSA_Owiz_645	TATAAAAGAAATCGGTGGAATCAAAAAAGTTAAACAACGTCTAAAAGAACTAGGAGATA	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	blaZ
MRSA_Owiz_646	AATAATAAAAGCGGAGATACTTTAATTAAAGACGGTGTTCCAAAAGACTATAAGGTTG	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	blaZ
MRSA_Owiz_647	ATTGTGGTCGTGTCAACAATCAATATGGTCAATTCGACAATATGTTTCCTTCTATTGT	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	ccrA

MRSA_Owiz_648	TATTTTTATCAGCTTGGCCGAACTTGAATCAGATAACATTGGAGAACAAGTCAGAAATG	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	ccrA
MRSA_Owiz_649	TCACGCTTTGTCTGTGATTTTAAAGGCATCAATGCACAAACACTTGAAGATAAAGTAT	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	ccrA
MRSA_Owiz_651	GGCTACAAACCTCATAGTAAAGATCCTAGAGATTATTTCGTTCCAGACAGTGAGTTACCA	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	gamma hemolysin
MRSA_Owiz_652	CTGAAGACATCGGTAAAGGAAACGATGTAGAAATTATCAAAAGGACAGAAGATAAAACAA	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	gamma hemolysin
MRSA_Owiz_653	GGTCAACACATTATGGTAACAGTTATTTAGATGGTCACAGAGTCCATAATGCATTTAAAA	4,5	Staphylococcaceae	Staphylococcus	*S. aureus*	gamma hemolysin
MRSA_Owiz_656	TTACAACTAATGAAACAGAAAGTCGTAACTATCCTCTAGGAAAAGCGACTTCACATCT	4,5	Staphylococcaceae	Staphylococcus	MRSA	mecA
MRSA_Owiz_657	GTGAATTATTAGCACTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGG	4,5	Staphylococcaceae	Staphylococcus	MRSA	mecA
MRSA_Owiz_658	ATTTCTGAAGACTATATCAAACAACAAATGGATCAAAATTGGGTACAAGATGATACCTT	4,5	Staphylococcaceae	Staphylococcus	MRSA	mecA
MRSA_Owiz_663	TGGAAGCAAGCCATAGCAGAATATGAAAAACGTTTAGGCCCATACACCAAGATAGACATC	4,5	Staphylococcaceae	Staphylococcus	MRSA	Sscmec orfx
MRSA_Owiz_664	GATCAAACGGCCTGCACAAGGACGTCTTACAACGTAGTAACTACGCACTATCATTCAG	4,5	Staphylococcaceae	Staphylococcus	MRSA	Sscmec orfx
MRSA_Owiz_665	AGAAAAAGAAGGCCAACGAATACTAGCCAAAATCAAACCACAATCCACAGTCATTACA	4,5	Staphylococcaceae	Staphylococcus	MRSA	Sscmec orfx
Mycobacterium_02	TGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGG	5	Mycobacteriaceae	Mycobacterium	*Mycobacterium*	16s_rRNA_Mycobacterium_bovis_AF2122/97 gi|31617962:155282-156818 Mycobacterium bovis subsp. bovis AF2122/97 complete genome; segment 5/14_228-285
Mycobacterium_03	TGGTGGGGACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCA	5	Mycobacteriaceae	Mycobacterium	*Mycobacterium*	16s_rRNA_Mycobacterium_bovis_AF2122/97 gi|31617962:155282-156818 Mycobacterium bovis subsp. bovis AF2122/97 complete genome; segment 5/14_1130-1188
M. tuberc+bovis_21	CGGCAATGGTGGCAGCGTCGAGCACACTGGCGCTACCGGCTCCTCTGCGAGCGGCGGCA	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	pe_pgrs5_Mycobacterium_bovis_AF2122/97 gi|31791177:362336-364156 Mycobacterium bovis AF2122/97, complete genome_960-1018
M. tuberc+bovis_22	GACGGCGGCAACGCCTCCACGAGTGGGGGCATCGGGATCGCGCAAACCGGGGGCG	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	pe_pgrs5_Mycobacterium_bovis_AF2122/97 gi|31791177:362336-364156 Mycobacterium bovis AF2122/97, complete genome_856-910
M. tuberc+bovis_84	TCGTTGTGAACAAGGCTGTGTCCTCGGCGCAAGCCCGTATCGCGGCACGTAAGGCACG	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	gyrB_Mycobacterium_bovis_AF2122/97 gi|31791177:5123-7267 Mycobacterium bovis AF2122/97, complete genome_1331-

						1388
M. tuberc+bovis_86	GCGGTTCTGCAAAAAGCGGTCGCGATTCGATGTTCCAGGCGATACTTCCGCTGCGCGG	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	gyrB_Mycobacterium_bovis_AF2122/97 gi|31791177:5123-7267 Mycobacterium bovis AF2122/97, complete genome_1508-1565
M. tuberc+bovis_90	GCAAGCCCGTATCGCGGCACGTAAGGCACGAGAGTTGGTGCGGCGTAAGAGCGCCACC	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	gyrB_Mycobacterium_bovis_AF2122/97 gi|31791177:5123-7267 Mycobacterium bovis AF2122/97, complete genome_1359-1416
M. tuberc+bovis_96	CGCAGTTCGAGGGCCAGACCAAGACCAAGTTGGGCAACACCGAGGTCAAATCGTTTGTGC	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	gyrB_Mycobacterium_bovis_T704 gi|6729234|dbj|AB014184.1| Mycobacterium bovis gyrB gene for DNA gyrase B subunit, partial cds, strain T 704_764-823
M. tuberc+bovis_98	AGGGCACCGGGCACGAGGTGGAGATCGCGATGCAATGGAACGCCGGGTATTCGGAGT	5	Mycobacteriaceae	Mycobacterium	*M. tuberculosis + M. bovis*	gyrB_Mycobacterium_bovis_T704 gi|6729234|dbj|AB014184.1| Mycobacterium bovis gyrB gene for DNA gyrase B subunit, partial cds, strain T 704_512-568
M. bovis_12	TGCAGAAGGTCTGTAATGAACAGCTGACCCACTGGTTTGAAGCCAACCCCACCGACTCGA	5	Mycobacteriaceae	Mycobacterium	*M. bovis*	gyrB_Mycobacterium_bovis_T704 gi|6729234|dbj|AB014184.1| Mycobacterium bovis gyrB gene for DNA gyrase B subunit, partial cds, strain T 704_821-880
M. bovis_03	TGGCTGGGCTTCGGCGGGGCCGGGGGCATCGGCGGAATCGGCGGTAACGCTAACGGGGG	5	Mycobacteriaceae	Mycobacterium	*M. bovis*	pe_pgrs9_Mycobacterium_bovis_AF2122/97 gi|31791177:837525-839918 Mycobacterium bovis AF2122/97, complete genome_2092-2150
M. avium_04	GCACGGCACCTCCACCCGCAAAGTCGACGATTTGGTCAAAGCCCTCGGCGCGGAC	5	Mycobacteriaceae	Mycobacterium	*M. avium*	Map_MAP0104 gi|41406098:110876-112111 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_330-384
M. avium_05	GTGTTCCTCGACGCCACTTACTGCAAGGCTCGGGTCAACCATCGGGTGGTCTCCCAAGCG	5	Mycobacteriaceae	Mycobacterium	*M. avium*	Map_MAP0104 gi|41406098:110876-112111 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_487-546
M. avium_08	CTTCGACGTCGGTGACTCCGAGGACGGCGCGTTTTGGACGGCGTTTCTGCGCTCACTGA	5	Mycobacteriaceae	Mycobacterium	*M. avium*	Map_MAP0104 gi|41406098:110876-112111 Mycobacterium avium subsp. paratuberculosis K-10, complete

						genome_597-655
M. avium_24	GATCTGGAGCACCAACCCCCTCGAACGACTCAACAAGGAGATCAAACGCCGCACCGACG	5	Mycobacteriaceae	Mycobacterium	*M. avium*	Map_MAP0849c gi|41406098:c872640-871405 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_993-1051
M. paraTB_48	TCGAGCAGCTTGACTCAGATGCGGTGGATGGACCTGGAAGCGAACTGCTTGAGAAGGGC	5	Mycobacteriaceae	Mycobacterium	*M. paratuberculosis*	Map_MAP0853 gi|41406098:875615-876274 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_455-513
M. paraTB_49	CGGGCTCGCCGTGATGTTGTTGCAAGCGTCCGTGATGGTCGAGCAGCTTGACTCAGATGC	5	Mycobacteriaceae	Mycobacterium	*M. paratuberculosis*	Map_MAP0853 gi|41406098:875615-876274 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_417-476
M. paraTB_50	CGGTGGATCGTCTCGCTAACGGGCCGCAACGTGTGGTTGAGCGTCTCACCTGCTGAACCA	5	Mycobacteriaceae	Mycobacterium	*M. paratuberculosis*	Map_MAP0853 gi|41406098:875615-876274 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_322-381
M. paraTB_51	GGAGGCGACGCGGAGGGCCAGAGCGTCGCTCTTGGTGATGAATATCCGAAGCGGTGTGGG	5	Mycobacteriaceae	Mycobacterium	*M. paratuberculosis*	Map_MAP0853 gi|41406098:875615-876274 Mycobacterium avium subsp. paratuberculosis K-10, complete genome_45-104
M. microti_01	GCGCGCCAGCGGTAACGCGTGGGATGTGTTTCCGTCCCTCTCGGGGTTTTGGGTC	5	Mycobacteriaceae	Mycobacterium	*M. microti*	gi|77543390|gb|DQ217928.1| Mycobacterium microti insertion sequence IS6110 nonfunctional transposase (Rv2814c) gene, complete sequence, and transposase (Rv2815c) gene, complete cds_2624-2678
M. microti_02	CTGACGACACGGGGCCGTGGGGCACTTACGGCGACGGCGCACATGTTTCCGTCCC	5	Mycobacteriaceae	Mycobacterium	*M. microti*	gi|77543390|gb|DQ217928.1| Mycobacterium microti insertion sequence IS6110 nonfunctional transposase (Rv2814c) gene, complete sequence, and transposase (Rv2815c) gene, complete cds_2750-2804
M. microti_04	CGGGGCGGTTCAGAGAGGGGACGGAAACATTTTGAGCGCGAACTCGTCCACAGTCCCCC	5	Mycobacteriaceae	Mycobacterium	*M. microti*	gi|6013442|gb|AF189829.1|MTDRLN2 Mycobacterium microti strain 16240 direct repeat locus and insertion sequence IS6110, 3'-sequence_774-832
M. microti_05	GCCAGGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACGCATATCGCCCGCCA	5	Mycobacteriaceae	Mycobacterium	*M. microti*	gi|6013442|gb|AF189829.1|MTDRLN2 Mycobacterium microti strain 16240 direct repeat locus and insertion sequence IS6110, 3'-sequence_982-1037

M. canettii_16	GTCGAACGCTTCTTCCGCACCCTCAACAGCGAACTGGACGTACGCCCCATCCGGCATCGG	5	Mycobacteriaceae	Mycobacterium	*M. canetti*	gi|53747881|emb|AJ619854.1| Mycobacterium canettii insertion sequence ISMyca1_1475-1534
M. canettii_17	GATCACTCACCCCGACTATCCCGGTGAGCGGCTGGTGTGCTGCCACAACCCCGCCC	5	Mycobacteriaceae	Mycobacterium	*M. canetti*	gi|53747881|emb|AJ619854.1| Mycobacterium canettii insertion sequence ISMyca1_1090-1145
M. canettii_19	CGCTGAACAAGGCAGCACGCAAACGCACCGAAGACAACCAACCGGTGCACAGCTTCACCA	5	Mycobacteriaceae	Mycobacterium	*M. canetti*	gi|53747881|emb|AJ619854.1| Mycobacterium canettii insertion sequence ISMyca1_1701-1760
M. canettii_20	GCACAAGCTGGACAGACTGGACCGGGCGCTTAAGGGCTTGCCGCCCGCGGACTGG	5	Mycobacteriaceae	Mycobacterium	*M. canetti*	gi|53747881|emb|AJ619854.1| Mycobacterium canettii insertion sequence ISMyca1_292-346
PUUV_UPS_S28	TGCAGCCTCTGGAGACCCCACATCACCTGATAACATTGATTCTCCTAATGCGCCATGGGT	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S29	AGAAGTCCTCTTTCTACCAATCTTACTTACGCCGGACTCAATCAATGGGAATTCAGCTTG	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S30	ATGCCTACTGCCCAGTCCACCATGAAAGCTGAAGAACTTACACCCGGACGGTTCCGTACA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S31	AGGAATCCCCCTTCATAAAGCCAGAAGTTAAACCCGGGACACCAGCACAGGAGATAGAAT	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S32	TGTGGCTGACATTGATAAGTTAATTGACTATGCTGCCTCTGGTGACCCTACATCACCTGA	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S33	CATTGACTCTCCTAATGCACCATGGGTCTTTGCCTGTGCACCAGATCGGTGTCCACCAAC	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S34	TGACGTTAATAAAAACACACTGCAAGCCAGGCAACAGACAGTGTCAGCACTGGAGGACAA	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S35	CAGAGGAACTCACACCTGGGCGGTTCCGTACAATAGTATGTGGTCTGTTCCCTACTCAAA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_S36	AGAGAGCAGTGGAGGTGGACCCAGATGACGTTAACAAGAACACACTACAAGCAAGGCAAC	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_UPS_L37	ATGTGCTGGACAGTGCTATCCATCCTGATTCATTGTCTACTGACCTACAACTCATGAGGG	3	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUV_UPS_L38	ATCACTGAGTCTTTAATTGCACATGCAGGGCTTAAGCGATCTAAGTATTGGTCTGCCCAT	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUV_UPS_L39	TAGAGCAGTGGATTGACTCGTTCTGCTCAAGAGGGGTTTTGCCAGTTGATCCTGAGATTC	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUV_UPS_L40	ATTGTGCCAAATCAGCCTACAAGCACCATATTTGCCTTCAACCATTGCTTCAGACCAATA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase

PUUV_UPS_L41	GAAGGCACTGAGGTGGGCTTCAGGAGAGAGTGAAATCCAGTCCTCATTAGGTCACTCTAT	3	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUV_UPS_L42	ATACGGACACTCTAGAAAGCCTAGGAACTTCCTCTTGTGTCAAATAAGCTTACAAGCACC	3	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUV_UPS_M43	ATGAGCCTATGCAAGGGTGCTACCGGACTTTATCCCTCTTTAGATATAGGAGTCGGTTCT	3	Bunyaviridae	Hantavirus	Puumala hantavirus	G1 and G2 glycoproteins
PUUV_UPS_M44	GCAGTAAGTGCCTTACGCATTGCTGGTAAGGTAACTGGTAAAGCGCCATCCACAGAAGCA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	G1 and G2 glycoproteins
PUUV_M45	TTAGTCCAGTTTGTCTGTATCTGCTTCTCCAGGGTCTATTACTATGTAATACAGGGGCTG	3	Bunyaviridae	Hantavirus	Puumala hantavirus	G1 and G2 glycoproteins
PUUV_M46	TGCAGTGAGTGCAATGCGTATTGCTGGAAAGGTGACAGGAAAGGCGCCATCAACAGAATC	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	G1 and G2 glycoproteins
PUUV_M47	GATCAAAGGATTCAAGTAAATTATGAAAAAACATACTGTGTTTCTGGGCAGCTTGTTGAA	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	G1 and G2 glycoproteins
PUUV_M48	ATTATATCTGTCTTATAGGGAGTAGTTCAGAGCCCTTATATGTTCCAGCATTAGATGATT	3	Bunyaviridae	Hantavirus	Puumala hantavirus	G1 and G2 glycoproteins
PUUV_S49	AACAGCAGCTTGTTGTTGCCAGACAAAAACTTAAGGATGCAGAAAGAGCAGTGGA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_S50	CTACGAGAAAGACTGGAATGAGTGACTTGACAGATATCCAAGAGGATATAACCCGCCATG	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_S51	TTGCCAGACAAAAACTTAAGGATGCAGAAAGAGCAGTGGAAGTGGACCCAGATGACGTTA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_S52	ACTGGAGGACAAACTCGCAGACTACAAGAGAAAGATGGCAGATGCTGTGTCCCGGAAAAA	3	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_S53	ATACAATCGGAGTTTATGTAATAGGGTTCACACTTCCTATCATCCTTAAAGCTTTATACA	3,5	Bunyaviridae	Hantavirus	Puumala hantavirus	N protein
PUUV_L54	AGAAGGTTATCATATACAGGATGTCTTAAACAATCAAGTGGTTGTTGAGCAACAACTCTT	3	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUV_L55	GAAGAAAATCCCAGATTTCTACTTGCTCATGCATACCATAATTTAAAGGATGTAAGATAT	3	Bunyaviridae	Hantavirus	Puumala hantavirus	RNA dependent RNA polymerase
PUUVUPS_545	GATAGGGTTCACACTTCCTATCATCCTTAAAGCTTTATACATGCTCTCAACGCGTGGGAG	4	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_546	TGACTTGACAGATATCCAAGAGGATATAACCCGCCATGAACAGCAACTTATTGTTGCCAG	4	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_547	TGATTCGCCTAATGCACCATGGGTCTTTGCATGTGCACCAGACCGATGCCCACCAACATG	4	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_548	ATGACGTTAATAAAAACACACTGCAAGCCAGGCAACAAACAGTGTCAGCACTGGAGGACA	4,5	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein

PUUVUPS_549	TTATATGTTTCTATGCCTACTGCCCAGTCAACTATGAAAGCAGAAGAACTCACACCTGGC	4,5	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_550	AGATGGTAGATCACTTCCATCTTGGTGATGATATGGATCCAGAGCTCAGAGGTCTTGCAC	4	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_551	TGGATACTAAACCTACTGACCCGACTGGGATTGAACCTGATGACCACCTCAAGGAGAGAT	4	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_552	AGAGTTCATGGAAAAAGAGTGCCCATTCATAAAGCCTGAAGTAAAACCAGGCACACCAGC	4	Bunyaviridae	Hantavirus	PUUV	nucleocapsid protein
PUUVUPS_553	TGCAGTGAGTGCCTTACGCATTGCTGGGAAGGTTACAGGTAAAGCACCATCCACTGAATC	4	Bunyaviridae	Hantavirus	PUUV	glycoprotein
PUUVUPS_554	GGGAGTAGCTCAGAGCCATTGTATGTTCCCACGCTGGATGATTATCGGTCAGCTGAGGTT	4,5	Bunyaviridae	Hantavirus	PUUV	glycoprotein
PUUVUPS_555	CCACACATCTCTACAAGTGCATTGGAGTGGATTGACCTAGATAGTTCACTTCGGGATCAC	4,5	Bunyaviridae	Hantavirus	PUUV	glycoprotein
PUUVUPS_556	CAATATAAGCTCACCTACTTGCCTGATAAACCGTGTTCAAAGATTCCGTGGTGCTGAACA	4	Bunyaviridae	Hantavirus	PUUV	glycoprotein
PUUVUPS_557	TAATCCCCCTGATTGTCCTGGTGTAGGGACAGGCTGCACTGCTTGCGGTGTATATCTGGA	4	Bunyaviridae	Hantavirus	PUUV	glycoprotein
PUUVUPS_558	ATGGCCCTCTCTCAGCCAAGCCATACTTATGATATAGTGACCATAATGGTTCGCTGTTTT	4	Bunyaviridae	Hantavirus	PUUV	glycoprotein
PUUVUPS_559	AGGCTGATCTTGAACTGGATTTCTCATTACCTTCATCTGCTAGCTATACTTACCGAAGAC	4	Bunyaviridae	Hantavirus	PUUV	glycoprotein
R.typhi_Owiz_668	TCTATGTTACAAGCATGTAATGGTCCGGGTGGTATGAACAAACAAGGGACTGGAACAC	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	17kda
R.typhi_Owiz_669	GCATTACTTGGTTCTCAATTCGGTCACGGTAAAGGACAACTTGTCGGAGTAGGTGTAGG	4,5	Rickettsiaceae	Rickettsia	*R.typhi+prowazekii*	17kda
R.typhi_Owiz_670	AGCTTTAGAATCTGCTCCTAGCGGTAGTAACATAGAATGGCGCAATCCAGATAATGGCAA	4,5	Rickettsiaceae	Rickettsia	*R.typhi+prowazekii*	17kda
R.typhi_Owiz_675	CAGTAAAGTCTATTGATCCTACACCGCTAAGATCATTATCGTTAATAGCAGCACCAGCA	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	ompB
R.typhi_Owiz_676	ACATTACTATATTTACACAACCTAATGCAATATGCATGACATATATTCCATGCTTTAC	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	rtpg04
R.typhi_Owiz_677	AAAATTCCTATCTCTTAGTGCTTTGACATTACTATATTTACACAACCTAATGCAATATG	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	rtpg04
R.typhi_Owiz_678	TGATCATTAAATGCAGCAGTCTTGAGTTAGATATTGCAATTTTGAAGACACAAGAAAA	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	rtpg04
R.typhi_Owiz_681	AGAAAAATGTTTAGAACAGACTACTCCAGAGCAACAAGAGTTGATCTTAGATGTATTG	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	sca-2
R.typhi_Owiz_682	CAGAGACAAAAGTATTTAAAAAAGAGATAGCTTTAGAAGAAAACGAAGCTATGGATGTGT	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	sca-2

R.typhi_Owiz_683	GTACACAAAGAAAAGATTGCAGATCTAACAGCAGAAACAGAGACAAAAGTATTTAAAA	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	sca-2
R.typhi_Owiz_686	CACAAATTCTATTACTTGATGAAATTTTTGCAGCAGGTGATAGCTATTTTATAGAAAA	4,5	Rickettsiaceae	Rickettsia	Multiple Rickettsia	rt0003
R.typhi_Owiz_687	TTTCCTTATCGCTTTTTGCATATGGTTACTGCATCTTATTTAACTACTTCTTTTGTGAT	4,5	Rickettsiaceae	Rickettsia	*R.typhi+prowazekii*	rt0207
R.typhi_Owiz_688	CCTTTAAATTGGTTAGCAATTATCTTTAATCCATCTTTTCCTTATCGCTTTTTGCATAT	4,5	Rickettsiaceae	Rickettsia	*R.typhi+prowazekii*	rt0207
R.typhi_Owiz_689	ATTTGCAGCTTTTCCTTTAGCCTATTCGATATTAATGTCTGCCTTATATATCCCTATCAT	4,5	Rickettsiaceae	Rickettsia	*R.typhi+prowazekii*	rt0208
R.typhi_Owiz_693	AGCTTAGAATTTTAGCTGGTGTGTAAGATCTTTATACTGTTATCTTAAAAATTCAAGA	4,5	Rickettsiaceae	Rickettsia	*R.typhi*	rtpg21
Salmonella_Owiz_695	TGAGATTCCAATCATCTTGGCTGATGGTCCGCTTTACTGGCGAGATATGACTCGTGCGG	4	Enterobacteriaceae	Salmonella	*S. enteritidis*	sen1961
Salmonella_Owiz_698	TTATCAGCGCGGGTATGTCTGGGGGCATGAAGAGCAGCAGGCATTCTTAAACGCAGTT	4	Enterobacteriaceae	Salmonella	Multiple	sen1961
Salmonella_Owiz_699	GTTTATAAAAAACGTCTCAATCAAAATCGCGATCGCGCAATCGTGTTGGCAAAGGGAGG	4	Enterobacteriaceae	Salmonella	Multiple	STM3777
Salmonella_Owiz_702	CATTGCAACCATAATCCTGAAACGTCCGTACTGGCACATTACCGGCTGGCGGGTACGTGC	4	Enterobacteriaceae	Salmonella	*S. enteritidis*	sen1390
Salmonella_Owiz_703	TTACGTAAAGCGGCGCGGGGCCTGATGTGTACGGTAAGAATTCCCGGCCATTGCAACC	4	Enterobacteriaceae	Salmonella	*S. enteritidis*	sen1390
Salmonella_Owiz_704	GTACTGGCACATTACCGGCTGGCGGGTACGTGCGGAACGGCGACAAAACCAAACGATAT	4	Enterobacteriaceae	Salmonella	*S. enteritidis*	sen1390
Salmonella_Owiz_705	GGGCGGGTAAAAATCGACGACTTCACGAAAACAGAAATTCGCCTGATGCACGCAGAGGGC	4	Enterobacteriaceae	Salmonella	*S. enteritidis*	sen1390
SalmonellaUPS_709	TGGCGCAGAAGTTAGGTTGTCGATGAGAAGCGCTATACGGCGCGTAGAAAGATAACGGAG	4,5	Enterobacteriaceae	Salmonella	Multiple	endonuclease
SalmonellaUPS_710	TTACCATCATCATGCCGGACGAAGATAGCGATTTTCGTCTGTGTCGAAGGTTGTGCGCCA	4,5	Enterobacteriaceae	Salmonella	Multiple	endonuclease
SalmonellaUPS_711	TTGCGACTATCAGGTTACCGTGGAGGCTATCGAACATAAAGCGAAGCCAGTGCTGACGCT	4,5	Enterobacteriaceae	Salmonella	Multiple	endonuclease
SalmonellaUPS_733	TCACCATCCCCAGGCCCATATACATGCTCTAATCGCATGTTTACAAATGAAATGTCATGC	4,5	Enterobacteriaceae	Salmonella	*S.typhimurium*	CDP-abeguose synthase
SalmonellaUPS_734	CCACATCATCTACAAAAATAAAGTCTCTTATCTGTTCGCCTGTTGTACATTTCACGCAAC	4,5	Enterobacteriaceae	Salmonella	*S.typhimurium*	CDP-abeguose synthase
SalmonellaUPS_735	CCAGCACCAGTTCCAACTTGATACTCAGTATATGAAGGTACTTCTTTTCTATTTTCTAAT	4,5	Enterobacteriaceae	Salmonella	*S.typhimurium*	CDP-abeguose synthase
SalmonellaUPS_736	ATGCAGAATCAATTGATAACTCCTCGACTAATAATTCGATATTATCCCAACTGCACCATC	4,5	Enterobacteriaceae	Salmonella	*S.typhimurium*	CDP-abeguose synthase

SalmonellaUPS_737	ATGCCCAATTTCATCAAAGTGTCTTTTAGTAATTATATAAGGCCGCATATGTTGATAATT	4,5	Enterobacteriaceae	Salmonella	*S.typhimurium*	CDP-abeguose synthase
SEOV_UPS_L1	ATTTGGGAGCATCGCCTCAAATCTCACAACTTGCAGTCTCACTTAGTACGAGTAAGGTTG	3	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_UPS_L2	TAGGAGAGCCTGTAGAATCTAGTGAACTAGCAGTCCAACAACTATATTGTCAAGACGCAG	3	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_UPS_L3	GATAGCCCACGGTTTCTTTTGGCACATGCTTTTCATACTATCCGTGATGTTCGCTACCAA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_UPS_L4	TAACTCATTGACAGCCGTGGAATGCATGGATTACTTGGATAGGCTCTATGCTGTTCGGCA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_UPS_M5	CCCTTTAATCTGGAGGGGCCTAATTGACTTAACGGGATACTATGAGGCAGTCCACCCTTG	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_UPS_M6	CAACTGGCCTCCAAGCCAGTGCACCACATTTGGATAAAGTAAATGGTATCTCTGAGTTAG	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_UPS_M7	AATCCCCCTTGTCCCTCTCTGGACAGATAATGCTCATGGCGTTGGGAGTGTTCCTATGCA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_UPS_M8	AATAGGTTTAGAGCAGCTGAGCAGCAGATCAGCTTTATCTGCCAAAGGGTCGATATGGAT	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_UPS_S9	TCACACCTGGGAGGTTCCGCACTGCAGTTTGTGGGCTATATCCTGCACAAATAAAAGCAA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_UPS_S10	GATGGAGCCTAAAGAGTTTCAAGCCCTCAGGCAGCATTCAAAAGATGCCGGTTGTACACT	3	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_UPS_S11	GGCTTGACACACCCTGTAAGTTCATGGTAGAGTCTCCCATTGCCGGGAGTTTATCTGGGA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_UPS_S12	ATGGGTGTTTGCTGGGGCCCCTGATAGGTGTCCACCAACATGCTTGTTTGTCGGAGGGAT	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_UPS_S13	GTGAGCCCTGTCATGAGTGTAGTTGGGTTTTTGGCACTGGCAAAAGATTGGACATCTAGA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_UPS_S14	GAAAACCCAAACATCTGTATGTGTCAATGCCAAACGCCCAATCCAGCATGAAGGCTGAAG	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_UPS_S15	ATATTGAGTCACCATCGTCAATATGGGTGTTTGCTGGGGCCCCTGACAGGTGTCCACCAA	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_L16	AGGGATCTGAAGGAGTTTACTATTAACTCATTGACAGCCGTGGAATGCATGGATTACTTG	3	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_L17	ATTTGGATTCAATACTTGCAGATGGTGATATCTTATGGGGTGTCTCAAAAGTTATGAGCC	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_L18	AACAAATGAGTTACATCCTGGGGCTCTTCAAGTTTTTAATGGATCTTTCTGGTGAGACAT	3	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_L19	GATATCTCTTCTCCAGATGTTATGAAGAAAGTGGCACCTATGTTGTATAAAAGTGCAAAA	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase

SEOV_L20	TGTACAAAAATTGTGCATTGAGGACAGGTTTGAGGACAAACCAGGCCATCAACCACAAGT	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	RNA dependent RNA polymerase
SEOV_M21	ATTATATCTGTATTATTGGTGGAAACTCTGCTCCTGTATATGCCCCCGCTGGTGAAGACT	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_M22	TTTCAGGGCAGATAGAGGCAAAAATCCCTCATACAGTGAGCTCTAAAAGCTTGAAATTGA	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_M23	GCTGGGTATTGATTCCTGCATGCACATTAGCTATTCTTTTAGTCCTAAAGTTTTTTGCAA	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_M24	AAAGCCGGTAGGAACAGCCTTTAAAATTATAAGTGTAAGATATAGTAGAAAAGTGTGCGT	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_M25	CCTACCCATTAATCTTCACACCATGTATCGAATTTTAAACACTTTACCATTAAACAACTT	3	Bunyaviridae	Hantavirus	Seoul hantavirus	G1 and G2 glycoproteins
SEOV_S26	AGCATGAAAGCTGAAGAGATAACACCTGGAAGATTCCGCACGGCAGTCTGTGGGCTATAT	3	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOV_S27	ATGGCGGAATCTCCCATTGCCGGGAGTTTATCTGGGAATCCTGTGAATCGTGACTATATC	3,5	Bunyaviridae	Hantavirus	Seoul hantavirus	N protein
SEOVUPS_530	ACATATTGAGTCACCATCGTCAATATGGGTGTTTGCTGGGGCCCCTGATAGGTGTCCACC	4	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_531	GGCTTGGCGCACCCTGCAAGTTCATGGCAGAGTCTCCTATTGCTGGGAGTTTATCTGGGA	4,5	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_532	AGCCCTGTCATGAGTGTAGTTGGGTTTTTGGCACTAGCAAAAGACTGGACATCTAGAATT	4,5	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_533	GGATTGCAGCAGGGAAGAACATCGGGCAAGACCGGGATCCTACAGGGGTAGAGCCGGGTG	4	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_534	GAGATAACACCAGGAAGATTCCGCACTGCAGTATGTGGGCTATATCCTGCACAGATAAAG	4,5	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_535	GCTTGTTTGTTGGAGGGATGGCTGAGTTAGGTGCCTTCTTTTCTATACTTCAGGATATGA	4	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_536	TCAGCACTAAGCTACGGGAATACACTGGACCTGAATAGTCTTGACATTGATGAACCTACA	4	Bunyaviridae	Hantavirus	SEOV	nucleocapsid protein
SEOVUPS_537	GCACGTCTACCTGTCACTTTGGAGACCCTGGTGATGTCATGGGTCCAAAGGATAAACCAT	4,5	Bunyaviridae	Hantavirus	SEOV	glycoprotein
SEOVUPS_538	TTTTTCCGGGATTATTACATCACCGCATGGAGAAGACCATGATCTCCCTGGCGAAGAAAT	4,5	Bunyaviridae	Hantavirus	SEOV	glycoprotein
SEOVUPS_539	ACAGGTTGTACTGCTTGTGGGTTATATCTCGATCAATTGAAGCCGGTAGGAACAGCCTTT	4	Bunyaviridae	Hantavirus	SEOV	glycoprotein
SEOVUPS_540	AAATCCCCCTTGTCCCTCTCTGGACAGATAATGCTCATGGTGTTGGGAGTGTTCCTATGC	4	Bunyaviridae	Hantavirus	SEOV	glycoprotein
SEOVUPS_541	CCTTTAATCTGGAGGGGCCTAATTGATTTAACAGGATACTATGAGGCAGTCCACCCTTGC	4	Bunyaviridae	Hantavirus	SEOV	glycoprotein

SEOVUPS_542	AAGGCTGTCGTCAGTGCATTGAAGAGAGGCATGTACGCCATAGCAAGCATAGAGACAATC	4	Bunyaviridae	Hantavirus	SEOV	glycoprotein
SEOVUPS_543	TGATTGGTCTTGGTCCTTACAGAGTCCAGGTCGTTTATGAAAGGACATACTGCACTACGG	4	Bunyaviridae	Hantavirus	SEOV	glycoprotein
SEOVUPS_544	TTGGCTGAGAATCCTTGTAAAGTAGGGCTCCAGGCAGCAAACATAGAAGGTGCCTGGGGT	4	Bunyaviridae	Hantavirus	SEOV	glycoprotein
S.monil_740	ACTTTCTAAAAACAGCTCTGTTAAATCTTTTCCTATTACACTAACTTTTGAAACTTTAAC	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	Rhoptry protein
S.monil_741	TTTACCTTTAAATTCTATTTTCATGCCAGCACTTCTACTTGAAGCATATAGATACATCTT	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	Rhoptry protein
S.monil_742	TGCTAAGAGAGAGCTTTGCATCCTATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCT	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monil_743	GTCGCAAGCGTTATCCGGAATTATTGGGCTTAAAGGGCATCTAGGCGGTCTAACAAGTTG	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_744	GTATGTCGCAAGCGTTATCCGGAATTATTGGGCTTAAAGGGCATCTAGGCGGTCTAACAA	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_745	GTCGACAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGAAACCCCTATCA	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_746	AGCATGTGGTTTAATTCGACGCAACGCGAGGAACCTTACCAGGTCTTGACATACTCGGAA	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_747	TAAAGGCCTACCAAGGCTATGATAGGTAGCCGGCCCGAGAGGGTGAACGGCCACAAGGGG	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_748	GAAAAGCTGTGGCTCAACCATAGTCTTGCCTACAAAACTGTCAGACTAGAGTACTGGAAA	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_749	TCTGATCCAGCAATTCTGTGTGCACGAAGAAGGTTTTCGGATTGTAAAGTGCTTTCAGTA	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_750	ATGACGTCAAGTCATCATGCCCCTTATGATCTGGGCTACACACGTGCTACAATGGGTAGT	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_751	AGGCATCTACTATTAATGAAAGGAGAGATTGCTAAGAGAGAGCTTTGCATCCTATTAGCT	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	16s rRNA
S.monilUPS_752	AGCATCTGAAATATTAGCCTCTGGTACTATCAATGGTACTTCATCATCCATACGCCATGC	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	aspartate-semialdehyde dehydrogenase
S.monilUPS_753	AATTTAGGTGCAAACTCTAATGAAGTTGAACCTCCTGCTGAAAATAGGGCAAAGTCTATA	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	aspartate-semialdehyde dehydrogenase
S.monilUPS_754	CTATGTGAGGTATAAGGTTAAATGCTATTTGTGAAGGAAAATTAGTTGAAGCTTCCCCTT	4,5	Fusobacterium	Streptobacillus	*S. moniliformis*	aspartate-semialdehyde dehydrogenase
ToxoplasmaUPS_359	TGGTCCGTCGTATATCAGGCCTTCTGTTCTGTTCGCTGTCTGCCTAGGGCACCCTTACTG	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene
ToxoplasmaUPS_360	TATTACCCGCTGGCAAATACAGGTGAAATGTACCTCCAGAAAAGCCGCCTAGTATCGTGC	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene

ToxoplasmaUPS_361	TCCGTCGTAATATCAGGCCTTCTGTTCTGTTCGCTGTCTGTCTAGGGCACCCTTACTGCA	2,4,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene
ToxoplasmaUPS_362	GCTGCCGCTGTTTTGCAAATGAAAAGGATTCATTTTCGCAGTACACCAGGAGTTGGATTT	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene
ToxoplasmaUPS_363	TTCATTTTCGCAGTACACCAGGAGTTGGATTTTGTAGAGCGTCTCTCTTCAAGCAGCGTA	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene
ToxoplasmaUPS_364	TTCATTTTCGCAGTACACCAGGAGTTGGATTTTGTAGAGCGTCTCTATTCAAGCAGCGTA	2,4,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene
ToxoplasmaUPS_365	CCGCCGTGAAGCAATCGAAGAATTTCCGAAGTCAAGAAGAGCGAACCTTCTGTCATCTCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	C22-8 gene
ToxoplasmaUPS_366	GCTGAACGGAAAATGCAGCAAACCTGTGAACTCGCACAGCTGTAGCGACGAGCACTACCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	C22-8 gene
ToxoplasmaUPS_367	CTTTGTGCAGGGTTTTGGAGAGATTGAAATTCCTCGAGAAGCAAATTCGGAGGTGCCACT	2	Sarcocystidae	Toxoplasma	*T. gondii*	C22-8 gene
ToxoplasmaUPS_368	CGCGCGTTGCACCCGGAACATGTGAATGTGTTTGATTATTAAAGATGAAAACAGGGGGTC	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-6 gene
ToxoplasmaUPS_369	AGCGTAACTTCTGTCCTTTAACTGTCTCCACAGTTGCTGTGGTCTTTGTAGTCTTCATGG	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-6 gene
ToxoplasmaUPS_370	AAGACGACGTTACTCCTCTGTTCAAGAACCACAAGCGAAGGTGCCTAGCAAACGAACACA	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-6 gene
ToxoplasmaUPS_371	GAACCCCTAACCACCGGCCAGAATGTGGGCACTGTGTTAGGCTTCGCAGCGCTTGCTGCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-7 gene
ToxoplasmaUPS_372	ATGAGTCGAATCCGAAATTCTGACTTTTTCGATGGTCAAGCACCCGTTGACAGTCTCAGA	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-7 gene
ToxoplasmaUPS_373	CCGGCTGTTGGTATGGGTGCATCGTATTTTGCCGCTGATAGACTTGTGCCGGAACTAACA	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-7 gene
ToxoplasmaUPS_374	GCCGTAGCGTTCCTTGGCCTGGGTATCAAGAGGACGTACCGACATTTTTCCCCACGCAAA	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-7 gene
ToxoplasmaUPS_375	GGCGAAGATGGGGAGGATGCCCGCCAGTAGGATATGGGGGCTAATAAAAGTGAGTAGGAG	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-7 gene
ToxoplasmaUPS_376	TTCCCCACGCAAAAACAGATCACGGCAGCCTGCACTCGAGCAAGAGGTGCCTGAATCAGG	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA-7 gene
ToxoplasmaUPS_377	AGGAGAAGCTGATCTCTGCCTCTCTCCTCGCCTTCTTGTCTATCAGCGGGGTGGCGGCGC	2	Sarcocystidae	Toxoplasma	*T. gondii*	L358 gene
ToxoplasmaUPS_378	CAGAAAGAGGACATGCATGCGAGTGTGTCGTCCGGAAGAGCAGAGACTCGCACCCTTGCA	2	Sarcocystidae	Toxoplasma	*T. gondii*	L358 gene
ToxoplasmaUPS_379	CATGGGCGGAGAAAGCGAAACCTTCCTGCTGCGCGGTTTCCGTCGCTGTCTCGTCGCTCG	2	Sarcocystidae	Toxoplasma	*T. gondii*	L358 gene
ToxoplasmaUPS_380	AAGAACCTGTTAGAGTGCACCCGTACATCAGTATTCAGACCATCGCCTTCTCAGCGTATG	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC-1 gene

ToxoplasmaUPS_381	TTTGAATGGTACGAGCGCACTCAGTCGTCCTCTTTTTCAAGGCTTCTATCTGAGTTTACT	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC-1 gene
ToxoplasmaUPS_382	GCAGAACTCTGCCAAAGCGGACTTCGTAAAATGTGTGTGCCCTCTAGCCGGATAGTAGCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC-1 gene
ToxoplasmaUPS_383	AAGAGCCAGCAGGACCCGCAGTGCTTACGATAGGTCTGGGACACAAAGGCCGTCTCGCTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC-1 gene
ToxoplasmaUPS_384	TGCTAGACGTCGCACCGGCTGAAAGCTGTAGAAAATTTAGTTATTTTCCTGTCAGCTAGC	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC-1 gene
ToxoplasmaUPS_385	GTATAGTAGGTGGAGTGAGGAGTGGTTTCAGGCGTGCAAGAGAAGGTGTCGTTGGGGGAG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 1 gene
ToxoplasmaUPS_386	GGCATACGCGGCAGCTTCCTCATGTCCGTAAAGCCACACGCAAACGCTGATGACTTCGCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 1 gene
ToxoplasmaUPS_387	CCTTTCGAGCCACAATGGAGTCCCCGCTTATCCATCGTATGCACAGGTATCGCTCTCTTC	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 1 gene
ToxoplasmaUPS_388	CTGCAAACTCATTGTTCGCGTTCCGGGTGCCGATGGCCGTGTCACATCTGGGTTTGACCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
ToxoplasmaUPS_389	TTCTCTGTTACAAGTGCGTTGCCGAAGCAGGTGCTCCCGCTGGTCGAAATAATGATGGTT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
ToxoplasmaUPS_390	CCGCGAAAGGTCCTGCTACCTACACACTGTCTTACGACGGTACTCCCGAAAAACCTCAGG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
ToxoplasmaUPS_391	CGGGTTGCAGTTCTAGGAACTGAGTTGTGATTGTGCACAATTGCGGTGTGACACCTTCTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
ToxoplasmaUPS_392	CTCGTTCCAATCTTTGTCTTGTCGGAACTATGAGTTTCTCAAAGACCACGAGCCTAGCGT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
ToxoplasmaUPS_393	CCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAGGTGATGTCTTTTATGGCAA	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
ToxoplasmaUPS_394	CGAATGGCTTCCGTTGCCCTGGCGTTCCTTCTCGGTCTCCTTGTGCATGTGGCTGCCTAA	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
ToxoplasmaUPS_395	CGCAGGGAAATCGAGAAGTAAGATAACCTATTTTGGCACGCTCACTCAGAAGGCTCCGAA	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
ToxoplasmaUPS_396	CCAATGGTCGAAGGCAAGAGAGTGACCTGCGGGTATCCCGAGTCCGGCCCCGTGAATCTC	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
ToxoplasmaUPS_397	ACCCACGAGTATGTCACATTGATGCCAAGGACCAGGACGACTGCGAGCGCAACAGGGGCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
ToxoplasmaUPS_398	CCAGTGTTTCTAGTTATGTAGCAGTACAAACTCCTCATGTGAAACGTTCAGAACGCATCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
ToxoplasmaUPS_399	TTTGCTCTTGCACTGTTGTTTTGTACACGCTGCGCAACTGCACGATACATGTCGTTTGAG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
ToxoplasmaUPS_400	GCATGGAGATGTTAAATTGAACAACATGATGATCGATGTCCACGGCTTTGGGCATATGCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene

ToxoplasmaUPS_401	GCTTAGACGATGGGACGCTGTATGCAGCGAAGGTGCCGTACAGCCAGATCGCCCCGAATG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
ToxoplasmaUPS_402	CCAGTGTTTCTAGTTATGTAGCAGTACAAACTCCTCATGTGAAACGTTCAGAACGCATCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
ToxoplasmaUPS_403	GACTCGTTGTCGTGTCTAAAGAAGTCATGCTGCTGCCATTAATTGATGGCTCCCCATCGA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
ToxoplasmaUPS_404	TCTCCGCCGCAACGATTGACACATCGAAAGCCATCTCTATCTGGGGTGGTCGTTACCGAA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
ToxoplasmaUPS_405	GATGGGGGAGGGCTTGCTTCGTCGGTCTTTAAGTTCGGAGACTGCCCTGAATTCAGTCGC	2	Sarcocystidae	Toxoplasma	*T. gondii*	BSR4 gene
ToxoplasmaUPS_406	CGAAAAAGCGCCGATTCTGCCTAAATCAGATGTCAAAGCATCATCTTGCCGTGTGCTTGT	2	Sarcocystidae	Toxoplasma	*T. gondii*	BSR4 gene
ToxoplasmaUPS_407	TCCCTTAGCGCCACCGTGAGTTGTTTGGGGCAAGGCAACACATTTGTACCAGCAGATCTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	BSR4 gene
ToxoplasmaUPS_408	ACTTTTGAGTGTTCTGCTACGCAGGCACTGAGCATATCTACAATACCGACTACTATTGGG	2	Sarcocystidae	Toxoplasma	*T. gondii*	BSR4 gene
ToxoplasmaUPS_409	GCTAACCGTAGCGGGTTCAGATGCGAAGTGTGATTTCTTTACGCCAACCACACAAGATTC	2	Sarcocystidae	Toxoplasma	*T. gondii*	BSR4 gene
ToxoplasmaUPS_410	AAAAACACACTGACCATTAATTGCGGTAGCGATGGTTCTCTCCAGCCGACGACATACGCA	2	Sarcocystidae	Toxoplasma	*T. gondii*	BSR4 gene
ToxoplasmaUPS_411	TGCCGCGCCCACACTGATGTCGTTCTTGCGATGTGGCGTTATGGCATCGGATCCCCCTCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
ToxoplasmaUPS_412	TCCATTTTCGCCATGGTGACCGGACTTATTGGCTCTATCGCAGCTTGTGTCGCGTGAGTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
ToxoplasmaUPS_413	TTGAGCTCCTTGATTCCTGAAGCAGAAGATAGCTGGTGGACGGGGGATTCTGCTAGTCTC	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
ToxoplasmaUPS_414	AGCCAGTCACGTTTCCATTTTTGCCATGGTGATCGGACTTATTGGCTCTATCGCAGCTTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
ToxoplasmaUPS_415	TGCCGCGCCCACACTGATGTCGTTCTTGCGATGTGGCGCTATGGGATCGGATCCCCCTCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
ToxoplasmaUPS_416	CTCCGAACTGGTACCGCTGCTCTTCAACGAGGGCGAAAGAAGAGGTCGTAGGACATGTGA	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
ToxoplasmaUPS_417	GCGATACCCGAAAGAGGATCGGCTCCTCCCATTGCAAGCCATGGAAACTCCTGAGTACGA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 8 gene
ToxoplasmaUPS_418	GGCAACATTTACACACGCGCTCAATGCGTGGCAACTGGGTCTTAGCATATACCGGGTTTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 8 gene
Toxoplasma_419	CCGCAGGTAAATCGAGAAGTAAGATAACTTATTTTGGCACGCTCACTCAGAAGGCTCCGA	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
Toxoplasma_420	AGGCGCGACGTCGTCGTACCCGCGAGTATGTCACATTGATGCCAAGGACAAGGGCGACTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene

Toxoplasma_421	GCGAAGCAGTACTGGTACAAGATAGAAAAGGTGGAGAACAACGGCGAGCAATCCGTTCTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
Toxoplasma_422	CCGGGAACCCCAGAAAGTGGGGGAGAGGCGGAGGCGGCCATCCAGGAAGCGGAGGATCGC	2,4	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 3 gene
Toxoplasma_423	TCCCGCTGGTCGAAATAATGATGGTTCTAGCGCTCCGACGCCTAAAGACTGCAAACTCAT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 2 gene
Toxoplasma_424	ATGACTTCGCCTCCGACGACAACTACGAACCGCTGCCGAGTTTCGTGGAAGCTCCTGTCA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 1 gene
Toxoplasma_425	CGGAAGAGCAACAGGAGGGCCCTCAGGAGCCGCTGCCACCGCCGCCGCCCCCGACTCGGG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 1 gene
Toxoplasma_426	TGACGCGCCTCCGCAGGCGCGGCAGAACTAATGGCGAGGAGGGCAGGCCCCTACTGGGCG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 1 gene
Toxoplasma_427	TCTCTCGCCGCTTCTTCGTCCGAAACGAGTCGCGAGAGAGGCGGCAGGCGAATGCAGGAG	2	Sarcocystidae	Toxoplasma	*T. gondii*	L358 gene
Toxoplasma_428	GTGGCGGCGCGACATTGCAGCGTCGACGCGCAGCCGAGCGAAGGAAGCGGGGAAGACGAA	2	Sarcocystidae	Toxoplasma	*T. gondii*	L358 gene
Toxoplasma_429	GGTTCCGACGCTGAAGTGACTGACGACCACATCTACGAGGAGCACACTGATCGTAAAGTG	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA 7 gene
Toxoplasma_430	GAATTCTGCCGGAACTAACAGAGCAGCAACAGACAGGCGACGAACCCCTATCCACCGGCC	2	Sarcocystidae	Toxoplasma	*T. gondii*	GRA 7 gene
Toxoplasma_431	CGGTGGTGTTAAGCAGACCCCTTCGGAAACCGGTTCGAGCGGTGGACAGCAAGAAGCAGT	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	GRA 6 gene
Toxoplasma_432	TCGAAGAACAGGGTACAAGACGGCGTTACTCCTCTGTTCAAGAACCACAAGCGAAGGTGC	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	GRA 6 gene
Toxoplasma_433	AGGCAGAGGTGAAGGAGGAGGCGAGGATGACAGGCGCGCGTTGCACCCGGAACATGTGAA	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	GRA 6 gene
Toxoplasma_434	ATTCCCGCCGTGATGCAATCGAAGAATTTCCGAAGTCAAGAAGGGCGAACCTTCTGTCAT	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	C22-8 gene
Toxoplasma_435	TTGCATCATAACAAGAGCTGTATTACCCGCTGGCAAATACAGGTGAAATGTACCTCCAGA	2,4,5	Sarcocystidae	Toxoplasma	*T. gondii*	B1 gene
Toxoplasma_436	GCAGGCATAGAAACCGGGGAGGGTCTCCTACGGCAGGGCAACCTTCTCAATCCACACTAC	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_437	ACACAGTAGCGGACCGGGAGGATGGTTTCCAACAGAGTCAATATATACGCTATGGAGTTC	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_438	CGATTGACACATCGAAAGCCATCTCTATCTGGGGTGGTCGTTACCGAATTTCAAGAGCCA	2,5	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_439	GTGCCGACGCCTGCGTCGCTTGGTCAAGAAAATCCTCTTTTTCCTGGTCAGAGCGCTACA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_440	AGCTTGCCTTCAGTCATTCTAGTTATGTATCAGTACAAGCTTCTCTTGCGAAACGTTCAG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene

Toxoplasma_441	GGATGTTCGGGATAAGCTTGTGGCTAAAGGGCTCACACTTACAGAGACTGTGAGCCAATA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_442	GGATGTTCGGGATAAGCTTGTGGCTAAAGGGCTCACACTTACAGAGACTGTGAGCCAATA	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_443	CAGCCTTTCGAATCTGCCGAGTTCCTTCTGGTCGAGAGTTGAACACCTTTCGCGCCTTCG	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 16 gene
Toxoplasma_444	CCCAGATAAAAAATCGACAGCCGCGGTCATTCTCACACCGACGGAGAACCACTTCACTCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
Toxoplasma_445	GATTCCTGAAGCAGAAGATAGCTGGTGGACGGGGGATTCTGCTAGTCTCGACACGGCAGG	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
Toxoplasma_446	ACGCAGACGTTTGTGGTCGGTTGCATCAAGGGAGACGACGCACAGAGTTGTATGGTCACA	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
Toxoplasma_447	TTCCGGGACGACGCTGACTGGTTGCAACGAGAAATCGTTCAAAGATATTTTGCCAAAATT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
Toxoplasma_448	GGGGATCGCCTGAGAAGCATCACTGTACCGTGCAACTGGAGTTTGCCGGGGCTGCAGGGT	2	Sarcocystidae	Toxoplasma	*T. gondii*	SAG 1 gene
Toxoplasma_450	GAGAAGGCCAGCTTGCTGTACTCGGTTTATGCTGAGTGTGCGAGCAACTGCGGAACAACG	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC 1 gene
Toxoplasma_451	TCGGGGCCTTGTATTGAATCTACTTTCTGAGGTTCTTGCTTATCTCTTTAATGCAGCATG	2	Sarcocystidae	Toxoplasma	*T. gondii*	MIC 1 gene
Toxoplasma_452	TGTAGCGCTCTGCAACAACCAAGACCTCACAACATCTGCCAGATGGCGACGAAGCTCGCT	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 8 gene
Toxoplasma_453	TCATGCGCGACCTGCTGAAACATGAAAAAGAGCTGATTGGATACTGTCGCGAAGAAGCGT	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 8 gene
Toxoplasma_454	TGCAGAGCAAAGGACTTGTGCATGGACGCTTCACACCGGATAACCTTTTTCTTATGGGGT	2	Sarcocystidae	Toxoplasma	*T. gondii*	ROP 8 gene
T.gondiiUPS_755	TCGTCCCATGAAGTCGACCACCTGTTTCCTCTCTTCACTGTCACGTACGACATCGCATTC	4	Sarcocystidae	Toxoplasma	*T.gondii*	B1
T.gondiiUPS_756	GGTCCGCCCCCACAAGACGGCTGAAGAATGCAACATTCTTGTGCTGCCTCCTCTCATGGC	4	Sarcocystidae	Toxoplasma	*T.gondii*	B1
T.gondiiUPS_760	CGAATGGCTTCCGTTGCCCTGGCGTTCCTTCTCGGTCTCCTTGTGCATGTGGCTGCCTAA	4	Sarcocystidae	Toxoplasma	*T.gondii*	SAG3
T.gondiiUPS_761	CGCAGGGAAATCGAGAAGTAAGATAACCTATTTTGGCACGCTCCTCAAGAAGGCTCCGAA	4	Sarcocystidae	Toxoplasma	*T.gondii*	SAG3
T.gondiiUPS_762	ACCTGCGGGTACTCCGAGTCCGGCCCCGTGAATCTCGAGGTGGACTTGTCAAAGAACGCG	4	Sarcocystidae	Toxoplasma	*T.gondii*	SAG3
T.gondiiUPS_763	ATCGTCATATGATCAAATGGTAACTACAATCGCTGGTGCGGCCGCTTTGATGGGCCTGTT	4	Sarcocystidae	Toxoplasma	*T.gondii*	BSR4
T.gondiiUPS_764	GCCATGGTGATCGGACTTATTGGCTCTATCGCAGCTTGTGTCGCGTGAGTGATTACCGTT	4	Sarcocystidae	Toxoplasma	*T.gondii*	SAG1

T.gondiiUPS_765	TTGAGCTCCTTGATTCCTGAAGCAGAAGATAGCTGGTGGACGGGGGATTCTGCTAGTCTC	4	Sarcocystidae	Toxoplasma	*T.gondii*	SAG1
T.gondiiUPS_766	TGCCGCGCCCACACTGATGTCGTTCTTGCGATGTGGCGCTATGGCATCGGATCCCCCTCT	4	Sarcocystidae	Toxoplasma	*T.gondii*	SAG1
T.gondiiUPS_768	GGTGGGAATGAAGGCAGAGGTTACAGAGGCAGAGGTGAAGGAGGAGGCGAGGATGACAGG	4	Sarcocystidae	Toxoplasma	*T.gondii*	GRA6
T.gondiiUPS_769	TGCACCCGGAACGTGTGAATGTGTTTGATTATTAAAGATGAAAACAGGGGGTCTATGCGC	4	Sarcocystidae	Toxoplasma	*T.gondii*	GRA6
TrichinellaUPS_770	AAGAGGCAAGTCGTAACAAAGTAGATCTTCTGGAAGGAGGACCTAGACCCAGAGCATAGT	4	Trichinellidae	Trichinella	T. spiralis	cob ATP6
TrichinellaUPS_771	CCTCGATGTTGAATCAAGAATTACTGGTTGTAGAAGACCAGCAAGTTTCGTCTGTTCGAC	4	Trichinellidae	Trichinella	T. spiralis	cob ATP6
TrichinellaUPS_772	TTACCCATACTGAAGCAGAGATCCAGAAAACTTCTCTTATGCAGATCGTCTTTCATCCCC	4	Trichinellidae	Trichinella	T. spiralis	cob ATP6
TrichinellaUPS_773	CAACACTCACAACAGGATGAGTAGCCTCTTGAAGAGCAGATGCATATTACTCCATATGAT	4	Trichinellidae	Trichinella	T. spiralis	cob ATP6
TrichinellaUPS_774	AGCCTCTGAATATTTCACAACATAAGATACGCCATCCGATTCGTCTCACTTCCATTCCGA	4	Trichinellidae	Trichinella	*T. spiralis*	cob ATP6
TrichinellaUPS_775	ACCTCCTGCCCAATGAAAATAATTAAATGGCCGCGGTAACTTGACCGTGCAAAGGTAGCA	4	Trichinellidae	Trichinella	*T. spiralis*	cob ATP6
TrichinellaUPS_776	TACGCCTACCTAATAGCATCTTGACCCACTAAATTCCTTATGCACATATTGCCCGTCACC	4	Trichinellidae	Trichinella	*T. spiralis*	cob ATP6
TrichinellaUPS_777	TGCAAAACCACTTGCCATAGTTAAAGATCTAAGTGATAAATAGTGTGCCAGCTATCGCGG	4	Trichinellidae	Trichinella	*T. spiralis*	cob ATP6
TrichinellaUPS_778	CCAATTCTAACCTTAACAACCAAAATAACTCCTACACAATAACATCTAACATACTCCACA	4	Trichinellidae	Trichinella	*T. spiralis*	cob ATP6
TrichinellaUPS_779	AATACGTGATTTGAGTTTAAACCGTCGCAAGACAGGTTGGATTCTCTTTAAGACTGCAAC	4	Trichinellidae	Trichinella	*T. spiralis*	cob ATP6
TrichinellaUPS_780	CTGGATGCTCCTTTGCAAACGCGAGTTGCTGAACTATTAATAAAATACGCTTACGTAAGT	4	Trichinellidae	Trichinella	*T. spiralis*	glycoprotein
TrichinellaUPS_781	TGTCCACGGTGCTTCCTACTAATATTGCGTCGATTATTAAGCTCTACCTTCGTCAACTTC	4	Trichinellidae	Trichinella	*T. spiralis*	glycoprotein
Trichinella_782	TCTTCATCAGGGTTTATATCGTGTCTGCGGAGTGAAGTCCAACATTGAGCAAATCTGTGA	4	Trichinellidae	Trichinella	*T. spiralis*	glycoprotein
Trichinella_783	TGCTTCCGTCTGCTAATTATAGAACATTGAAACTGCTCTGCTTGCATCTGAATCGGTAAT	4	Trichinellidae	Trichinella	*T. spiralis*	glycoprotein
Trichinella_784	GTCTCCAACTAATCTTGGAATAGTCTTTGGCCCAAGTTTACTGTGGATAGATGAAGGATT	4	Trichinellidae	Trichinella	*T. spiralis*	glycoprotein
Yersinia_Owiz_269	TTGATAACCCATGACCGCTCAACGCCAGTAACTGGAAGTCTACTTCCCTACGTCGAGACA	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence

Yersinia_Owiz_271	CTGATTTGGCCTTGAACAAGGCAGACATGGCAGCGTTACAAAGTATTATTGACCGACTCA	1,4,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_272	ACTAAATTATTGGAGTCGGTCACCCGCGGCGCGGCAGGATCTCAACTGATATCAAATTAT	1,4	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_273	CATGGCAGCGTTACAAAGTATTATTGACCGACTCAAAGAAGAGTTATCCCATTTGTCAGA	1,4,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_274	CAAGAGAAAATCAAAGAAACAGAAGAGAATGCCAAGCAAGTCAAGAAATCCGGCATCGCA	1,4,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_275	GCGGCGCGGCAGGATCTCAACTGATATCAAATTATGTTTCAGTGCTAACGAAGTTTACGC	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_277	TATCCCAAGAGGCAATGAAAATATTAGGGCCGATACTCACTGCGATTGAAGTCGCATTGA	1,4	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_292	TGGTCTCGGTGACGGGATTATGGCAGATATGTATACTTTAACGATTCGTGAAGCGGGTCA	1,4,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|325667790|gb|CP002247.1| Yersinia enterocolitica subsp. palearctica 105.5R(r) plasmid 105.5R(r)p, complete sequence
Yersinia_Owiz_293	CGCAATATGTATGAAAGCAAAGGAAGTTCAGCGGTAGGAGATGACTCCAAATTACGGCCG	1,4,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_294	CGTACTGCGCAACTGATTGGCGCAATGTGCATGAATGATAGTCGTAATAGTCAGTTAAGC	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|325667790|gb|CP002247.1| Yersinia enterocolitica subsp. palearctica 105.5R(r) plasmid 105.5R(r)p, complete sequence
Yersinia_Owiz_295	GGTCGGTAACACTCGTACCATAGCGTGCCAGTATCCGCTACAATCTCAACTTGAAAGCCA	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|325667790|gb|CP002247.1| Yersinia enterocolitica subsp. palearctica 105.5R(r) plasmid 105.5R(r)p, complete sequence
Yersinia_Owiz_296	CCAGTGGAGCCAGAGAGTCAGAAAAAGTATTTGCTCAAACTGTACTAAGCCACGTAGCAA	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence
Yersinia_Owiz_297	CCGCCAGAGTGGTACCTATGGCAGTATCACTGTAGAGTCTAAAATGACTCAGCAAGTTGG	1,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|325667790|gb|CP002247.1| Yersinia enterocolitica subsp. palearctica 105.5R(r) plasmid 105.5R(r)p, complete sequence
Yersinia_Owiz_311	TAGTCAGCGGCACTCTGCAGTCTATAGCTGATATGGCAGAAGAGGTAACATTTGTCTTCT	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	>gi|317374452|gb|HQ612242.1| Yersinia pestis strain C790 plasmid pCD1-pMT1, partial sequence

Yersinia_Owiz_316	AACTCATTCTGGGGTAATTCTATGTATCATTATCCTCTGGGGGTGGGGCAGCGTTTTAGA	1,4	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|16082691:12610-13578 Yersinia pestis CO92 plasmid pCD1, complete sequence_880-939
Yersinia_Owiz_317	TGAATTCCACTTTTCTGATAAGGAAAAGTTCCGCAAATGGTTTACTAACTCATTCTGGGG	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|16082691:12610-13578 Yersinia pestis CO92 plasmid pCD1, complete sequence_834-893
Yersinia_Owiz_318	TGGTACGACGGAATCAGAAGGGCTGGATCAATTATTAAACGCTATCCTTGATACTCATGG	1,4	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|16082691:12610-13578 Yersinia pestis CO92 plasmid pCD1, complete sequence_663-722
Yersinia_Owiz_331	CAGTAATGAAGAGCTTAAAAATATCATAACACAGTTGGAAACTGATATAGCGGATGGATC	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|32470270:58301-59167 Yersinia enterocolitica 8081 plasmid pYVe8081, complete sequence_69-128
Yersinia_Owiz_332	TAAATCCATTATAGATGGAAAGGAATTATCAGTTTCGGTACATAAAAAGAGAATAGCTGA	1,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|32470270:58301-59167 Yersinia enterocolitica 8081 plasmid pYVe8081, complete sequence_783-842
Yersinia_Owiz_339	TCCTAAAGAACGTGAGCAAGTAGGCAGACGAGTAAAGTATGAACTTCATCATCTGAAGCC	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|51594359:181435-181854 Yersinia pseudotuberculosis IP 32953, complete genome_270-329
Yersinia_Owiz_342	GAATTGGAACTTATTCTTCGTCATTATGTTTATGGACAATCTAAATCTTCTATTGCTCGA	1,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|51594359:183509-183880 Yersinia pseudotuberculosis IP 32953, complete genome_196-255
Yersinia_Owiz_344	TTTACAGCGCGCAGCAAGAGAAAAAGAATTGGAACTTATTCTTCGTCATTATGTTTATGG	1,5	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|51594359:183509-183880 Yersinia pseudotuberculosis IP 32953, complete genome_171-230
Yersinia_Owiz_352	TTCAGGAGAATGCCGGTAGTGAGAATATAGGTTATGAGGATGCAGAAAAACCTATCCAGT	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|51594359:1436462-1437826 Yersinia pseudotuberculosis IP 32953, complete genome_1175-1234
Yersinia_Owiz_355	AAGTGGCCTCTGTTGAGATCCCTCTGTCAGAGTTACTGACGGTGAATAATGACTCGGTGA	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|51594359:3983362-3983775 Yersinia pseudotuberculosis IP 32953, complete genome_206-265
Yersinia_Owiz_358	CGGATCCCTGTCACCTTAACACTGGAAGTGGCCTCTGTTGAGATCCCTCTGTCAGAGTTA	1	Enterobacteriaceae	Yersinia	Multiple Yersinia	gi|51594359:3983362-3983775 Yersinia pseudotuberculosis IP 32953, complete genome_181-240
YersiniaUPS_796	GTGGTGTCTGGCATCATGGCTGCTACTTCTACGGTTGCTAGTGCTTTTTCTATAGCGAAA	4	Enterobacteriaceae	Yersinia	Generic *Yersinia*	plasmid pCD1-pMT1
YersiniaUPS_797	CAAGACAGACAGCCCAATTATCACGACCGGTTCACAGCTTGATGCCATCACTACAGAGAC	4,5	Enterobacteriaceae	Yersinia	Generic *Yersinia*	plasmid pCD1-pMT1
YersiniaUPS_798	TTTATGAAAGATGTCCTGCGCTTGATTGAACAATATGTTAGCAGTCATACTCACGCCATG	4,5	Enterobacteriaceae	Yersinia	Generic *Yersinia*	plasmid pCD1-pMT1

YersiniaUPS_799	ATGCGTTTAATGCCGCAACGCAGCCGTTAGGACAAATGGCAAACAGTGCGATTCAAGTTC	4	Enterobacteriaceae	Yersinia	Generic *Yersinia*	plasmid pCD1-pMT1
YersiniaUPS_800	GCGAGGCAAGCTTATCTCGGTCACAGGTGCCAGAATTGATCAAACCGAGCCAGGGAATCA	4	Enterobacteriaceae	Yersinia	Generic *Yersinia*	plasmid pCD1-pMT1
YersiniaUPS_801	TAACCCATGACCGCTCAACGCCAGTAACTGGAAGTCTACTTCCCTACGTCGAGACACCAG	4	Enterobacteriaceae	Yersinia	Generic *Yersinia*	plasmid pCD1-pMT1
Y.enteroco_Owiz_155	GGGGCGTACTTTGATTTTGGCCGCGTCACGTATAGATAGCATTGATGATTTGATTGCTCG	1,4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|325667790|gb|CP002247.1| Yersinia enterocolitica subsp. palearctica 105.5R(r) plasmid 105.5R(r)p, complete sequence
Y.enteroco_Owiz_156	TCCGGCTCTATTACCCGAGGTGCTGGCATTACGTCAGGATGATGCACTCAAGTTGGCT	4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE3228
Y.enteroco_Owiz_157	AAAAACACAAGATTCAACGCTGGCAACGGCGGGTTACGCCCGGTTATTTCTCCAGC	4,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE3228
Y.enteroco_Owiz_158	GCAGCAGGCGTTATTGCACGCTAACTGGGTCGCAGCCATGACTCACGAAGCATTGC	4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE3230
Y.enteroco_Owiz_159	GGTTTGCCGCAAGCCCAGCTTGAACAAGCACTGATTGCGCTGCGAGAATCGCGTC	4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE3230
Y.enteroco_Owiz_160	GGGTCGCAGCCATGACTCACGAAGCATTGCGTCTGGTAGAGGAAGACCGCGAGTTATGTG	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|122087364|emb|AM286415.1| Yersinia enterocolitica subsp. enterocolitica 8081 complete genome
Y.enteroco_Owiz_161	AACAAACCTTGCCACAACGCCAGGAATGGCTGTCCGCCGATACGGTGGAATTGCTGATT	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|122087364|emb|AM286415.1| Yersinia enterocolitica subsp. enterocolitica 8081 complete genome
Y.enteroco_Owiz_162	CAGCAGTAATGGTGTGGGCTACACCTTACGCGCATTAATTGAGCGCCGTCTGAGCTCGC	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|122087364|emb|AM286415.1| Yersinia enterocolitica subsp. enterocolitica 8081 complete genome
Y.enteroco_Owiz_166	GGCACCTGACTCTGAGGTGTATCGGCGCGCTAAACTGATGTTGGCCATGACAAAACCAG	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_170	TGGCACCAGTATCGCAATGGGGTGGCAAAATGATCATTGGGCCACGGATATTGGT	4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE4072
Y.enteroco_Owiz_171	CGCATGTATTTCAGCCAACAAAAGGCCGCACAAGGCTACGCGCTATTGGAGAAAGTGGC	1,4,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_172	CTACCCAGAAACTTGCTTCGTTAGACCAACAATATCCGGGCAACGTCGCTCTGCGCATGG	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|122087364|emb|AM286415.1| Yersinia enterocolitica subsp. enterocolitica 8081 complete genome
Y.enteroco_Owiz_173	GCGCTAAACTGATGTTGGCCATGACAAAACCAGAGGTGCGTCAGCAATTGCAAGAGGC	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798

Y.enteroco_Owiz_175	ATTGCTCGCAAAGAGCTGGCTAATCAGCAAACGATGTTGGCCGATCCAACCTACCAGGC	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_176	GTGGCGCGGTTACCGGGTCAGGAAGTCCTGGCTACCCAGAAACTTGCTTCGTTAGACCAA	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:c4447607-4444122 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_478-537
Y.enteroco_Owiz_177	GATGGCACCGAATGATGTCTGGATAACTTACCGGTTAGCACAAGCATTGGCGCAAGATGG	1,4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_178	CGCATTGACCCATTTTCTTGATGTGTTTTCAACTGGCCCACAAGCAGATATTGCTCGCA	1,4,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_182	CACCGACACTGCCGACAGATAATGACAGCTATACCTTACTGACACGTAATCAGGCCACGG	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_183	ATCACAGGATAATGCGCTGGTGTTTCGTGACTCGGCCCGTTTAGCACAATCACAAGGGC	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_185	CGGGGCGATAACCGCGTTACGTCAGCAACCTGCGAATACACAAATTGACCTGACACTGG	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_188	TGGGCGAGGCGACTAATAAAGATGAGTTGGTTAAGCAGTCACTGTATCGCCTGAACATGA	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_190	ATCAGCGCTTGAAATTAGATCAAGTGCTGACATTGGCGCGTCAGCTACGGGAAAATGGTG	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|330862146|emb|FR718688.1| Yersinia enterocolitica W22703 biovar 2, serovar O:9, contig 7180000001798
Y.enteroco_Owiz_194	TTGAGATGAATGCCAGCACATTTGCGAATACCAAGGGTGTATATAAGGAGAAGTTCGGT	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:c4447607-4444122 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_2501-2559
Y.enteroco_Owiz_196	AAACGATGTTGGCCGATCCAACCTACCAGGCGCGGTTAAGTGGGCTGGCGAAAGTT	4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE4072
Y.enteroco_Owiz_198	ATTCCGGTGTCTTTGGTAACTATCTGCAAAACATCCAGGATGACGGCAATATGCGCGCAC	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_947-1006
Y.enteroco_Owiz_200	CTCGTCACACGACATTCCCAGCATCTTTACCCGGTGACGCGCAACCCTTTGATTTATTA	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_629-687
Y.enteroco_Owiz_201	TCCCAGCATATCGTGTCCACCGCTGGCGTAATTGGCTAGCCATACCCATCGGTAT	1,5	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia

						enterocolitica subsp. enterocolitica 8081, complete genome_170-224
Y.enteroco_Owiz_204	TCATGAATATCATACCGTTACATGATGGTAACCGTTTCGTCGGTACCAACAAAACGGCGG	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_1139-1198
Y.enteroco_Owiz_205	CGCACCGATGATGTCGCAGAAAGGTATTAGCAATCAATTGGCCTCCTTTGACGGC	4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	YE4078
Y.enteroco_Owiz_208	TAGCGCCGAAAATGTGGACTGGCAAACACTGACACAAGGTTTACCCGAAACCGCCTTAAT	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_1503-1562
Y.enteroco_Owiz_209	ACCGCTCATTTGAATGCTTTTTATGAGCAAGAGAAAACTCGTCACACGACATTCCCAGCA	1	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_592-651
Y.enteroco_Owiz_210	GAACAGCTTTCGTCATTCTGGTAGGCTACCTGTTTATTTCACAATGGATTCGCGTCACCG	1,4	Enterobacteriaceae	Yersinia	*Y. enterocolitica*	gi|123440403:4456536-4458188 Yersinia enterocolitica subsp. enterocolitica 8081, complete genome_356-415
Y.enteroco_Owiz_211	GCCGTTTCATTAACTGGGCGATGATAGGAACAGCTTTCGTCATTCTGGTAGGCTACCTG	4	Enterobacteriaceae	Yersinia	Y. enterocolitica	YE4078
Y.pestis_Owiz_97	TACTTTTATCTCCTGCACAAAGAACGATAGCCCGACGAATCGGATCGAACGCATAAAAGG	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_98	GAACGCTACTCTCCTTCCTGTAGGAAGCTCAACATCCAAGCTTAATTTGCCTCCCATTGC	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_100	AGGGCATGGAGAAGGTCAAATTAGACATGGAACGCTACTCTCCTTCCTGTAGGAAGCTCA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_102	TCGTGTCTGGCTTTCAGGTGAACGACTGGCAATCACCTCATCTAATGTTCTCATTACTTG	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_103	TCAATCGGAAACCCCATAATGTACATCAGTTTAAAATTACTCCGGCCAGGCAGATCCACA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_106	GGCCTGGGTAACTCAGGTCCGTAAACCTGTAGCTTTTTCAAATCAGCCAAAACCTTTTCC	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_107	ACCGACACACAGTCTGAGTACAGATACATGTCACGATGATGCAGGATTAGCGGAAGAGTG	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_109	GAACAGGCGGAACCCGGACAGGGGGAGAACGAATCGCTAAATAATTTTCGTAGTTGTATT	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_110	CACACTCCACATATCACTGACGGAGCACAACGGAATAGTGAACAAACAACAACAAACTGC	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_111	ACCGACACACAGTCTGAGTACAGATACATGTCACGATGATGCAGGATTAGCGGAAGAGTG	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_112	GAACAGGCGGAACCCGGACAGGGGGAGAACGAATCGCTAAATAATTTTCGTAGTTGTATT	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP

Y.pestis_Owiz_113	TCGTAGGTCGTTATAGGAAAATTCTTAGCACCATTCCGGAACAATCAGAACAGCAGGCCA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_114	TCGTAGGTCGTTATAGGAAAATTCTTAGCACCATTCCGGAACAATCAGAACAGCAGGCCA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_115	TGGCGGATCTTATTTGAATATTCACTGTCTTTCTCCATCTCCGTATCAATCGGAAACCCC	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_116	GGTCTGTAACTGCTTAATGTACTTCCGGAAAAGAGAAAAGCCGGAACACCACCTGAACCA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_117	TACAGATCATATCTCTCTTTTCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGG	1,5	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_118	TCATTGTCTCATGGCATCTATGGGAAACAGACAACCTTCAAGCAAACCGAGTTTACAAA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_119	TGAGGGGCTCATGGTTATTTTAACAATCCACTATCGATATCTTTTTGCACCAGAGCGCCC	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_120	TGTTGTCCGCTAGGACGATGCGATTTCGGTTATTATTCAGAATGTCTTCGTTCTCTTTC	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	
Y.pestis_Owiz_121	TGTCCGGGAGTGCTAATGCAGCATCATCTCAGTTAATACCAAATATATCCCCTGACAGC	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	
Y.pestis_Owiz_122	TTTCTGTTCGTAAGACACGGTCCCTTCAGTTTTTGAAATTTTACTTTCCTGGCGGATCTT	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_123	TGTGAGGGCGTATGATAAGGACTGAATCGATGGTTAATATGTCTAGTCCTGACTTTTGCA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_124	CCGGAGATACGCAGTCATATTTTTTACACAATTCTCTAATCCCGACAAGGTCGTAGGTCG	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_125	TCGCTCAAAAGATCATATTTCCCTGGATATTTTCCACCGTTTCTTATGTGAGCAAAGTCA	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_126	CCGGAGATACGCAGTCATATTTTTTACACAATTCTCTAATCCCGACAAGGTCGTAGGTCG	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_127	GTGGAGATTCTGTCTCTATTGGCGGAGATGCTGCCGGTATTTCCAATAAAAATTATACTG	1,5	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_128	TTCAGAATGTCTTCGTTCTCTTTCTTATCGAGATGTTCAATAGATTCGGCAATCGTTCCC	1	Enterobacteriaceae	Yersinia	*Y. pestis*	pPCP
Y.pestis_Owiz_129	GAATCGCGCCCGGATATGTTTTAACGCGATTTTCAGACTCAGACAAATTCAGCAGAAT	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	
Y.pestisUPS_785	AATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAGGCCAGTATCGCAT	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP1
Y.pestisUPS_786	AATGATGAGCACTATATGAGAGATCTTACTTTCCGTGAGAAGACATCCGGCTCACGTTAT	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP1
Y.pestisUPS_787	TAAATTCAGCGACTGGGTTCGGGCACATGATAATGATGAGCACTATATGAGAGATCTTAC	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP1

Y.pestisUPS_788	AGCCCGACCACTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP
Y.pestisUPS_789	TCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTC	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP
Y.pestisUPS_790	AAAGGACAGCATTTGGTATCTGTGCTCCACTTAAGCCAGCTACCACAGGTTAGAAAGCCT	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP
Y.pestisUPS_791	AAGGAGTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAG	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP
Y.pestisUPS_792	TTTGTACCGAGAACCTTTCACGGTATCGGCATATGGCCTGGGTAACTCAGGTCCGTAAAC	4,5	Enterobacteriaceae	Yersinia	*Y.pestis*	pPCP
Y.pestisUPS_793	GAACAAAGAAATCCTGGCTGCCCGTAGCCAAGACGACGTCATCCCCCACAAGGTTCTCAC	4	Enterobacteriaceae	Yersinia	*Y.pestis*	pMT
Y.pestisUPS_794	CGGTTACAGCATCAGTGTATTTACCTGCTGCAAGTTTACCGCCTTTGGAACCAATTGAGC	4	Enterobacteriaceae	Yersinia	*Y.pestis*	pMT
Y.pestisUPS_795	AGGAGAGATATCAAAATCTCTAGAATCCTTGCCAATCACTTTTGTAGTGAATTGGTGGTT	4	Enterobacteriaceae	Yersinia	*Y.pestis*	pMT
Y.pseudo_Owiz_214	GTTGGTCAGTTTATTATTAGTAATCGCGAGCGTATGAGTGTAGGTAAGGCCCCTAAAGCT	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|186696370|gb|CP001048.1| Yersinia pseudotuberculosis PB1/+, complete genome
Y.pseudo_Owiz_216	TTGGGCCAGAGATAACAGTTGCCTCGATTATCCGCATATTTCTAGTGGTTTTAAAGGAT	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:183509-183880 Yersinia pseudotuberculosis IP 32953, complete genome_39-97
Y.pseudo_Owiz_218	ATCCGCATATTTCTAGTGGTTTTAAAGGATTAGTCACAAGAAACAAACCCGCTGAGTCCT	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:183509-183880 Yersinia pseudotuberculosis IP 32953, complete genome_68-127
Y.pseudo_Owiz_219	CTATTGCTCGATTAAAAAAATGCAATGAGCGTGAAATAAGACGCCAGTTACAAATTGCA	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:183509-183880 Yersinia pseudotuberculosis IP 32953, complete genome_245-303
Y.pseudo_Owiz_220	TAACGGCATTGGTAGCTTCTTTTCTATTACTTCTTGTGCAGTGGAGTAACCTGTCAAAG	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:184491-184826 Yersinia pseudotuberculosis IP 32953, complete genome_29-87
Y.pseudo_Owiz_222	ATGATATGTCCAATCGTGTTGCTACTGCTCGTCAATTAGAGAATGAGAGGGTATTACGTA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:184491-184826 Yersinia pseudotuberculosis IP 32953, complete genome_173-232
Y.pseudo_Owiz_224	TTTTGTTGTCTTAAGTTCGGCTGGTGTCAAAATGAGCAGGATTGGGCTTAACTATGATG	1,4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:287857-288525 Yersinia pseudotuberculosis IP 32953, complete genome_273-331
Y.pseudo_Owiz_226	GGACGCTTTTAGCCGAACTCAACGAAAGCTTGGAAGGGTACACACCTGATTTATTT	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0244
Y.pseudo_Owiz_227	TTGAGACTTCACCAAAAGGTGAGCATTTTGTTGTCTTAAGTTCGGCTGGTGTCAAAAT	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0244

Y.pseudo_Owiz_228	ATGAACCAGGTCGATGTGCAGAAAGACATTAGTGATTTGATTGTTAACGCCTTCCC	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0244
Y.pseudo_Owiz_229	TTTAGCGAAAGAACCATAGACCATGGGCCAACAGGAACGTTAGGTGTCTTAATTCT	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0244
Y.pseudo_Owiz_231	TCATGAACCACAGAGCAGGATGCTCGATTTAAGGGTTGTGGTACCTTTCAGTAATTTG	1,4,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:287857-288525 Yersinia pseudotuberculosis IP 32953, complete genome_486-543
Y.pseudo_Owiz_233	GAAGAGGCTTTTTCTGCGGAATTACCCTCTTTTCCAAGTTTGGAAAATTTTGTCTCACTT	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:288538-289740 Yersinia pseudotuberculosis IP 32953, complete genome_742-801
Y.pseudo_Owiz_234	GTTGATGATTATAGGTCCATTGATGATGAAGCCATCGAACGGGTAGCTTCTGATTGTAG	1,4,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:288538-289740 Yersinia pseudotuberculosis IP 32953, complete genome_373-431
Y.pseudo_Owiz_236	ATCAGCGTAAGCCGGAGTCGGTGAGGCTGTTGCCCAGATGTATTAATCTATTACT	4,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_237	CCAGAAAGACTAACCCAAATGCGAGAAGCTCGAGGGTTATCCAAAATAAACTTGGGTA	4,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_238	CAGTCTCGGCAAGGTTTTAAATTGCCCTGTGAGTTGGTTTACCAAAGTTGCTTATG	4,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_239	GCCCATGAACTCGGCCATTTGGTTTTACATCGATACGTAAAACGCCTTGATAGTATTA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:288538-289740 Yersinia pseudotuberculosis IP 32953, complete genome_628-685
Y.pseudo_Owiz_242	GCGACGGCCAAAGATTTGTGTAAATCGTCAGAAGTTTATATGGGATGGTTACAAGAGA	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:288538-289740 Yersinia pseudotuberculosis IP 32953, complete genome_256-313
Y.pseudo_Owiz_243	AATTTGCTGCCTGTTTTATGCTACCTGAAGAGGCTTTTTCTGCGGAATTACCCTCT	4,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_244	AGAGAAAATAATCCCGTTTTCTTTAGAACATTGTCGGCGACGGCCAAAGATTTGT	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_245	GGTGGCGTAAAGGTGAGCCCTTAGATGATCAGCGTAAGCCGGAGTCGGTGAGGCT	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_246	ATGAAGCCATCGAACGGGTAGCTTCTGATTGTAGAAAGAGATGGGGACTCGGTAT	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0245
Y.pseudo_Owiz_247	CTCTTGAACATCATAGTAATCTTGTTCCCTGGCAGCAAATATGTAGGGTTACGGGGGC	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_383-440
Y.pseudo_Owiz_248	GAGGAACGACAGAAGCTATTAATTTAGTCGCGACAAGCTGGGGGAAGAGCTATTTGC	4	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	YPTB0557

Y.pseudo_Owiz_249	TCTACTGAGTAACCGAACGCGGGTTTTAGCCCTTTCTCATGTATCTAATGTGCTGGGTAC	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_501-560
Y.pseudo_Owiz_250	GAAAAAACTATTGTATTGATCGATGGCGCGCAGGCTGTACCACATATTTCCATTAATGTG	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_628-687
Y.pseudo_Owiz_251	CGAACCATCAGGGGTATTACAGATAGATTCACTTGAGCATCTACTGAGTAACCGAACGCG	1,5	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_462-521
Y.pseudo_Owiz_252	CGATCAACTCTCACAATTCTCGTGTATTCGCATATTAGGTCAGGTACCACTAAAAGCTGC	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_990-1049
Y.pseudo_Owiz_257	AAATAGTGGTGGATTGGATGTGGATTTTATTCGCGATCAATTTCCGATTCTAAAAAAACA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_18-77
Y.pseudo_Owiz_258	ATGATCAAATGCAGCAAGGTAAAAAAGAAAAAACTATTGTATTGATCGATGGCGCGCAG	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_602-660
Y.pseudo_Owiz_259	TATTCCGGCCAGAAATATTGGATTAGCATTAGATAAGCAAGGTATTGCGGTACAGGTCGG	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:658664-659932 Yersinia pseudotuberculosis IP 32953, complete genome_1074-1133
Y.pseudo_Owiz_262	TGAATGCTCAGTATGGTTTAGACGTCATGTATATACTGACGGGGAAACGAACCCCTCCA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:2215440-2215808 Yersinia pseudotuberculosis IP 32953, complete genome_176-234
Y.pseudo_Owiz_263	CATGTATATACTGACGGGGAAACGAACCCCTCCAGATACTATCTCTACAGAAGAGCTGAA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:2215440-2215808 Yersinia pseudotuberculosis IP 32953, complete genome_201-260
Y.pseudo_Owiz_265	CTAAAATGGAAAGGTAAGAATGGCTATTCTGAATCCGAAGTTTCTAATAAACGTAAAGCA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:2228366-2229091 Yersinia pseudotuberculosis IP 32953, complete genome_541-600
Y.pseudo_Owiz_266	ATTAGACCACCAGAAAGAAAGAAATTCATTACCAATATAAAATCGCTATGCTCAAGTTCA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:2228366-2229091 Yersinia pseudotuberculosis IP 32953, complete genome_409-468
Y.pseudo_Owiz_267	CCAGACTTCATAATACTTAACCTCGTTTTACAGTTTATTAGACCACCAGAAAGAAAGAAA	1	Enterobacteriaceae	Yersinia	*Y. pseudotuberculosis*	gi|51594359:2228366-2229091 Yersinia pseudotuberculosis IP 32953, complete genome_373-432

9.2.1 Illumina library preparation

In addition to PCR and qPCR testing of the *Y. pestis* results, sequencing (with enrichment) was performed on a selection of the array-positive samples. This was done to determine if there was any of the pathogen present within the samples. The method used was also more sensitive than standard PCR or qPCR so it would be able to detect trace amounts of pathogen in the samples. The solution based hybridisation capture was performed by colleagues at the University of Berlin (Macé et al., 2013).

DNA extracts of 30 suspected *Y. pestis* positive rodents were used for Illumina library preparation. A negative control library of 10 *Y. pestis* negative rodent samples was also prepared and indexed separately to prevent any contamination introduced during the experiment. The DNA was fragmented to a size between 200 and 250 bp using a Covaris M220 in 50 µl screw cap microtubes following a protocol of peak power set at 50, a duty factor of 20, at 200 cycles for 200 s. DNA fragmentation produces heterogeneous ends, comprising a mixture of 3' overhangs, 5' overhangs and blunt ends. T4 DNA polymerase was used to convert the overhangs from fragmentation into blunt ends which were required for adaptor ligation. In addition, T4 Polynucleotide Kinase was used to phosphorylate the 5' ends of the DNA fragments. Blunt ending of the DNA was performed after incubation at 25°C for 15 min, then 12°C for 15 min with the reagents given in Table 9.2.

Purification was then performed using a Qiagen MinElute column. 250 µl of Buffer PBI (5M Gu-HCl, 30% isopropanol) was added to each reaction and loaded into a column. This was then centrifuged for one minute at 13,000 x *g*, after which the supernatant was discarded. 750 µl of the wash buffer PE (10 mM Tris-HCl pH 7.5) was then added. This was then centrifuged for one minute at 13,000 x *g*, after which the supernatant was discarded. The column was then centrifuged once more for one minute at 13,000 x *g* to remove all of Buffer PE. 20 µl of 1 x EB (50 mM Tris-Cl pH 8.1-

8.2, 1.4 M NaCl) was added, and the mixture was then incubated for 5 min at 37°C. This was further centrifuged for one minute at 13,000 x *g*.

Each library contained a unique adapter to allow for subsequent discrimination among samples after the sequencing of the pooled libraries. Adapter ligation onto the DNA fragments was performed, and the reagents used are given in Table 9.3. This step added distinct sequences to the 5' and 3' end of each strand in the genomic fragment. These additional sequences were necessary for library amplification with the unique P7 index list. The adapter ligation took place at room temperature for 20 min.

Each index was added by PCR in 50 µl reactions (reagents shown in Table 9.4). The assay was performed in a Stratagene MxPro 3000p qPCR system. Cycling conditions were: 94°C for 2 min; 7 cycles at 95°C for 20 s; 60°C for 30 s; 72°C for 30 s; 72°C for 3 min; the samples were then held at 4°C. The unique P7 index list is given in Table 9.5. The DNA product was purified following the same method as described above. After indexing, the samples were effectively at little or no risk from cross contamination either from the other libraries or from laboratory DNA.

Table 9.2. Reagents required for blunt-ending of DNA

Reagent	**1 reaction (µl)**
New England Biolabs Taq2x Master Mix 2 (10x)	5
dNTP (10 mM)	0.6
ATP (10mM)	5
T4 Polynucleotide Kinase (10U/µl)	2
T4 Polymerase (3U/µl)	2
BSA (10 mg/ml)	4
Water	11.4
Template	20
Total	50

Table 9.3. Reagents required for adapter ligation

Reagent	**1 reaction (µl)**
Quick Ligase Buffer (2x)	20
Illumina adapter mix (100 µM Regular Adapter Stock)	1
Quick Ligase (5/U µl)	1
Template	18
Total	40

Table 9.4. Reagents required for PCR indexing

Reagent	**1 reaction (µl)**
Water	To 50 µl
Herculase II reaction buffer (5x)	10
dNTPs (25mM)	0.5
Illumina_P5 Index Primer (10 µM) (AATGATACGGCGACCACCGA)	1
Illumina_P7 Index Primer (10 µM) (CAAGCAGAAGACGGCATACGA)	1
Herculase II fusion DNA Polymerase	0.5
Template	6
Total	50

Table 9.5. Unique P7 identifying index sequences

Sample number	Index	Index Sequence
172	16	GTGTAT
C71	17	CGCTAT
190	19	GTATCT
C82	18	TGAACA
K14	20	CAGCTA
K3	21	ACATAC
K136	23	AGCATG
16	26	TGTGAC
97	28	TCTCGC
K24	29	TGATGC
325	30	GACGAC
K53	31	GACACT
C53	32	ACGCAT
110	33	GACCGG
K26	22	TGAGCC
29	24	CGATGA
31	25	TAGATG
25	27	TGCATA
214	34	GAGATA
K102	35	CTGACA
32	36	TGCAGG
124	37	ACTGCC
92	38	GCAACG
82	40	TGACGT
28	41	TGTCTG
172	42	ACGTGC
K57	43	TGATCC
60	44	CTCTAG
184	45	GAGAAG
27	46	CACGAA

PCR products of *Y. pestis* used as "bait" for capturing sequences from the Illumina libraries were generated at the University of Nottingham. The baits were prepared by using the PCR primers given in Table 9.6. The baits were then blunt-ended in Berlin by incubating a reaction mix (Table 9.7) at room temperature for 30 min. The blunt-ended DNA was biotinylated by ligating the Bio-T/B adapter sequences (Table 9.8) at room temperature for 15 min. After this incubation period the mixture was purified once again. 500 ng of the Bio-T/B adapter-ligated bait DNA was mixed in a volume of 5-50 µl with the same volume of BWT (2 M NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA ph 8.0, 0.1%

Tween20) buffer (2x). The mixture was heated for 1 minute at 98°C, and was then immediately placed on ice. 5 µl of M-270 beads were washed with 200 µl of BWT buffer (1x). This involved resuspending the beads in the buffer, then collecting the beads with a magnet and removing the supernatant. The buffer was then removed by pipetting. The beads were then washed with 200 µl of TET buffer (10 MM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 0.05% Tween-20). The bait mixture was then removed from ice and added to the beads, and this was then resuspended. The tube was rotated for 20 min at room temperature to allow the biotinylated bait to bind to streptavidin beads. The beads were collected with a magnet and the supernatant removed. The beads were then washed twice with 200 µl of BWT buffer (1x) heated to 50°C. The beads were then resuspended in 50 µl of TET buffer.

Table 9.6. PCR primers used for bait preparation

Primer name	Primer sequence	Target	Reference
F1	CAGTTCCGTTATCGCCATTGC	caf1	(Dupinay et al., 2014)
F2	TATTGGTTAGATACGGTTACGGT		
Yp1a	CTGACAGCTTTACAGTTGCAGC	pla	(Janse et al., 2010)
Yp2a	CACTCCTTTCGGGAAGTTTCCG		
Ypfur1	GAAGTGTTGCAAAATCCTGCG	fur	(Maricic et al., 2010)
Ypfur2	AGTGACCGTATAAATACAGGC		
3aF	TGTAGCCGCTAAGCACTACCATCC	Pla	(Daniels et al., 2003)
3aR	GGCAACAGCTCAACACCTTTGG		
YPtoxU	AGGACCTAATATGGAGCATGAC	Ymt	(Riehm et al., 2011)
YPtoxUR	CGTGATTACCAGGTGCAACA		
YPpla S	GTAATAGGTTATAACCAGCGCTT	Pla	(Riehm et al., 2011)
YPpla R	AGACTTTGGCATTAGGTGTG		

Table 9.7. Reagents required for blunt-ending of baits

Reagent	1 reaction (µl)
Bait DNA (1.3 µg)	76
Blunting buffer (10x)	10
dNTP mix (1mM)	10
Blunting enzyme mix	4
Total	100

Table 9.8. Reagents required for bait adapter ligation

Reagent	1 reaction (µl)
Blunt-ended DNA	15
Adapters Bio-T/B (50 µM)	1
Quick ligase buffer (2x)	20
Quick ligase	4
Total	40

The Illumina libraries were divided into 5 groups which contained 5-8 samples in each and pooled to equimolar amounts to a total of 2 µg in a microcentrifuge tube. The hybridisation mixture was prepared (Table 9.9) and incubated for 3 min at 95°C to denature the DNA. This was then transferred to a heat block and incubated for 30 min at 37°C. The mixture was then briefly centrifuged at approximately 13,000 x *g* to separate the supernatant. The TET buffer was then removed from bait-coated beads by magnetically collecting them and discarding the supernatant. 52 µl of the hybridisation mixture was added to the beads. This was then incubated in the oven at 65°C for 48 hours. After removing the tube from the oven, the beads were collected with a magnet and the supernatant discarded. The beads were then washed 3 times with 200 µl BWT buffer (1x). 200 µl of HWT (2.5 mM MgCl, 50 mM KCl, 15 mM Tris-HCl pH 8.0, 0.1% Tween-20) buffer was then added to the beads, which had been preheated to 60°C, then incubated at 60°C for 2 min on a heat block. The beads were collected and the supernatant discarded. The beads were then washed with 200 µl BWT buffer (1x). 100 µl of TET was then added and the suspension was transferred to a new tube. This was then incubated at 95°C for 3 min. After a 10 s vortex, the beads were collected and the

supernatant transferred to 500 µl PBI buffer. The mixture was then purified with a MinElute kit and the DNA eluted into water or TE buffer. The eluted libraries were then further amplified with a reaction mixture given in Table 9.10 with cycling conditions of: 95°C for 2 min; 10 cycles of 95°C for 20 s; 60°C for 30 s; 72°C for 30 s; a final annealing step of 72°C for 3 min and then the samples were held at 4°C. The resulting product was then sent for sequencing on an Ilumina MiSeq platform.

Table 9.9. Reagents required for the preparation of the hybridisation mixture

Reagent	Primer sequence	1 reaction (µl)
2 µg of pooled indexed library		17.8
BO1.P5.F (200 µM)	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	0.5
BO2.P5.R (200µM)	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT	0.5
BO3.P7.part1.F (200µM)	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC	0.5
BO4.P7.part1.R (200µM)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	0.5
BO5.P7.part2.F (200µM)	ATCTCGTATGCCGTCTTCTGCTTG	0.5
BO6.P7.part2.R (200µM)	CAAGCAGAAGACGGCATACGAGAT	0.5
Agilent blocking agent 10X		5.2
Agilent hybridisation buffer 2X		26
Total		52

Table 9.10. Reagents required for solution-based hybridisation capture

Reagent	1 reaction (µl)
Water	To 50 µl
Herculase II reaction buffer (5x)	10
dNTPs (25mM)	0.5
Illumina_P5 Bridge Primer (10 µM)	1
Illumina_P7 Bridge Primer (10 µM)	1
Herculase II fusion DNA polymerase	0.5
Template	6
Total	50

9.2.2. Solution-based hybridisation capture

The data provided by the University of Copenhagen National High-Throughput Sequencing Centre consisted of 35,899,896 150 bp Illumina paired end reads. The *R. norvegicus* genome (Rnor_5.0.74) was downloaded from the Ensembl FTP site (http://www.ensembl.org/info/data/ftp/index.html) and imported into CLC bio, Qiagen. A total of 84 Y. *pestis* reference sequences were extracted from the ensemble FTP site and imported into CLC using the fasta importer. An initial assessment with the CLC Create Sequencing QC Report tool was performed by Dr. Tom Giles at the Advanced Data Analysis Centre (ADAC) at the University of Nottingham on the quality of the data. The results indicated that the data required trimming because the average PHRED scores of some reads fell below the standard quality limit of 0.05. The data was trimmed with the CLC Trim sequences tool and 33,023,770 of the reads remained. The average read length was reduced from 150bp to 110bp.

The remaining reads were then mapped to the *R. norvegicus* genome with the CLC *Maps read to reference tool*, and 26,047,625 (~71.8%) aligned with the rat genome. Of the reads that did not map to the rat genome, on average 9.3% of the reads per sample (333,869) aligned to at least one of the 84 *Y. pestis* genomes. Further investigation revealed that only 6 of the samples had more than 5% alignments. The number of reads within the samples varied significantly and therefore the number of reads per average genome length was calculated. This indicated that most samples had around 10^{-5} reads per bp of genomic sequence.

As only a small number of the reads were found to map to the *Y. pestis* reference genomes, a *de novo* assembly was created to identify contigs that could represent DNA from other species. The reads from each sample that did not map to either *R. norvegicus* or *Y. pestis* reference genomes were selected, and the CLC *De Novo Assembly*

tool was run (minimum contig length was set to 300 bp). 6 of the samples, when assembled, had no contigs greater than 300bp in length. The reads from each of the other samples assembled into one or more contigs (938 contigs in total). The assembled contigs were compared against the NCBI non-redundant DNA sequence database using a BLAST search. Only 6 of the contigs had no BLAST results and 812 had e-scores of less than 1^{-10}. Further analysis showed that only 8,080 reads from a single sample aligned to *Chromobacter xylosoxidans* NBRC 15126 genome. All of these reads appeared to align within a 200 bp window.