

**Development and Evaluation of Novel Methods to Improve Phage-Based
Detection Methods**

By Anton Kubala, BSc (Hons)

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Supervisors

Prof. Catherine Rees

Dr. Benjamin Swift

Abstract

Mycobacterial pathogens, such as *M. tuberculosis*, *M. bovis* and *Mycobacterium avium ssp. paratuberculosis* (MAP), pose significant and emerging global health risks for humans while mycobacterial disease in domesticated animals result in billions of dollars in losses to the agrarian industries, especially the dairy industry. Mycobacterial diseases are seen as endemic and persistent, with a lack of diagnostic tools able to detect mycobacterial infections quickly and with high sensitivity being the main hurdle to combating endemic mycobacterial infections. Due to the typically slow growth of mycobacteria such as MAP and *M. bovis*, current culture-based methods are too slow and classic microscopy is neither sensitive enough to detect low numbers of cells in clinical samples nor does it allow specific identification of the mycobacterial species encountered. PCR-based detection assays for both MAP and *M. bovis* exist and are able to produce a conclusive result in a few hours but they have limited sensitivity and cannot differentiate between live and dead organisms. The most common tool for the diagnosis of mycobacterial pathogens in animals are ELISA assays which are rapid, low cost and are reasonably specific but have low sensitivity, especially in the early stages of infection. To address all these issues, the Actiphage[®] assay was developed which utilises bacteriophage D29 to specifically infect and lyse mycobacterial cells recovered from a clinical blood sample and then PCR is used to identify species-specific signature sequences in the released mycobacterial DNA. This approach allows highly specific and sensitivity identification of the mycobacterial cells present in a blood sample within 24 h.

This project was sponsored by PBD Biotech Ltd which was set up to commercialise The Actiphage[®] assay and the main aim was to develop the methods required to produce the kit reagents in a stable format and to further refine the methods used to process the blood samples. The first approach that was used to stabilise the bacteriophage was to freeze them while still embedded in the agar used to grow them in the laboratory. This proved to be successful and was found to be a method that could be generically used to create stable phage samples that could be stored at –20 °C. This work also provided good information about the characteristics of the excipients required to protect the phage from freeze-thaw damage, but ultimately was not practical for large scale manufacture of kits. Thus it was decided to try and freeze dry high titre liquid lysates of the phage.

To that end research was carried that allowed fast and inexpensive production of large volumes (10-1000 ml) of high titre ($> 1 \times 10^{10}$ pfu ml⁻¹) phage suspension as well as methods to allow this to be freeze dried in appropriate volumes with good shelf stability at room temperature. This provided a framework of knowledge to then develop a method for the production of a stable form of both MAP and *M. bovis* culture to be included as a positive control in the commercial kits. While it was possible to produce stable reagents, it was found that, unlike the phage samples, these needed to be stored at low temperature to maintain functionality. This led to the discovery that while the mycobacteria would survive freeze drying in high numbers, cells transitioned into a state that was not detectable by phage D29, possibly because they entered a stationary growth phase and were thus viable but non-detectable. However it was found that this process could be reversed by incubating the resuscitated cells for 2-3 h at 37 °C. Additional work was carried out on investigating phage host cell interaction in the presence of excipients and at low temperatures for the purpose of optimizing the efficiency and sensitivity of the phage based detection assay for the freeze dried reagents.

A second area of research focussed on optimizing the blood separation method employed for concentrating mycobacterial cells in animal blood samples. A method was developed to separate peripheral blood mononucleocytes (PBMCs) using ACK lysis buffer to specifically lyse the erythrocytes in the blood allowing the PBMCs to be isolated and concentrated by centrifugation. Replacing the standard density gradient centrifugation with ACK lysis made assay procedure cheaper, more reliable and faster thus increasing usability and sample throughput. It was then shown that this method could then be used for testing commercial cervid (farmed deer) blood samples and that the Actiphage® assay could successfully be used as an epidemiological tool for the diagnosis and monitoring of MAP in farmed deer, confirming that this assay has great potential for the control of mycobacterial diseases.

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Table of contents

Title Page	
Abstract	II
Acknowledgments	IV
Table of Contents	V
List of Figures	XIV
List of Tables	XVI
List of Abbreviations	XVII
1. Introduction and Literature Review	1
1.1. The Genus <i>Mycobacterium</i>	2
1.1.1. The <i>Mycobacterium tuberculosis</i> Complex	8
1.1.2. The <i>Mycobacterium avium</i> complex	14
1.1.3. <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> (MAP)	15
1.1.4. MAP as a pathogen in farmed and wild animals	16
1.1.5. The pathology of Johne's disease	19
1.1.6. MAP as a pathogen in humans	22
1.2. Detection, Diagnosis and culture of <i>Mycobacteria</i>	23
1.2.1. Detection of <i>Mycobacteria</i> using culture-based methods	23
1.2.2. Microscopy	26
1.2.3. Nucleic acid based detection of <i>Mycobacteria</i>	27
1.2.4. The Tuberculin skin test	28
1.2.5. ELISA based assays for the detection of MAP and <i>M. bovis</i>	29
1.3. Bacteriophage	32
1.3.1. Phage biology and life cycle	32
1.3.2. Phage infection of host cells	39
1.3.3. Summary of key points	40
1.4. Application of bacteriophage for phage based diagnostics	41
1.4.1. Introduction	41
1.4.2. The two day phage amplification assay for the detection of <i>M. tuberculosis</i>	45

1.4.3. The two day phage amplification assay for the detection of MAP	47
1.4.4. The Actiphage® Blood assay for the detection of Mycobacteria	49
1.5. Aims of this Thesis	51
2. Materials and Methods	52
2.1. Growth Media	53
2.1.1. Middlebrook 7H9 media and Media Plus	53
2.1.2. Middlebrook 7H10 agar	53
2.1.3. Nutrient agar and broth	53
2.1.4. BHI agar and broth	54
2.2. Solutions and buffers	54
2.2.1. Lambda buffer	54
2.2.2. Calcium chloride	54
2.2.3. Gelatine	54
2.2.4. ACK buffer	54
2.2.5. Virusol	54
2.2.6. Mycobactin J	55
2.3. Standard procedures	55
2.3.1. Phage plaque assay	55
2.3.2. Picking of plaques	55
2.3.3. Eluting phage from agar plaques	56
2.3.4. Production of <i>Mycobacterium smegmatis</i> lawn plates for phage titre	56
2.3.5. Production of non-mycobacterial bacterial lawn plates (<i>E. coli</i> , <i>S. enterica</i> and <i>L. monocytogenes</i>)	56
2.3.6. Production of Semi Confluent Lysis Plates (SCLPs)	56
2.3.7. Gram stain	57
2.3.8. Ziehl-Neelsen Stain	57
2.3.9. Production of artificial plaques	57

2.3.10. Freeze drying	58
2.3.11. Enumerating phage using the Miles and Misra technique	58
2.3.12. Extracting genomic DNA from MAP/BCG using boiled lysate method	59
2.3.13. Washing of culture to remove eDNA	59
2.4. Microorganisms used	60
2.4.1. Bacteriophage used	60
2.4.2. Bacterial strains used	61
2.5. Growth of bacterial cultures	62
2.5.1. Growing <i>M. smegmatis</i> culture	62
2.5.2. Growing BCG and MAP culture	62
2.5.3. Growing <i>E. coli</i> culture	63
2.5.4. Growing <i>S. enterica</i> culture	63
2.5.5. Growing <i>L. monocytogenes</i> culture	64
2.6. The two-day phage amplification assay	64
2.6.1. Two-day phage amplification assay controls	68
2.6.2. Two-day phage amplification for the enumeration of mycobacteria	70
2.6.3. Extraction and purification of DNA from agar plaques	71
2.6.4. Extraction of DNA from agar plaques using the Zymo™ Gel extraction kit	71
2.7. Blood processing methods	72
2.7.1. Processing of whole blood using Ficoll buoyancy gradient	72
2.7.2. Processing of whole blood using ACK lysis buffer	73
2.8. The Actiphage assay for the detection of Mycobacteria	73
2.8.1. Zymo™ clean and concentrator kit for the purification of mycobacterial DNA	74
2.9. PCR reactions	75
2.9.1. The IS900 end point PCR	75
2.9.2. Detection of MAP using qPCR	77

2.9.3. Detection of <i>M. bovis</i> using the Biosellal BioTK088 qPCR kit for the detection of MTBC organisms	78
2.9.4. Analysis and interpretation of qPCR results	78
3. Freezing of plaques as a method for preserving bacteriophage	79
3.1. Introduction	80
3.1.1. Aims and objectives	82
3.2. Results	83
3.2.1. Freezing of plaques for storing bacteriophage	83
3.2.1.1. Freezing of mycobacteriophage <i>D29</i> and <i>TM4</i> plaques and suspension	83
3.2.1.2. Viability over time of phage stored frozen in plaques	89
3.2.1.3. Investigation of critical components of plaques for Cryoprotection	91
3.2.1.4. Freezing of non-mycobacteriophage plaques and suspension	94
3.2.2. Drying of plaques for storing bacteriophage	99
3.2.2.1. Drying of plaques: initial experiments	99
3.2.2.2. Stability of cold dried plaques	101
3.2.2.3. Use of excipients to improve phage survival in artificial plaques	103
3.2.2.3.1. Trehalose	103
3.2.2.3.2. Glycerol	108
3.2.2.3.3. Gelatine	110
3.2.2.4. Freeze-drying of plaques and artificial plaques	112
3.3. Discussion	116
3.3.1. Freezing of plaques	116
3.3.2. Drying of plaques	119
3.4. Conclusion	121

4.2.3.2. Production of high titre TM4 phage lysate using the SCLP method	163
4.2.3.3. Freeze-drying of phage TM4 lysate with trehalose and stability studies	163
4.3. Discussion	166
4.3.1. Using SCLPs for the production of phage lysate	166
4.3.2. Production of a freeze dried bacteriophage D29 preparations	168
4.3.3. Conclusion	169
5. Freeze-drying of Mycobacteria	171
5.1. Introduction	172
5.1.1. Aims and objectives	174
5.2. Freeze drying of <i>Mycobacterium avium ssp. paratuberculosis</i>	176
5.2.1. Initial experiments: Freeze drying of MAP (ATCC) slope scrape material without excipients	176
5.2.2. Producing MAP culture in an active growth state	177
5.2.3. Freeze drying of MAP using excipients initial experiments	177
5.2.4. Freeze drying of MAP using Sorbitol as an excipient	179
5.2.5. Stability of freeze-dried MAP preparations containing excipients stored at room temperature	183
5.2.5.1. Results MAP preparations immediately after Freeze Drying	184
5.2.5.2. Calculating dilution efficiency of FD MAP culture dilutions	184

5.2.5.3. Effect of storage at room temperature on Freeze-dried MAP samples	187
5.2.6. Investigating effect of trehalose on phage D29 plaque formation	190
5.2.7. The presence of eDNA in freeze dried MAP preparations	195
5.2.8. Washing of MAP cells to remove eDNA	200
5.2.9. Stability of freeze-dried MAP preparations	203
5.2.9.1. Freeze-dried MAP tested without storage	205
5.2.9.2. Effect of storage and temperature on Freeze-dried MAP samples	205
5.2.10. Pre incubation as a means to increase the number of detectable MAP cells	209
5.3. Freeze drying of <i>Mycobacterium bovis</i> (BCG)	213
5.3.1. Introduction	213
5.3.2. Results	213
5.3.2.1. Initial experiments on freeze drying <i>Mycobacterium</i> <i>bovis</i> (BCG) with and without trehalose	213
5.3.2.2. Freeze drying <i>Mycobacterium bovis</i> (BCG) using trehalose as an excipient	216
5.3.2.3. Evidence for the presence of eDNA in freeze-dried <i>M. bovis</i> (BCG) samples	220
5.4. Discussion	223
5.4.1. Freeze drying of MAP and <i>M. bovis</i> (BCG)	223
5.4.2. Pre-incubation of freeze dried culture as a means of increasing detectability	224

5.5. Conclusion	227
6. Evaluation of ACK lysis buffer for the isolation of MAP from blood samples for use phage based detection assays	229
6.1. Introduction	230
6.1.1. Aims and Objectives	232
6.2. Results	233
6.2.1. Phage based detection of MAP in deer blood using end point PCR	233
6.2.2. Use of ACK buffer for the Isolation of peripheral blood mononucleocytes	238
6.2.2.1. Initial experiments on cattle blood	238
6.2.2.2. Determining lysis time of deer blood when treated with ACK buffer	245
6.2.3.2. Comparing the ACK lysis buffer and Ficoll buoyancy gradient method for isolating white blood cells from deer	249
6.2.3. Detection of MAP in deer blood treated using the ACK lysis buffer method	252
6.2.4. Comparing the Actiphage assay + qPCR to MAP ELISA assay	254
6.3. Discussion	254
6.3.1. The use of ACK buffer for the isolation of PBMCs from deer blood	254
6.3.2. Detection of MAP infection and JD in deer using the Actiphage® Rapid Blood Test kit	255

6.4. Conclusion	257
7. General discussion and future work	258
7.1. Impact of the Covid19 pandemic on this project	259
7.2. Production of high titre phage lysate using SCLPs	261
7.3. Preservation of bacteriophage	263
7.4. Freeze-drying of MAP	265
7.5. The use of ACK lysis buffer for the isolation of PBMCS intended to be used in the Actiphage® Rapid Blood assay kits	267
7.6. Conclusion	269
8. Bibliography	270
9. Appendices	318
Appendix 1: Paper published: Development of a method to detect <i>Mycobacterium paratuberculosis</i> in the blood of 2 farmed deer using Actiphage® Rapid	319
Appendix 2: Paper under review for publication: Freezing of plaques as a novel method for storing Bacteriophage	330
Appendix 3: Presentation slides 4 th international scientific conference, 2018, Nizhny Novgorod, Russia	346
Appendix 4: Presentation slides Phages 2019 symposium, 2019, Oxford, UK	351

List of Figures

1.1.	Image of ZN stained mycobacteria	3
1.2.	Schematic diagram of the mycobacterial cell wall	5
1.3.	Phylogenetic tree of MTBC organisms	10
1.4.	Image of <i>M. smegmatis</i> colonies	25
1.5.	Schematic representation of a Myovirus and a Siphovirus	34
1.6.	Schematic diagram illustrating the lysogenic and lytic pathways of phage replication	38
1.7.	Schematic diagram of the Fast Plaque phage amplification assay	44
1.8.	Schematic diagram of the Actiphage blood assay	50
2.1.	Schematic overview of the two-day phage amplification assay	66
2.2.	Interpreting results of the two-day assay	67
2.3.	Examples of positive and negative results of the FPTB assay	69
3.1.	Comparison of phage D29 survival in liquid suspension or embedded in plaques when storing at refrigeration or frozen at -20 °C	87
3.2.	Comparison of phage TM4 survival in liquid suspension or embedded in plaques when storing at refrigeration or frozen at -20 °C	88
3.3.	Effect of long term frozen storage on stability of phage D29	90
3.4.	Comparing the effect of gelatine and agarose on cryoprotection of phage	93
3.5.	Evaluation of plaque storage in different phage morphologies	97
3.6.	Stability of dried artificial plaques containing 10% trehalose at room temperature	107
3.7.	Comparing phage viability in freeze-dried natural and artificial plaques during room temperature storage	115
3.8.	Schematic diagram comparing the molecular structure of Agarose, Sucrose and Trehalose	118
4.1.	Determining elution time of phage D29 plaques	131
4.2.	Determining the effect of volume of elution medium on final phage concentration	135
4.3.	Image of Semi Confluent Lysis Plate	138
4.4.	Comparing titres of phage suspensions obtained from 1st and 2 nd elution of cubed SCLPs	140
4.5.	Monitoring stability of freeze-dried phage D29 stored at room temperature	151
4.6.	Monitoring viability of freeze-dried phage TM4 stored at ambient temperatures	165
5.1.	Room temperature stability of freeze dried MAP containing various excipients	189
5.2.	Effect of trehalose on the efficiency of plating of Bacteriophage D29	192
5.3.	Repeat of experiment to determine effect of trehalose on the efficiency of plating of phage D29	194
5.4.	Determining the presence of eDNA in freeze dried MAP preparations	199
5.5.	Effect of washing on eDNA levels present in FD MAP cultures	202

5.6.	Comparing detectability of freeze dried MAP culture stored at room temperature using the two-day phage amplification and Actiphage® Rapid assay	204
5.7.	Effect of pre-incubating freeze-dried MAP cells on detection using the phage enumeration assay	211
5.8.	PCR Efficiency plots for the diluted <i>M. bovis</i> BCG samples	219
5.9.	Effect of washing on the detectability of BCG culture using the Actiphage® Rapid phage detection assay	222
6.1.	Detecting MAP in 40 deer blood samples	235
6.2.	Gel image of non-amplified deer DNA	237
6.3.	Visual comparison of PBMCs obtained using ACK lysis buffer or Ficoll gradient	239
6.4.	Comparison of Actiphage qPCR test results using ACK lysis buffer or Ficoll gradient for isolation of PBMCs	242
6.5.	Comparison of PCR Efficiency when using samples prepared using either the ACK or Ficoll method	244
6.6.	Deer blood in ACK lysis buffer before and after blood lysis	246
6.7.	Effect of lysis time of deer blood in presence of ACK buffer	248
6.8.	Distribution of positive Actiphage MAP test results among commercial deer herd production groups	253

List of Tables

1.1.	Diagnostic tests available for the detection of JD	31
2.1.	List of bacteriophage used	60
2.2.	List of bacteria and growth media used	61
2.3.	P90 and P91 Primer sequence and DNA target	76
3.1.	Comparing phage D29 survival in plaques or suspension at refrigeration and freezing conditions	86
3.2.	Details of phage, host strains and media used	95
3.3.	Titres of different phage recovered from plaques stored at 4 °C or – 20 °C	98
3.4.	Effect of varying trehalose concentration on phage survival	105
3.5.	Effect of varying glycerol concentration on phage survival	109
3.6.	Effect of varying gelatine concentration on phage survival	111
4.1.	Effect of volume on total number of phage eluted	134
4.2.	Using freeze-dried D29 in the two-day phage amplification assay to detect <i>M. smegmatis</i>	154
4.3.	Using freeze-dried D29 in the two-day phage amplification assay to detect MAP	157
4.4.	Comparing freeze dried phage preparation with liquid phage lysate when used in the Actiphage assay	161
5.1.	Two-day phage amplification assay detectability of freeze-dried MAP containing sorbitol	182
5.2.	Number of MAP cells detected after Freeze-drying culture on day 0 and dilution efficiency calculations	186
5.3.	Summary of storage trial results of FD MAP cultures	206
5.4.	Summary of data for initial <i>M. bovis</i> BCG samples	215
6.1.	Comparing qPCR results of deer blood samples treated using ACK lysis buffer and Ficoll	251

List of abbreviations

ACK	Ammonium-Chloride-Potassium
BCG	Bacillus Calmette-Guérin
BHI	Brain Heart Infusion
Btb	<i>Mycobacterium bovis</i>
bTB	Bovine tuberculosis
CFU	Colony Forming Units
Ct	Cycle threshold
Cq	Cycle quantification
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
E.o.P	Efficiency of plating
EUR	Euro
FAS	Ferrous ammonium sulphate
FD	Freeze-Dried
FISH	Fluorescence In Situ Hybridization
FPTB	Fast Plaque Tuberculosis (assay)
GBP	Great British Pound
HIV	Human Immunodeficiency Virus
IFU	Instruction for Use
IMS	Immuno-Magnetic Separation
IRGM	Immunity-related GTPase M
JD	Johne's Disease
LAM	Lipoarabinomannan

LOD	Limit Of Detection
LPS	Lipopolysaccharide
MAC	<i>Mycobacterium avium</i> Complex
MAH	<i>Mycobacterium avium ssp. hominissuis</i>
MAP	<i>Mycobacterium avium subsp. paratuberculosis</i>
MDR	Multi Drug Resistant
MP	Media Plus (Middlebrook 7H9 media containing OADC supplement)
Mtb	<i>Mycobacterium tuberculosis</i>
MTC	<i>Mycobacterium tuberculosis</i> Complex
NTM	Non-Tuberculous Mycobacteria
NZD	New Zealand Dollar
OD	Optical Density
PBMC	Peripheral Blood Mononucleocyte
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PNA	Peptide Nucleic Acid Probes
qPCR	Quantitative Polymerase Chain Reaction
RBP	Receptor Binding Protein
RNA	Ribonucleic Acid
RO	Reverse Osmosis
RT	Room Temperature
SCLP	Semi Confluent Lysis Plate
SDR	Single Drug Resistant

SEM	Scanning Electron Microscope
SGIM	Scanning Gallium Ion Microscope
SHIM	Scanning Helium Ion Microscope
Tb	Tuberculosis
TDR	Totally Drug Resistant
TST	Tuberculin Skin Test
USD	United States Dollar
UV	Ultraviolet
WBC	White Blood Cells
WHO	World Health Organization
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen

Chapter 1

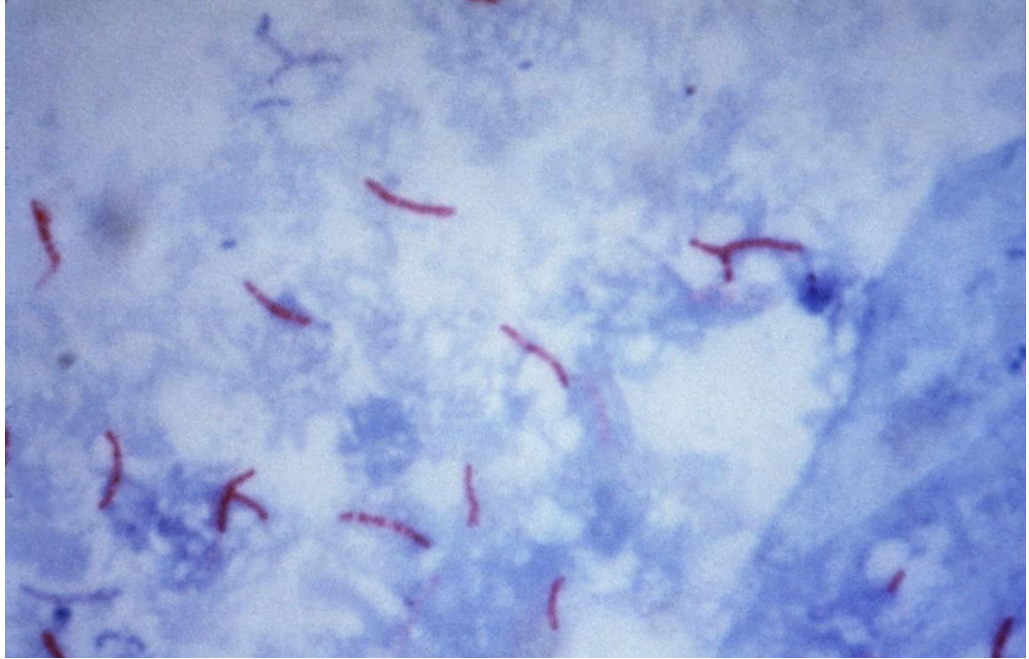
Introduction and Literature Review

1.1. The genus *Mycobacterium*

The genus *Mycobacterium* is part of the family of the *Mycobacteriaceae*, which is part of the order of *Actinomycetales* (Stackebrandt *et al.*, 1997; Gupta *et al.*, 2018) these organisms are aerobic, acid fast, non-motile rods. The genus *Mycobacterium* encompasses a large number of clinically and economically relevant pathogens, most notably *M. tuberculosis* which causes tuberculosis in humans and results in the death of ~2 million people annually (Almeida DaSilva and Palomino, 2011). Other important pathogenic mycobacteria include *M. bovis* (the causative agent of bovine tuberculosis) and *Mycobacterium leprae* (the causative agent of leprosy) (Schuenemann *et al.*, 2013; Wilen *et al.*, 2015). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is another member of the slow growing group which causes Johne's disease in cattle and has been suspected of causing Crohn's disease in humans (Liverani *et al.*, 2014; McNees *et al.*, 2015).

One of the defining features of the Mycobacteria are their cell walls which are rich in high molecular weight lipids, most notably mycolic acid. Although genetically part of the Gram-positive group of bacteria, the waxy, lipid-rich cell wall of Mycobacteria prevents the penetration of the chemicals used for cell classification using the Gram staining procedure. Therefore, mycobacteria are visualized and identified using acid fast staining. The most common acid fast staining method used for the identification of mycobacteria is the Ziehl-Neelsen stain (ZN) which detects ability of the waxy cell walls to retain carbol fuchsin and therefore the cells appear red (Fig. 1.1), typically on a blue background when visualised in clinical specimens. This waxy, cell wall rich in high molecular weight hydrocarbons, is not present in any other group of Gram-positive bacteria (Cook *et al.*, 2009) but is found in other members of the mycolata which is a distinctive suprageneric taxon that includes the genera *Corynebacterium*, *Nocardia* and *Rhodococcus*, as well as *Mycobacterium* (Sutcliffe, 1998).

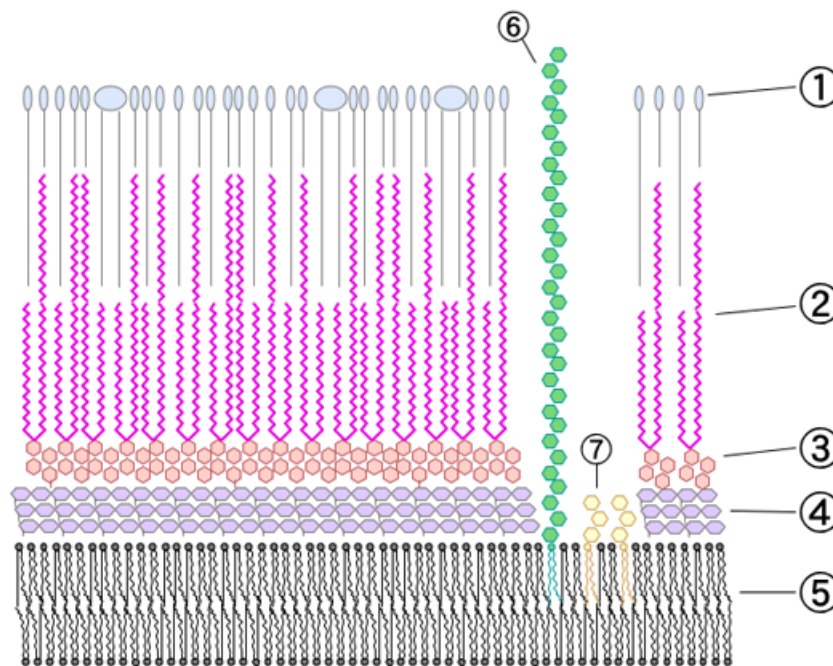
Figure 1.1. Image of ZN stained mycobacteria



Mycobacterium tuberculosis (red) stained using Ziehl-Neelsen staining at 1000x magnification. Open source Image kindly provided by Dr. G.P. Kubicka, CDC.

The mycobacterial cell wall is composed of a relatively thin peptidoglycan layer surrounding the cell membrane with a layer of arabinogalactans serving as an anchor for a thick outer layer consisting of hydrophobic mycolic acid strands (Fig. 1.2). Exopolysaccharides (EPS) and glycolipids are also present on the mycobacterial cells surface (Brennan, 2003). The thick hydrophobic outer layer of the mycobacterial cell wall contains comparably few porins thus not allowing easy permeation of chemicals, including water (Gygli *et al.*, 2017). This is believed to contribute to the ability of Mycobacteria to survive in the environment by protecting the organism from desiccation and antimicrobial agents allowing mycobacterial species to survive >50 weeks in the environment (Whittington *et al.*, 2004). The waxy nature of the cell wall also hinders the penetration of antimicrobial compounds, such as antibiotics and chemotherapy agents therefore conferring a high degree of intrinsic resistance to antimicrobial agents. This contributes to the difficulty when trying to treat mycobacterial infections, since there is only a limited range of antibiotics which can be effectively used against these type of bacteria (Jarlier and Nikaido, 1994).

Figure 1.2. Schematic diagram of the mycobacterial cell wall



Schematic diagram showing cross sectional organisation of a mycobacterial cell wall. Numbers represent relative position of: 1, Outer lipid layer; 2, Mycolic acid; 3, Arabinogalactan; 4, Peptidoglycan; 5, cell membrane; 6, Exopolysaccharides (EPS); 7, Glycolipids. (Adapted from Tambe, 2006.)

The intrinsic resistance to antimicrobial agents found in mycobacteria is compounded by drug inactivation by the cells, such as enzymatic degradation. The most well characterised example of this is beta lactamase which hydrolyses the beta lactam ring found in antibiotics such as penicillin, methicillin and aztreonam rendering *M. tuberculosis*, and also *M. bovis* and MAP, virtually resistant to beta lactam antibiotics (Almeida DaSilva and Palomino, 2011; Gygli *et al.*, 2017; Ghielmetti and Giger, 2020).

Two of the most widely used drugs for the treatment of tuberculosis (ethambutol and isoniazid) target the biosynthetic pathways for the production of arabinan and mycolic acid (Johnson *et al.*, 2006; Gygli *et al.*, 2017). Today the antibiotic Rifampicin is also commonly used in the treatment of tuberculosis and other mycobacterial disease due to its ability to block RNA synthesis by binding to the RNA polymerase and physically blocking extension of the RNA molecule thus preventing RNA and protein synthesis (Johnson *et al.*, 2006). However, when Rifampicin was first widely used in the 1960's and 1970's it was quickly discovered that antibiotic resistance developed very fast (Prasad *et al.*, 2018). This is the reason why today Rifampicin is almost never given alone but rather as part of a multi-drug therapy which commonly also include ethambutol and isoniazid (Gandhi *et al.*, 2010).

Resistance against these two first line drugs developed rapidly as well with drug resistance in *M. tuberculosis* being categorized into four groups. A) Single drug resistant (SDR-TB), usually only resistant to rifampicin B) Multi drug resistant (MDR-TB) resistant to rifampicin and isoniazid, C) extensively drug resistant (XDR-TB) which possess resistance against rifampicin, isoniazid and at least one second line anti mycobacterial drug such as ethambutol or pyrazinamide. The fourth category are the totally drug resistant (TDR-TB) strains of *M. tuberculosis* which possess resistance against all common first and second line anti mycobacterial drugs (Singh and Chibale, 2021). According to Singh and Chibales (2021) there were around 500,000 new rifampicin resistant cases in 2019, of which 78 % were also MDR-TB and resistant to isoniazid. They also report a total of 12,350 global cases of XDR-TB in 2019.

Hence understanding the basis of drug resistance is of growing importance, not only to help treat infections, but also for the development of novel treatments in the future.

The reduced permeation of chemicals through the mycobacterial cell wall is also conducive to its lifestyle as an intracellular pathogen as it protects the *Mycobacterium* from the hostile environment within the phagosome of a macrophage. However, this is not the only reason these bacteria are able to survive initial uptake into macrophages. Other mechanisms - including the inhibition of phagosome maturation and acidification - are more relevant to mycobacterial survival within macrophages. In particular, inhibition of phagosome maturation into phagolysosomes is a key aspect of successful mycobacterial infection of macrophages (Awuh and Flo, 2017). The exact mechanism of this process varies between different pathogens and is not yet fully understood. However, the compartmentalization of the mycobacterial pathogen inside the phagosome and the secretion of effector proteins, (mainly lipoproteins, and lipids such as lipoarabinomannan (LAM) and phosphatidyl inositol mannoside) into the phagosome, appears to mask the presence of the pathogen. Despite this, nutrients like iron and lipids, required by the mycobacterial cells for growth, continue to be delivered to the phagosome (Thoen *et al.*, 2006; Awuh and Flo, 2017). Prevention of phagosome maturation requires constant excretion of mycobacterial effector proteins, and eventual detection and destruction of the mycobacterial pathogen by the phagosome will resume once inhibitor proteins are no longer secreted by the pathogen (Awuh and Flo, 2017).

The genus *Mycobacterium* contains a very large number of diverse species, most of which are non-pathogenic, but some species of *Mycobacterium* – as described earlier - are of great clinical relevance. Members of the genus can be grouped in two categories depending on their growth characteristics in the laboratory; fast growers (for example *Mycobacterium smegmatis*) produce colonies in under 7 days and slow growers (such as *Mycobacterium tuberculosis*, *Mycobacterium leprae* or *Mycobacterium bovis*) that can take

weeks or months to produce colonies (Cook *et al.*, 2009). The intracellular pathogenic Mycobacteria generally belong to the slow growing group, while the fast-growing mycobacteria are commonly environmental organisms with only few opportunistic pathogens belonging to that group. The reasons why some mycobacteria are slow growers while others have doubling times similar to that of *E. coli* or *Salmonella* are not fully understood, however it has been proposed that the high energy cost of producing large quantities of mycolic acid (up to 40 % of total weight in *M. tuberculosis*), as well as the low nutrient permeability of the mycobacterial cell wall, are limiting factors for mycobacterial growth (Lewin and Sharbati-Tehrani, 2005). A recent study by Bachmann *et al.* (2020) compared the genomes of >150 mycobacterial species and has found that there appears to be a significant difference in the amount of amino acid transport proteins found in the genomes of fast and slow growing mycobacterial species, with the slow growers possessing significantly less amino acid transporters which may be a limiting factor in bacterial growth. However, it is evident from the levels of Mycobacteria that can be detected in clinical samples, that even the slow growing bacteria have the capacity to grow to high levels inside their host. For instance, Imirzalioglu *et al.* (2011) calculated that the amount of MAP present in samples taken from naturally infected animals was in the range of $\sim 2 \times 10^4$ to 6×10^7 bacteria per gram of inflamed gut tissue and $\sim 1 \times 10^7$ to 2×10^9 bacteria per gram of faeces.

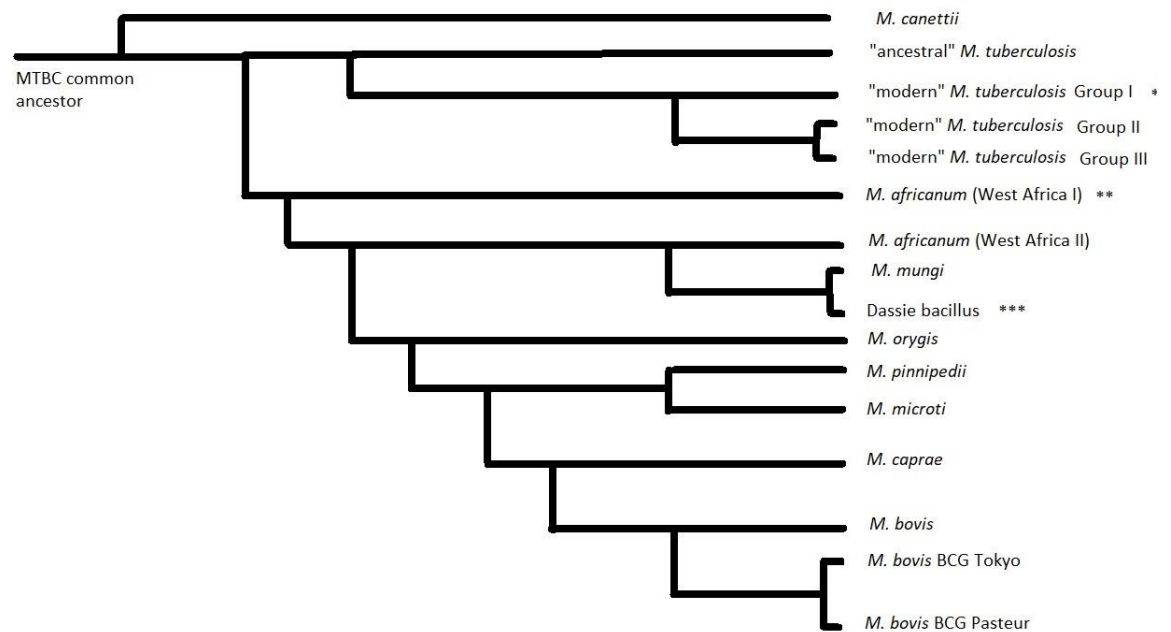
1.1.1. The Mycobacterium tuberculosis Complex

The *Mycobacterium tuberculosis* complex (MTbC) consists of multiple different species and sub-species, including *M. tuberculosis* and *M. bovis*, but also includes a number of less well-known organisms that are primarily associated with disease in animals, such as *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium microti*, and *Mycobacterium pinnipedii* (Ernst *et al.*, 2007). The MTbC organisms are strikingly similar at the genetic level, sharing the same 16s rRNA sequence

(Böddinghaus *et al.*, 1990) and cause disease in a very similar fashion (droplet infection through the lungs followed by dissemination through the lymphatic system) but vary greatly in their host preference. For instance, *M. canetti* can cause tuberculosis in humans, although this is not common. *M. caprae* and *M. microti* are known to cause tuberculosis in different mammals, such as cats, llamas, pigs and voles (Niemann *et al.*, 2002). *Mycobacterium pinnipedii* meanwhile causes tuberculosis in marine mammals such as seals and sea lions and is suspected to be transmissible to other animals such as cattle and sheep (Roe *et al.*, 2019). An illustration of the phylogeny of MTBC organisms is provided in figure 1.3.

The most significant mycobacterial human pathogens today are *M. tuberculosis* (the causative agent of tuberculosis), *M. bovis* (the causative agent of bovine tuberculosis) and *Mycobacterium leprae* (the causative agent of leprosy) (Schuenemann *et al.*, 2013; Wilen *et al.*, 2015). Interestingly, despite the wide host tropism of these other members of the MTBC group, humans are the only known reservoir for *M. tuberculosis* which is also the main causative agent of tuberculosis in humans.

Figure 1.3. Phylogenetic tree of MTBC organisms



Phylogenetic tree depicting the genetic relationship between MTBC organisms based on regions of difference and single nucleotide polymorphisms. Adapted from Brosch et al., 2002 and Rodriguez-Campos et al., 2014. The distances between certain branches may not correspond to actual phylogenetic differences calculated by other methods.

* 3 "Modern" *M. tuberculosis* strains due to mutations in the *katG*⁴⁶³ and *gyrA*⁹⁵ codons as described by Sreevatsan et al., 1997.

Group I: *katG*⁴⁶³ CTG, *gyrA*⁹⁵ ACC

Group II: *katG*⁴⁶³ CGG, *gyrA*⁹⁵ ACC

Group III: *katG*⁴⁶³ CGG, *gyrA*⁹⁵ AGC

** *M. africanum* West Africa I lacks RD9 region while West Africa II also lacks RD7, RD8 and RD10 regions (Brosch et al., 2002).

*** Dassie bacillus is a mycobacterial pathogen in South African hyrax or dassie (*Procavia capensis*). Phenotypically similar to *M. microti* but phylogenetically not closely related and more aligned with *M. africanum* and *M. mungi* (Rodriguez-Campos et al., 2014)

M. tuberculosis results in life threatening disease in humans affecting a broad range of organs, but most significantly is associated with disease of the lungs. These infections are often chronic and eventually fatal due to tissue damage caused by granuloma formation. Approximately one third of the global population is suffering from *M. tuberculosis* infection at any given time and the organism causes around 2 million deaths annually (Almeida DaSilva and Palomino, 2011). Especially in developing countries, tuberculosis remains a major public health problem accounting for ~7 % of total deaths and being the most common cause of death from a single pathogen in adults (Zaman, 2010).

Due to this organism being part of the slow growing mycobacterial group (*M. tuberculosis* grown in the laboratory has a doubling time of 15 - 20 h and visible growth on agar takes up to 12 weeks to develop), samples have to be incubated for extended periods of time, making culture a less than optimal method of diagnosis of infection in a clinical setting (Lange and Mori, 2010). The issue of long cultivation times is compounded by the emergence of drug resistance, since drug sensitivity testing is becoming more and more necessary for successful treatment (Singh and Chibale, 2021) and is classically performed once colonies have been isolated from a clinical sample. This problem has been addressed by new developments in nucleic acid amplification-based methods which are able to detect the presence of mycobacterial DNA and allowing identification of putative pathogens in a fraction of the time required for culture based methods. Nucleic acid based detection assays like the GeneXpert MTB system amplify mycobacterial DNA using PCR and also allow antibiotic sensitivity testing (GeneXpert MTB/RIF) based on the identification of polymorphisms in the amplified bacterial genome. Originally the GeneXpert MTB/RIF assay was developed to the detection of Rifampicin resistance by identifying mutations in the RNA polymerase beta (*rpoB*) gene responsible for rifampicin resistance (Boehme *et al.*, 2010). Rifampicin resistance can also be used as a marker for Isoniazid resistance as, as discussed earlier, resistance to rifampicin alone is rare and > 90% of rifampicin resistant isolates are also isoniazid resistant (Ioannidis *et al.*,

2011). However, this company has now developed a system (Xpert MTB/XDR) that can detect mutations associated with a number of different antibiotic resistances, including isoniazid, fluoroquinolones, second-line injectable drugs (amikacin, kanamycin, capreomycin) and ethionamide, in a single test. A 2020 review by Bainomugisa *et al.* (2020) found that the Xpert MTB/XDR system offered significant benefits in terms of field operability and deploy ability compared to standard drug susceptibility assays. The Xpert MTB/XDR system was also able to detect fluoroquinolone resistance with 91.4% sensitivity and 98.5% specificity however it was also noted that the assay suffered from an inability to detect low level fluoroquinolone resistance caused by mutations (Bainomugisa *et al.*, 2020).

A vaccine against tuberculosis is available in form of an attenuated strain of *M. bovis* Bacillus Calmette-Guérin (BCG) and is named after the scientists who first described this strain in the early 20th century. This vaccine strain was first used to immunize a human against TB in 1921 and is still one of the most widely used vaccines in the world today. As of 2018, *M. bovis* (BCG) has been used by national vaccination programmes in over 180 countries with individuals usually being vaccinated a few days after birth. The BCG vaccine has been shown to have up to 80 % efficacy in preventing disease (Li *et al.*, 2021; Cho *et al.*, 2021). However, despite aggressive eradication and vaccination programmes tuberculosis, which can be caused by *M. tuberculosis*, *M. bovis* or other members of the MTBC group, remains a significant challenge to global health with some vaccine strains of BCG being found to be significantly less efficient at producing protection than the 80 % reported by Li *et al.* (Cho *et al.*, 2021).

M. bovis, like *M. tuberculosis*, is an intracellular pathogen and part of the slow growing group of mycobacteria, again making culture challenging and time consuming. *M. bovis* primarily causes tuberculosis in cattle (Bovine TB) but may infect a broad range of other animal species (Gormley *et al.*, 2006). Its large host range includes, besides cattle, also sheep (Perez de Val *et al.*, 2021), deer (Waters and Palmer, 2015), dogs (Ellis *et al.*, 2006), goats (Gonzalez-

Juarrero *et al.*, 2013) and many more. The ability of *M. bovis* to cause tuberculosis in most domesticated mammals makes it an economically relevant pathogen, especially for the cattle and dairy industry. An estimated 3 billion USD are lost to the global economy due to *M. bovis* infections and mass culling of diseased animals every year while between 2008 and 2009 the British government spent ~100 million GBP on eradication and control programs to combat *M. bovis* in the UK (Schiller *et al.*, 2010). However, apart from its economic impact on cattle herds, it also poses a considerable public health risk for humans because it is able to jump the species barrier and cause tuberculosis in humans (Perez-Lago *et al.*, 2014). Animal eradication programs have been undertaken around the world over the past decades, but eradication has proven difficult due to the organism being endemic in animal populations in many areas and in the presence of wildlife reservoirs, such as badgers or deer (Schiller *et al.*, 2010). *M. bovis* can be transmitted to humans via consumption of contaminated milk and other dairy products and by inhalation of droplets while being in the presence of infected animals (Perez-Lago *et al.*, 2014). It is also known that the organism can be transmitted between humans, again primarily by inhalation of expelled droplet aerosols (Sunder *et al.*, 2009).

As milk in developed countries is commonly pasteurized, infections by this route in countries that use these food chain interventions are now rare. However, in non-developed countries where milk pasteurization and infection control in dairy herds are less common, *M. bovis* is still a major cause of human TB. For example, one study found that up to 28% of human cases in Mexico and up to 9% of cases in India were caused by *M. bovis* (Olea-Popelka *et al.*, 2017) while only 0.5-1% of cases in western European countries were found to be caused by this organism. Interestingly, developed countries with a large meat and dairy industry, such as Ireland or New Zealand, were found to have above average rates for developed countries (2.7% and 3%, respectively) of human tuberculosis cases being caused by *M. bovis* (Perez-Lago *et al.*, 2014). Animals found to be infected with *M. bovis* in developed countries are

commonly culled as part of disease control programs, causing significant economic loss for farmers in countries that do not have TB-free status. For instance, in the UK between 2008 and 2018, over 400,000 animals were slaughtered because they were found to be infected with *M. bovis* (Uberoi, 2019).

1.1.2. The *Mycobacterium avium* complex

The *Mycobacterium avium* complex (MAC) consists of slow growing mycobacteria that are commonly found in the environment. Before the dawn of the genomic era, the MAC consisted of just *M. avium* and *M. intracellulare*. However based on findings from genome sequencing studies, *M. avium* was divided into three distinct subspecies. *M. avium* ssp. *avium*, *M. avium* ssp. *paratuberculosis* (MAP) and *M. avium* ssp. *silvaticum* (Turenne *et al.*, 2007). MAC bacteria can be found in the soil and in water and can tolerate exposure to disinfectants and UV light. For instance MAP cells have been found to survive in the environment and on surfaces for up to 55 weeks under ideal conditions (Whittington *et al.*, 2004). Mycobacteria, pathogenic and environmental, are also regularly detected in potable water supplies in developed countries due to their ability to resist biocides as well as ultraviolet radiation and ozonation commonly employed for the disinfection of drinking water (von Reyn *et al.*, 1994, Dowdell *et al.*, 2019). MAC organisms also form hard to remove biofilms inside water pipes, water outlets and shower heads (Tzou *et al.*, 2020).

M. avium ssp. *avium* and *M. intracellulare* have been known to be opportunistic pathogens in humans for decades, however the incidence of infections by these pathogens (termed non-tuberculosis mycobacteria or NTM) is low and there is no evidence for human-to-human spread of MAC NTMs. Symptoms of MAC infections in humans can be pulmonary disease and sub-acute lymphadenitis, but dissemination of the bacteria throughout the body has been reported (von Reyn *et al.*, 2002). Furthermore, in HIV patients, the infection can disseminate to the gastrointestinal tract, the central nervous system, the spleen, and the bone marrow (Inderlied *et al.*, 1993). Treatment

of MAC infections consists of long term antibiotic treatment but recurring infections even after antibiotic treatment are common (Hasegawa *et al.*, 2009).

Infections caused by NTMs in humans are usually a result of repeated exposure to the pathogen, for example by close proximity to animals or persistent presence of the organism in water or food (Inderlied *et al.*, 1993; Nishiuchi *et al.*, 2017). Alternatively, infection with MAC organisms are generally found in immunocompromised patients, such as those undergoing immunosuppressive therapy or suffering from other diseases that impair the immune system, such as leukaemia or AIDS (Jung *et al.*, 2017). Hence, since the onset of the HIV epidemic during the 1980s, the reports of MAC infections has increased considerably (Inderlied *et al.*, 1993; Von Reyn *et al.*, 1994). In 2014 the estimated incidence of pulmonary NTM infections in Japan was 14.7 per 100,000 population marking an ~2.6 fold increase from the case levels reported in 2007 (5.7 cases per 100,000population). NTM infections can be caused by a diverse range of non-MTBC organisms, but MAC organisms are the most common cause accounting for ~88 % of reported cases (Nishiuchi *et al.*, 2017). A similar trend was observed in the UK where the incidence of NTM infections rose from 5.6 per 100,000 population in 2007 to 7.6 per 100,000 population in 2012, again with the most frequently identified organisms being MAC (Shah *et al.*, 2016). Cystic fibrosis is another underlying health condition that is a risk factor for acquiring NTM infections (Adjemian *et al.*, 2018). The incidence of MAC and other NTM infections has continuously increased over the past 4 decades. In the 1980s in North America less than 2 cases of NTM infections were recorded per 100,000 people while a recent (2020) review on the topic reports incidences of > 14 cases per 100,000 people (Hannah *et al.*, 2020). Meanwhile a study conducted in South Korea reported an increase of NTM infections from 7 per 100,00 in 2001 to >55 in 100,000 in 2015. During this time period 75% of the NTM infections recorded were caused by MAC organisms (Ko *et al.*, 2018).

1.1.3. *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

Mycobacterium avium ssp. *paratuberculosis* (MAP) is another slow growing member of the MAC that primarily causes infecting cattle and other ruminants. Although taxonomically part of the *M. avium* complex (MAC) and a subspecies of *M. avium*, MAP differs from other species in this group in many genotypic and phenotypic characteristics. For instance, MAP possess a unique insertion sequence (IS900) in its genome in multiple copies, and this is commonly used as a signature sequence for the identification of MAP using polymerase chain reaction (PCR) assays (Turenne *et al.*, 2008).

One notable phenotypic difference is the very long incubation times required when these bacteria are cultured (up to 18 weeks for primary isolation), and MAP is also unique in that most strains require the addition of purified mycobactin J which is a metal ion chelator, to the culture media to allow growth in the laboratory (Merkal and McCullough, 1982). Mycobactin J is believed to help intracellular mycobacteria take up iron when growing in the nutritionally deprived environment that exists within the macrophage (Thorel *et al.*, 1984). MAP can naturally produce mycobactin J but the levels produced during *in vitro* do not seem to be sufficient to support good growth, and MAP may use alternative iron acquisition systems during *in vivo* growth (Wang *et al.*, 2016).

1.1.4. MAP as a pathogen in farmed and wild animals

MAP is the causative agent of Johne's disease - also known as paratuberculosis - in a large number of animal species including cattle, deer, sheep, antelopes, primate species, wild rabbits, badger, camels and many more. However, the economically most important species infected by MAP is cattle (Greenstein, 2003; Schiller *et al.*, 2010). Johne's disease is a chronic, inflammatory infection of the gastrointestinal tract which causes the intestinal wall to thicken resulting in reduced absorption of nutrients. As there is currently no cure available affected animals are culled resulting in significant economic loss as well as animal suffering.

Johne's is characterized by persistent diarrhoea and weight loss of the animal eventually leading to death. Poor nutrient absorption also drastically reduces milk yields and results in infertility of the animal (Ott *et al.*, 1999; Cho *et al.*, 2012). It was found that milk yield of animals infected with MAP was reduced by up to 19% compared to healthy individuals in the herd (Ott *et al.*, 1999). In addition, premature culling of infected animals, reduction of meat yield for slaughtered animals, reduced fertility, a sub-optimal feed conversion efficiency (*i.e.* due to the chronic wasting and weight loss, a larger amount of feed is consumed while a smaller amount of milk or meat is produced) and the overall increased susceptibility of the animal to other pathogens due to its weakened state add to the economic burden of this disease (Garcia and Shaloo, 2015).

Since Johne's disease is a worldwide problem, with infected herds found on every continent and in most regions of the planet, it represents a significant economic problem for any nation with large beef and dairy industries, including the USA, Canada, Argentina, the Netherlands and Ireland (Sohal *et al.*, 2015). To illustrate the scale of the problem, MAP-infected cows were found in approximately 32 % of Canadian and up to 68 % of US dairy herds (Cho *et al.*, 2012). In a study by Hill *et al.* (2003) it was found that approximately 50 % of all cattle herds in Alabama in the US contained MAP-positive animals, while the overall prevalence of MAP-infected animals was estimated to be 8 % of the total population. Ott *et al.* (1999) estimated that a Johne's-positive dairy herds in the USA experienced an average economic loss of \$100 per animal compared to a Johne's-free herd, mainly due to reduced milk yields and the cost of having to replace culled animals. In Europe the situation is similar, with an estimated 65 % of UK cattle herds containing at least one MAP-positive animal (Beasley *et al.*, 2011).

Johne's disease is most often transmitted through the faecal oral route but, as this is a systemic disease the organism can be excreted directly into the udder and can also be transmitted vertically via contaminated colostrum or via the placenta *in utero* thus infecting the new calves even before birth (Sweeney *et*

al., 2011; Cho *et al.*, 2012). Progression of the disease is slow (Magombedze *et al.*, 2017) and was found that animals not actively showing symptoms of Johne's disease can shed large numbers of viable MAP cells into the environment (usually $< 10^3$ cfu g⁻¹ of faeces; Garcia and Shaloo, 2015). Since clinical symptoms only become visible in the adult stage of the animal's life, it is likely by this point it has likely shed vast amounts of contaminated faeces into the environment.

MAP is only a notifiable disease on a state/regional level in many countries, such as Australia, Canada, and the UK (only Northern Ireland), and therefore it is very likely that MAP and Johne's infections in these countries are significantly underreported (Manning and Collins, 2001; Jordan *et al.*, 2020). It is likely that, due to the chronic and slowly progressing nature of the disease and the insufficient sensitivity in current testing methods, the real prevalence of Johne's disease in cattle herds is much higher than reported.

MAP has also been found to cause Johne's disease in wild and farmed deer and in ranches elk in Canada (Forde *et al.*, 2015). In cervids, MAP causes the same characteristic, slowly progressing wasting symptoms found in cattle (Fawcett *et al.*, 1995; Nugent *et al.*, 2011). This is concerning on two distinct levels. First of all, since deer farming is an industry mainly targeted towards meat production, the economic impact of Johne's disease on the deer industry is likely to be comparable to that identified for the beef industry. A report by the Deer Industry of New Zealand (Anon, 2015) estimated that annual losses for large scale deer farms due to reduced meat yields and replacement costs for culled animals can total more than 10,000 NZD a year.

Besides the economic damage of MAP inflicted on the deer farming industry, there is concern that wild deer could act as a reservoir for MAP in the wild and potentially spread the pathogen to other deer or cattle herds. This has been a major concern in regard to the spread of *M. bovis* as it was shown that wild deer can act as a reservoir for *M. bovis* and that it is possible for wild deer, as well as other wild animal species such as reindeer, elk or antelope, to cause outbreaks of *M. bovis* infections in farmed animals (Waters and Palmer, 2015;

Crispell *et al.*, 2020). There have been a limited number of studies on the prevalence of MAP in wild deer with one study finding that up to 30 % of wild red deer (*Cervus elaphus*) in Spain tested positive for MAP using an ELISA assay (Reyes-Garthia *et al.*, 2008). A second Spanish study looking at MAP prevalence in fallow deer (*Dama dama*) using post-mortem examination found that almost 30 % of fallow deer carcasses examined showed typical paratuberculosis lesions. These lesions were subsequently stained using Ziehl Neelsen staining and revealed the presence of mycobacteria which were confirmed to be MAP by PCR (Balseiro *et al.*, 2008). Further credibility was given to the hypothesis of wild deer posing an infection risk to farmed animals including cattle when Raizman *et al.* (2005) found that wild deer in Minnesota (USA) would graze and also defecate upon the pastures also used for grazing cattle, representing a point of contact between deer and cattle. Since 4 % of samples of deer excrement collected during this study were found to be MAP-positive, this confirmed a potential point of pathogen transmission between wild and domesticated species sharing the same environment. This was supported by the fact that the farms where the MAP-positive deer faeces were also found to have MAP-positive cows in their herds, however the study was not able to draw a conclusion whether the infections in cows were a result of cow and deer contact or purely coincidental (Raizman *et al.*, 2005). Nevertheless, further research and surveillance of MAP prevalence in farmed and wild deer will be relevant for determining the true impact of wild animal and farm animal contact.

1.1.5. The pathology of Johne's disease

It is generally thought that MAP infection predominantly happens during the first months or even days of an animal's life due to its close proximity to potentially contaminated faeces and milk and that susceptibility of the animal to MAP is at its highest immediately after birth (Windsor and Whittington, 2010; Sweeney, 2011; Cho *et al.*, 2012). Once MAP has been ingested, the main site of infection seems to be the ileum where the MAP cells are taken up by specialized epithelial cells called M cells which are located within the

payer's patches of the intestinal mucosal surfaces. These M cells facilitate MAPs crossing of the epithelium of the small intestine. Following entry of MAP cells into the tissues underneath the epithelium and the mucosal surfaces the bacterial cells are ingested by macrophages through phagocytosis. At this point the progression of the infection is dependent on the ability of the macrophage to destroy the internalized MAP cells which some animals are likely able to do thus controlling the infection in an early stage (Sweeney, 2011).

However, MAP cells are able to evade destruction by the macrophage and even replicate within the macrophage and it was found that MAP cells were able to survive inside macrophages for up to 5 weeks (Whitlock and Buergelt, 1996). The exact mechanisms of MAP survival within macrophages are not fully understood (Ssekitoleko *et al.*, 2021) however the main survival strategy employed by MAP, as well as MTC mycobacteria, is to infect the phagosome of the macrophage and prevent phagosome maturation and phagolysosome formation thus avoiding exposure to bactericidal enzymes and free radicals normally produced by the macrophage to destroy internalized pathogens (Arsenault *et al.*, 2014). As prevention of phagolysosome maturation is a crucial part of bacterial survival within the macrophage environment it is unsurprising that MAP employs multiple simultaneous strategies to disrupt phagolysosome formation mainly targeted at phagosome and lysosome membranes to prevent fusion of the two units. A secondary means of preventing phagosome maturation by MAP cells is through inhibiting the acidification of the phagosome which is required for optimal action of the lysosome's hydrolytic enzymes (Arsenault *et al.*, 2014; Ssekitoleko *et al.*, 2021). Nevertheless, in the early stages of infection, the host animal is generally able to contain the spread of the pathogen and of the disease only progressing slowly while being limited to the tissues of the small intestine and the surrounding lymphoid tissue. This early "eclipse" phase is characterized by the absence of clinical symptoms of Johne's disease and may last for two or more years (Sweeney, 2011). The long latency period of the disease with

symptoms not becoming visible for years after the infection makes control of the disease challenging. Therefore, routine testing of animals within herds to identify infection before they reach a clinical stage of infection when animal-to-animal spread becomes more likely, is of great importance for the control and possible eradication of Johne's disease.

The continuous presence of MAP antigens in the tissues of the epithelium of the small intestines attracts more macrophages to the area forming characteristic swollen lumps (granulomas) in the infected tissues. Although these granuloma help the animal's immune system to contain the infection in the gut and prevent a systemic spread of the pathogen, at a certain point the immune system's ability to contain the infection becomes severely reduced. The exact mechanisms of this are not fully understood but the release of immune suppressive cytokines including interleukin-10 from infected macrophages has been proposed (Lybek *et al.*, 2009; Fiorentina *et al.*, 2021). As a result of the immune system no longer containing the infection to the granulomas the pathogen starts spreading more rapidly, with dissemination of the pathogen to adjacent tissues. Its spread through the lymphatic system, combined with more and more granuloma formation, leads to granulomatous inflammation of the infected tissues in the small intestine and the clinical manifestation of Johne's disease symptoms (Whitlock and Buergelt, 1996; Sweeney, 2011; Rathnaja *et al.*, 2017). This point of the infection is characterised by increased shedding of MAP organisms in the animal's faeces and MAP antibodies may be detected in serum. The granulomatous inflammation due to influx of macrophages causes the characteristic swelling of the intestinal walls giving it a corrugated appearance and results in malabsorption of nutrients and hypoproteinaemia leading to weight loss and the characteristic "wasting" aspect of the disease and milk yields decrease. This stage is also characterized by the watery "pipe stream" diarrhoea shed by the animal.

The terminal stage of the disease is characterized by visible signs of malnourishment and appetite loss of the animal. At this point, the disease has

also become systemic with granuloma formation in lymph nodes outside the gastrointestinal tract. During the late/terminal stage of the disease the animal sheds large volumes of faeces which are heavily contaminated with MAP cells into the environment posing a significant risk of spreading the disease to new hosts in the herd and the animal will eventually die due to overall weakness or dehydration (Sweeney, 2011; Rathnaja *et al.*, 2017).

1.1.6. MAP as a pathogen in humans

MAP has been suspected to be one of the causes of Crohn's disease in humans, an inflammatory bowel disease affecting millions of people worldwide (Liverani *et al.*, 2014; McNees *et al.*, 2015). A link between this organism and Crohn's disease has been made since the early 20th century by a Scottish surgeon named Thomas Kennedy Dalziel, who noted that the clinical appearance in some of his patients was similar to that found in animals suffering from Johne's disease. Notably this was before Crohn's disease caused by MAP infections was first described by Crohn *et al.* in 1932. Both diseases are characterized by inflammations of the mucosal surfaces of the ileum and small intestines with symptoms including abdominal pain, diarrhoea, bleeding, bowel obstruction and weight loss (McNees *et al.*, 2015). The symptoms of Crohn's disease can vary from mild to severe and can be lifelong. However, the exact cause of Crohn's disease remains fiercely debated and there are a plethora of theories regarding the origin of the disease and what pathogenic microorganism - if any - may be the underlying cause (Naser and Collins, 2005; Naser *et al.*, 2015; McNees *et al.*, 2015). However, there has been mounting evidence for MAP being a factor in the development of Crohn's disease; for example, Naser *et al.*, (2004) managed to isolate MAP from breast milk and peripheral blood macrophages of Crohn's patients. Additionally, multiple studies employing PCR-based detection of the MAP-specific IS900 sequence found that this sequence was significantly more prevalent in samples taken from Crohn's patients and patients suffering from

ulcerative colitis than in the healthy control group (Lisby *et al.*, 1994; Mishina *et al.*, 1996).

Due to mounting evidence of MAP being a factor in the development of Crohn's disease and its widespread prevalence among dairy herds combined with MAPs ability to be transmitted through milk and dairy products (Gerrard *et al.*, 2018) a precautionary principle with the aim of removing MAP from the food chain should be adopted. This would involve routine testing not only of dairy herds but also of bulk milk and especially of powdered milk for use in infant formula.

1.2. Detection, Diagnosis and culture of Mycobacteria

1.2.1. Detection of Mycobacteria using culture-based methods

The diagnosis of mycobacterial diseases caused by any of the slow-growing mycobacteria (e.g. *M. tuberculosis*, *M. bovis* and MAP) is challenging as the long incubation periods required mean that using traditional culture-based identification methods is not practical. However, these organisms can be routinely cultured in laboratories, and when doing this the most common culture media used for the classical clinical detection of Mycobacterial species are Löwenstein-Jensen and Middlebrook 7H9, 7H10 or 7H11 media (Waterson, 2000). Löwenstein-Jensen medium contains egg suspension as well as 0.4 g of Malachite Green per litre serving as an antimicrobial agent to prevent growth of non-mycobacterial organisms. Middlebrook 7H9, 10 and 11 media should be enriched with OADC supplement containing oleic acid, albumin and dextrose in order to allow fastidious organisms such as *M. tuberculosis*, *M. bovis* and *M. avium* to grow. Middlebrook 7H9 medium contains no malachite green while 7H10 does. Middlebrook 7H11 medium is used for the isolation and sensitivity testing of *M. tuberculosis* and contains only 25% of the malachite green found in 7H10 agar as well as additionally containing casein enzymic hydrolysate to facilitate the growth of more fastidious *M. tuberculosis* strains (Cohn *et al.*, 1968). Colonies formed on these media are characteristically off-white to orange in colour with a rough,

dry appearance (See Fig. 1.4). However, some species do produce characteristic yellow-orange pigments, in particular when grown under stress conditions (Stevenson *et al.*, 2002; Cunningham *et al.*, 2004; Saviola and Felton, 2011). It may take up to 8 weeks for *M. tuberculosis* and up to 12 weeks for MAP before visible growth appears on these solid media (Whipple *et al.*, 1991), which is clearly not ideal when using culture as a primary diagnostic test before treatment can be initiated. Even grown in liquid broth, slow growing *Mycobacteria* may take up to 21 days to develop visible growth and then further tests are required to confirm the identity of the organism detected. Nevertheless, world-wide, culture-based approaches on solid media are still by far the most commonly used method for the detection of *Mycobacteria* in clinical or other samples due to its high sensitivity and low cost (Waterson, 2000).

Figure 1.4. Image of *M. smegmatis* colonies



Mycobacterium smegmatis colonies on Middlebrook 7H10 media. *M. smegmatis*, like other *Mycobacteria* such as *M. bovis* and MAP, form irregular, colonies with a rough or dry surface and are of off-white to light orange colour. Some strains of MAP can be pigmented, with colours ranging from yellow to orange (Stevenson et al., 2002).

Due to the difficulties performing conventional culture, automated or semi-automated “rapid” culture methods have been developed that rely on a machine detecting growth of the organism in a selective liquid media before it would be visible to the naked eye. An original commercial example of this would be the BACTEC 460TB (Beckton Dickinson, USA) system which used a broth in which the only available carbon source was radio labelled ^{14}C -palmitate which was converted to radioactive $^{14}\text{CO}_2$ by the metabolism of actively growing mycobacteria. The radioactivity in the gas phase due to the labelled CO_2 was then detected by a sensor within the apparatus. This system was replaced by the BACTEC 960/MGIT (Beckton Dickinson, USA) which detected oxygen consumption of mycobacteria growing in selective media with detection of growth being based on an oxygen-quenched fluorescence indicator. In this case the detection of increasing levels of fluorescence is used to detect and quantify mycobacterial growth (Zanetti *et al.*, 1997). These automated systems can shorten the time for culture-based detection considerably, but detection times are still relatively long (20-40 days), and further diagnostic tests are required to confirm the identity of the pathogen detected (Stabel, 1997; Gumber and Whittington, 2007).

1.2.2. Microscopy

Besides culture, microscopy is still the main method used for the detection of mycobacteria in clinical samples. As described earlier, Mycobacteria are acid fast bacteria (AFB) and are thus commonly visualized using the Ziehl-Neelsen (ZN) staining method which involves first dyeing the bacterial cell wall with carbol fuchsin and then a washing step using acid alcohol so that the cells are stained red (Yahya Noori *et al.*, 2017; see section 2.3.8). The main benefits of microscopy over alternative detection methods are the very low cost and that it allows same day detection. The sensitivity of microscopy however is within 50 – 70 % relatively low since a cell density $\sim 5 \times 10^5$ cells ml^{-1} per sample is required to ensure a cell is detected in a field of viewing when using x1000 magnification (Waterson, 2000). Microscopy only allows for a presumptive identification of bacteria and an indication of the infection status of the

patient. By default, ZN staining and microscopy does not allow differentiation between *M. tuberculosis*, other MTC organisms or non-tuberculous mycobacteria (NTM).

1.2.3. Nucleic acid based detection of Mycobacteria

Nucleic acid based rapid detection of Mycobacteria can be done by targeting specific sites on the 16S – 23S rRNA strands or insertion sequences. Insertion sequences commonly used for the detection of pathogenic mycobacteria are: IS1081 for *M. tuberculosis* and *M. bovis*, IS6110 for *M. bovis* and IS900 for MAP (Ahmed *et al.*, 1998). Spoligotyping (spacer oligotyping) uses the conserved direct repeat sequences as targets for *in vitro* DNA amplification which results in hybridization patterns allowing simultaneous detection and strain level identification of *M. tuberculosis* (Kulkarni *et al.*, 2005) and other mycobacteria such as *M. bovis* (Tekle, 2020). Nucleic acid based methods are commonly used for the detection of mycobacteria in clinical and veterinary samples. The main advantage of nucleic acid-based detection systems compared to culture based methods is the short time, weeks to months for culture and hours to days for nucleic acid methods, in which results can be obtained. Also, PCR-based detection should have a high sensitivity since theoretically a single genome copy is enough to be detectable. However, in practice these detection systems come with some severe drawbacks. The sensitivity of DNA based methods are often times considerably lower than that of culture based approaches and seems to be dependent on the quality of the sample and the number of mycobacterial cells present (Waterson, 2000). Furthermore, nucleic acid based detection cannot differentiate between live and dead cells (Soejima *et al.*, 2008; Zeng *et al.*, 2016) and can thus not give any indication whether an infection is currently in progress or already cleared. A further issue, especially regarding the developing world and potential field use, would be the high cost and the need for advanced laboratory equipment associated with many nucleic acid-based detection methods. This is especially true for testing carried out in the context of herd

screening and surveillance as hundreds to thousands of samples need to be analysed on a regular basis. Nevertheless, advances have been made in recent years that have not only reduced the cost of performing nucleic acid based detection but also increased the sensitivity of these methods. For example, Park *et al.*, (2016) were able to detect sub clinical faecal shedding of MAP in dairy cattle using a RT nested IS900 PCR obtaining results comparable to those obtained when using an ELISA based method on the same samples. In a further study by Park *et al.*, (2017) the group used RT PCR to detect host biomarkers active during mycobacterial infection and found that PCR based detection of biomarkers could be a promising approach for the diagnosis of subclinical JD infections.

1.2.4. The Tuberculin skin test

The Tuberculin skin test (TST) also known as Mantoux test was developed by Robert Koch in 1890 based on the observation that killed *M. tuberculosis* cells and their extracts would cause a localised immune reaction in animals that are currently undergoing a Tb infection. It has stayed the main tool for diagnosing tuberculosis in humans and animals for over 100 years. To perform the tuberculin skin test tuberculin purified protein derivative (PPD) is intradermally injected. A swelling of the skin at the injection site after 48 – 72 hours indicates a localized immune response to the antigen and a positive test result. The advantages of the tuberculin skin test are its low cost, high market availability, high sample throughput. Overall, the TST is well suited to screening large populations in a short amount of time and explain why the TST is still commonly used to diagnose human Tb over 130 years after its invention (Palmer *et al.*, 2006; Lee *et al.*, 2011). However, the tuberculin skin test also suffers from some severe drawbacks. First and foremost, its tendency to produce false positive results in individuals previously vaccinated against Tb using the attenuated *M. bovis* BCG vaccine thus rendering the TST only useful for diagnosing non vaccinated individuals. It also cannot differentiate between an infection of *M. tuberculosis*, *M. bovis*, *M. avium* ssp. or the presence of non-pathogenic environmental mycobacteria (Menzies, 1999; Wang, 2002;

Kang, 2005; Swift and Rees, 2013; Overton *et al.*, 2018) making additional confirmatory tests necessary.

The TST is also the standard test for diagnosing tuberculosis in animals and its low cost and high sample throughput make it well suited for mass screening of herds of farmed animals like cattle or sheep. The sensitivity and specificity of the TST in animals is comparable to that in humans with (Wood *et al.*, 1991) reporting a sensitivity of 64% and a specificity of 96% while Whipple *et al.* (1995) reported 88% sensitivity and around 96-98% specificity depending on the stage of the infection. More recently Singhla *et al.* (2019) in a study on cattle in Thailand found the sensitivity of the TST to range between 75 and 95% and the specificity to be 83% on average. However, the TSTs inability to differentiate between infections by *M. bovis*, *M. avium* ssp. or non-pathogenic environmental mycobacteria reduces the confidence in the test results obtained requiring additional confirmatory tests depending on severity and stage of the infection (Menzies, 1999; Schiller *et al.*, 2010; Middleton *et al.*, 2021).

Overall, the tuberculin skin test is a cheap, high throughput, time tested and robust detection assay for mycobacterial pathogens in animals and humans. However, its variable sensitivity coupled with its tendency to cross react with the BCG vaccine given as a standard immunisation against *M. tuberculosis* in humans and inability to differentiate between MTBC organisms, MAP and non-pathogenic environmental mycobacteria leave a lot of room for improvement.

1.2.5. ELISA based assays for the detection of MAP and *M. bovis*

ELISA (Enzyme Linked Immunosorbent Assay) for the detection of mycobacterial pathogens use specific antigen proteins bound to a surface to indirectly detect antibodies present in an animal sample, usually blood but also milk (Sekyia *et al.*, 2013). When the animal is currently undergoing active mycobacterial infection its blood and milk contain large amounts of antibodies to fight the infection. During the assay the blood serum or milk

sample is exposed to surface bound antigens which the free antibodies in the animal sample will bind to. These bound antigen antibody complexes can then be visualized by adding a further antibody bound to an enzyme or a fluorescent marker which binds to the specific antibody originally contained in the sample (Ahmed, 2010; Kennedy *et al.*, 2016). Although ELISA based assays for the detection of *M. bovis* have been developed and are commercially available they are not routinely used for diagnosing *M. bovis* in cattle (McCallen *et al.*, 2021). This is in part due to the significant variability in sensitivity depending on geographical region with Trost *et al.*, finding the IDEXX ELISA to be 77% sensitive in UK samples, 45% in US samples and only 9% in samples from Mexico (sample size $n \geq 120$ per region; Trost *et al.*, 2016). Unlike *M. bovis* ELISAs, MAP specific antibody ELISA assays are very popular for the diagnosis of Johne's. It offers a high degree of specificity of 85 to 100% (Clark *et al.*, 2008; Karuppusamy *et al.*, 2021) as well as being highly available on the market and having very low cost compared to culture based methods. While faecal culture of MAP can cost up to 35 GBP per sample a milk ELISA only costs about 3 GBP per sample (Nielsen, 2009) making it vastly more economic when mass testing herds of hundreds of animals. A comparison of the estimated costs of different diagnostic assays for the detection of MAP in veterinary samples is given in Table 1.1.

Table 1.1. Diagnostic tests available for the detection of Johne's disease.

Test	Sensitivity	Cost	Comments
Faecal smear	10-30%	£12.00	Rarely useful due to prohibitively low sensitivity, although short turnaround time may occasionally be useful.
Serology (ELISA)	20-90%	£6.90	Cheaper and more sensitive than faecal smear.
Faecal culture	30-65%	£40.00	Considered the 'gold standard' pre-mortem.
Faecal PCR	35-65%	£24.65	8-12 day turnaround time, cheaper than culture.

An overview of different diagnostic tools employed for the detection of MAP and their estimated cost and sensitivity. The cost is based on figures from the Veterinary Laboratories Agency (VLA), (Hudson, 2008).

1.3. Bacteriophage

With an estimated 10^{32} total phage particles bacteriophage are considered to be the most abundant life form in the biosphere (Wittbole *et al.*, 2014). The term bacteriophage derives from the Greek word “phage” which means “to eat”, therefore bacteriophage are “bacteria eaters” which is an appropriate name since bacteriophage (or phage) are viruses infecting bacteria. Scientists have been interested in phage for a long time as a tool to study bacterial evolution due to their role in horizontal gene transfer between bacteria and the study of phage biology has resulted in the development of a large number of molecular biology techniques. Their ability to infect and lyse bacterial cells has also led to their exploitation as therapeutic agents and this application has been the focus of much scientific research in recent decades (Clokier *et al.*, 2011). However, in the context of this thesis, one very important aspect of their study has been their ability to discriminate between different bacterial cell types to allow them to identify and infect only appropriate host cells which will support their replication, and this has led to their application in novel diagnostic methods (Rees and Loessner, 2021).

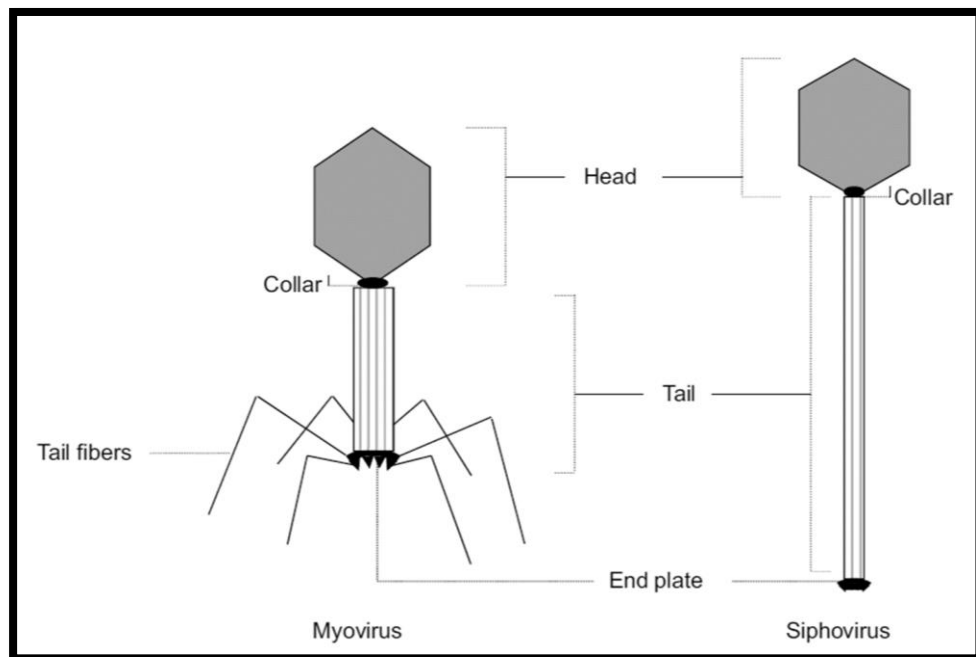
1.3.1. Phage biology and life cycle

Bacteriophage are typically small in size ranging from 30-200 nm in length, with a capsid shell made up of proteins protecting the genetic material inside. Bacteriophage are generally characterized and grouped in families based on three features, nucleic acid type (DNA or RNA), capsid structure and the presence or absence of a tail structure and characteristics of tail fibres. Phage morphology and presence or absence of tail and or tail fibres is not indicative of phage host range or life cycle. Most bacteriophage (96%) possess a tail and a wide variety of different tail and tail fibre arrangements have been reported (Brussow and Hendrix, 2002). The tail apparatus of these phage facilitate the infection process and are involved in both recognition events and phage adsorption on to the host bacterium and the delivery of the nucleic acid of the

phage stored in the capsid into the host cell which results in subversion of the host cell molecular machinery and reproduction of the virus (see Figure 1.5).

Most bacteriophage studied for therapeutic or diagnostic use belong either to the morphological groups *Myoviridae* or *Siphoviridae* which both possess double stranded DNA genomes packaged into icosahedral heads. Additionally, the *Myoviridae* are characterized by a contractile tail and a complex apparatus of tail fibres while the *Siphoviridae* possess a non-contractile tail and small less pronounced tail fibres.

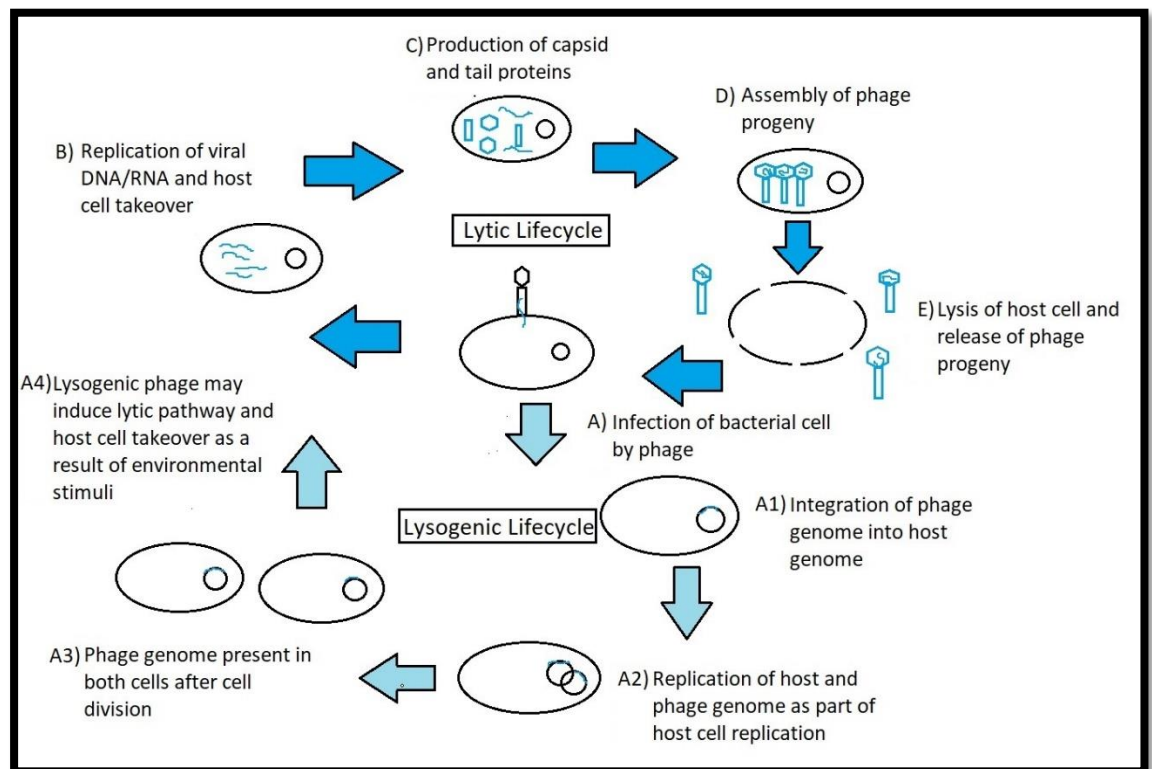
Figure 1.5. Schematic representation of a Myovirus and a Siphovirus



Schematic diagram of a Myovirus and a Siphovirus (adapted from Rees et al., 2014). Viral DNA is stored in the icosahedral head of the phage and travels through the tail to the end plate where it is injected into the host bacteria. Note the absence of elaborate tail fibres in Siphoviridae compared to Myoviridae.

In the first stage of the infection cycle, the phage adheres to the cell wall of the host bacterium and injects its nucleic acid into the cell cytoplasm. However, events that occur after this are more variable and phage can also be categorized based on their life cycle which is described as either lytic or lysogenic (see Fig. 1.6). The lytic life cycle is the simpler of the two and infection leads to reproduction of virus particles and eventual destruction (lysis) of the host cell. The first step of lytic phage infection is the subversion of the cell's biosynthetic pathways and replication of the phage genome. This is followed by production of phage structural proteins, such as those required for assembly of the capsid and tail structures. The newly replicated phage genomes are then packaged into the capsid, the tail is added, and the mature phage is assembled. Once assembly of progeny phage is completed, the last stage of the phage's life cycle is initiated, which is the production of enzymes such as lysins and holins which result in weakening of the cell wall structure, resulting in cell lysis and release of the new phage into the environment (see Ugorcakova and Bukovska, 2003). The initiation of holin and lysin production is strictly controlled and is timed to allow as many progeny phage to be produced and assembled as possible before the host cell is killed. The most common lysis mechanism is based on holins providing a specific transport mechanism for the lysin proteins, allowing them to cross the cell membrane and degrade the peptidoglycan of the host cell, destroying its structural integrity (Echols, 1986; Shi *et al.*, 2012), although variations of this basic model have also been described (see Young, 2014). The number of progeny phage particles released at the end of the infection cycle is variable (this is termed the burst size) but can be up to 100 new phage particles per infection event in DNA viruses and up to 20 000 in RNA phages (Herskowitz and Hagen, 1980; Kannoly *et al.*, 2023). It is worth noting that once an obligate lytic phage has irreversibly bound to a host cell, it is committed to the lytic pathway of replication and cannot alter, delay, or stop the process (Rakhuba *et al.*, 2010).

Figure 1.6. Schematic diagram illustrating the lysogenic and lytic pathways of phage replication.



Schematic diagram illustrating the lysogenic and lytic pathways of phage replication, with phage-related elements drawn in blue; lytic cycle phage are shown in dark blue; lysogenic phage are shown in light blue. Both life cycles start by phage infecting a host cell and injecting its genome (A) which is followed by replication of phage genetic material (B), production of phage capsid and tail proteins (C), assembly of new phage (D) and ultimately lysis of the host cell and release of progeny phage (E). When utilizing the lysogenic lifecycle, the phage does not immediately take over the host cell but integrates its genome into the host cells genome or by forming a plasmid (A1). The phage genome is subsequently replicated every time the host cell replicates (A2 and A3). The integrated phage genome may be stable for thousands of generations and the phage has the option to induce the lytic pathway (A4) and cause host cell takeover, assembly and lysis of the host cell based on environmental stimuli (adapted from Botsaris et al., 2013)

The second, and arguably more interesting, pathway is that of lysogenic or temperate phage. These phage can either choose to undergo the lytic pathway, as described above, or they can enter a dormant phase of existence. Often genetic material is integrated into the host bacterium's genome by recombination or, alternatively, by converting into a plasmid structure that is maintained stably inside the bacterium. This way the phage genome is replicated every time the bacterial cell divides and the phage does not have to re-infect a new host cell to replicate. This coexistence can be stable for thousands of generations and the phage, in addition to providing immunity from infection by similar bacteriophages, protects the host cell from further lytic infection by phage. The lysogenic phage may also carry genes that are expressed during the dormant phase which provide specific advantages for the host by altering its phenotypic characteristics, such as providing new virulence factors. This phenomenon is called lysogenic conversion (see Nanda *et al.*, 2015) and a well-known example of this is the production of phage-encoded virulence factors in the waterborne pathogen *Vibrio cholerae* which is responsible for causing cholera in humans (Reidl and Mekalanos, 1995) or the phage-encoded shiga-like toxin produced by some lysogenic strains of *Escherichia coli* (Beuthin *et al.*, 2013). This arrangement is beneficial to both the phage and the host cell because the host cell gains a competitive advantage over other bacteria of the same species which should result in a higher rate of reproduction, while the phage takes advantage of being reproduced itself at every cell division without the risks associated with having to find a new host cell to infect. Lysogenic phage also have the ability to revert to the lytic pathway in a process called pro-phage induction and initiate active phage replication resulting in cell lysis. This pro-phage induction is commonly induced when the host cell experiences environmental stresses, such as UV radiation or exposure to toxic chemicals, and is believed to be an evolutionary mechanism that allows the phage to escape the host cell before it dies, although there is also evidence that spontaneous induction of pro-phage also has a positive impact on the overall fitness of bacterial populations (Nanda *et al.*, 2015). It was recently shown that the transition from lysogenic

to lytic pathway can also be dependent on the abundance of phage particles in the environment due to a mechanism similar to quorum sensing where phage can sense small peptide chains released by other phage (Erez *et al.*, 2017). Erez *et al.* suggested that this mechanism allows the phage to decide if it wants to pursue a lysogenic or a lytic pathway dependent on the ratio of phage to host bacteria present in the environment. I.e. if a small number of phage encounter a large number of host bacteria it would be more advantageous to use the lytic pathway while if a large number of phage only have a small number of host cells available it would make more sense to use the lysogenic pathway in order to increase the chance of reproductive infection for the progeny phage.

1.3.2. Phage infection of host cells

The first step in phage-host cell infection is finding a suitable host cell to infect. Phage are non-motile and thus have no means of independent propulsion which renders them virtually completely dependent on diffusion and Brownian motion, i.e. the random movement of a particles due to collisions with other particles, to randomly encounter a suitable host cell (Kasman *et al.*, 2002; Simmons *et al.*, 2018). Therefore, the principal factor determining if a phage will find a host cell is the relative abundance of suitable host cells in its environment (Simmons *et al.*, 2018; Stone *et al.*, 2019).

Interestingly phage T4 has been observed to take advantage of sub-diffusion mechanics in mucosal environments by binding to strands of mucin molecules via their capsid proteins thus delaying diffusion of the phage particle out of the mucosal surface where its host cells reside. This allows the phage to maximise the time spent in the mucosal surface thus increasing the chance for random phage host-cell encounters (Barr *et al.*, 2015).

Once a phage particle has found a suitable host cell initial binding is carried out by Receptor Binding Proteins (RBP) situated at the ends of the tail fibres or alternatively on the base plate of the phage (see figure 1.5) to suitable receptors on the surface of the bacterial cell wall. Examples of such receptors include, but are not limited to, pili, flagellae, lipopolysaccharides (LPS) and cell surface proteins (Silva *et al.*, 2016; Stone *et al.*, 2019). Whether successful phage binding occurs is dependent on a variety of factors and these are commonly highly specific to the phage and the host cell in question, such as type, number, distribution and function of the bacterial receptors used for binding as well as the number of binding sites the phage can utilise for adsorption (Kasman *et al.*, 2002; Stone *et al.*, 2019). Additionally, adsorption of phage particles onto host cells is influenced by the growth state of the host cell if the expression of the receptor is regulated (e.g. the phage lambda LamB receptor; Howes, 1965), and can also be affected by physical factors that affect all protein binding events such as pH and salt concentrations in its environment. Overall, adsorption and binding of phage onto host cells is a

highly specific and complex procedure which is not yet fully understood (Jończyk *et al.*, 2010; Swift *et al.*, 2014; Simmons *et al.*, 2019). The initial adsorption of the phage onto the host cell mediated by RBPs is reversible and the infection process can be aborted at this stage. Whether this happens depends on a variety of factors relating to the host cell and environmental conditions, including the metabolic state of the cells (Baptista *et al.*, 2008; Thanki *et al.*, 2018).

If the infection process is not aborted at this point, the phage initiates irreversible binding which is associated with a conformational rearrangement of the phage structure, usually involving relocation of the baseplate close to the host cells membrane and injection of phage DNA into the host cell to initiate phage replication and host cell takeover. In the *Myoviridae*, delivery of the DNA into the host cells cytoplasm is also facilitated by the contraction of the phage tail structure (Thanki *et al.*, 2018; Stone *et al.*, 2019).

1.3.3. Summary of Key points

Bacteriophage are viruses that infect bacteria. They commonly possess a head which contains its genetic material and a tail which is used for injecting its genetic material into a suitable host cell. Phage are highly specific in their host range and will reliably infect their host cells but ignore bacterial species not suitable for productive infection. They use their tails and tail fibre apparatus to first reversibly attach to the surface of a suitable host cell. If the host cell and the phage are compatible, which is determined by a multitude of factors such as location, distribution and number of cell surface receptors but also the growth state of the host cell, irreversible phage binding occurs, and the phage injects its genetic material into the host cell through its tail. After injection most phage (including all phage species used in this thesis) undergo the lytic pathway and take over the host cells gene expression and reproductive machinery to copy the phage genome and build and assemble new phage particles that are eventually released resulting in the death of the former host cell. Some phage have the option of delaying the lytic pathway of phage replication by integrating their genome into the host cells genome

where it lays dormant, but is reproduced during host cell division, until the phage leaves this lysogenic pathway and initiates the lytic pathway as a result of environmental stimuli.

1.4. Application of Bacteriophage for Phage-based diagnostics

1.4.1. Introduction

Like all viruses, bacteriophage have a specific host range and many phage are typically characterized as having a narrow host range. This has allowed them to be exploited to develop methods for the identification of specific types of pathogenic bacteria. Phage typing is one of the oldest phage-based assays and has been used for many decades to differentiate bacterial strains of the same species (see Rees and Dodd, 2006). This method is based on a bacterial lawn of the target organism being challenged with different bacteriophage. If the bacterial strain is sensitive to the phage, the phage will lyse the cells and cause a characteristic zone of clearing called a plaque. A pattern based on the susceptibility of the target bacteria to the different phage allows the specific characterisation of a bacterial strain which reveals differences between different closely related isolates. Phage typing procedures exist for most clinically relevant bacteria including *Salmonella*, *Yersinia*, *M. tuberculosis*, *Staphylococcus*, *Escherichia coli*, *Campylobacter* and others (Ahmed *et al.*, 1987; Schofield *et al.*, 2012). Nevertheless, phage typing is not commonly used for the detection and diagnosis of clinical pathogens due to it requiring the maintenance of large libraries of phage isolates which limits its use to large scale reference laboratories. Additionally, the advent of nucleic acid-based detection and characterization methods for microorganisms in recent years and the always increasing availability of sequencing technology have made phage typing more or less redundant. The current gold standard for the diagnosis of bacterial pathogens is culture but there is a growing demand for specific detection and differentiation of pathogenic bacteria without the need for primary culture (Schofield *et al.*, 2012).

However, for use in diagnostic tests, phage with a wider host range are required, and several phage have been isolated that are specific for a single species, or even a whole genus of bacteria (Rees and Loessner, 2021). For example, one of the bacteriophage used for this study, phage D29, is able to infect many species of the genus *Mycobacterium*.

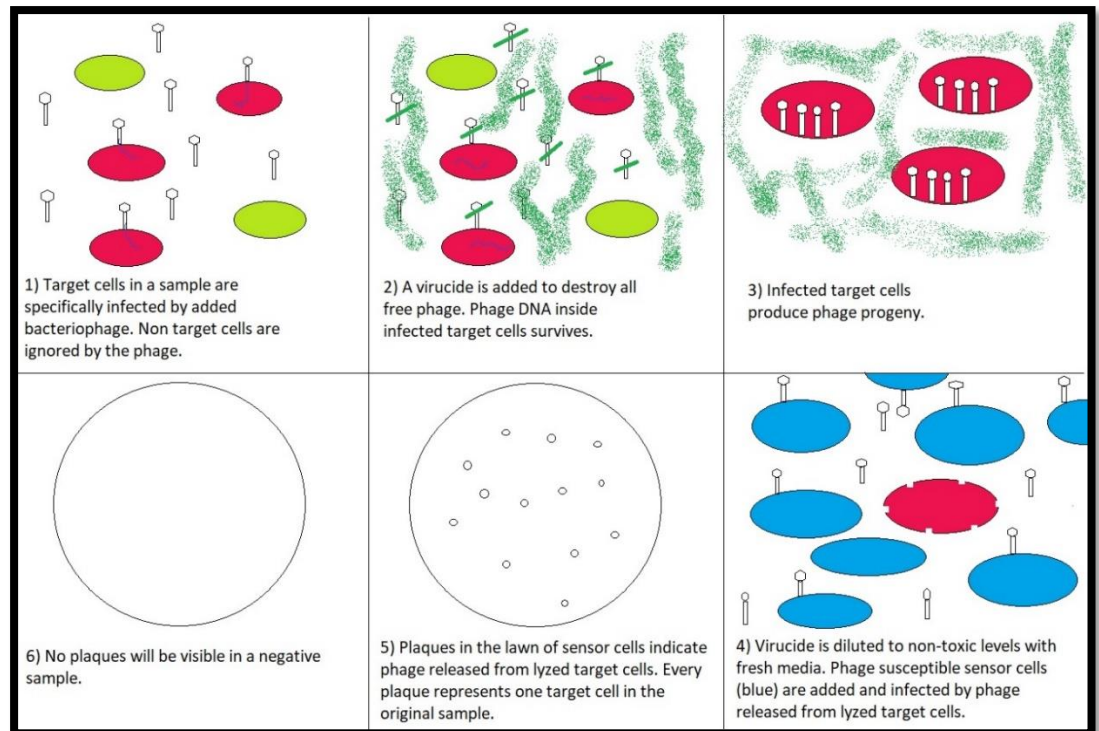
One well developed method for detecting pathogenic bacteria, including mycobacteria, using such broad host range phage is the phage amplification assay. It is again based on bacteriophage specifically infecting their host cell but the newly formed phage progeny themselves produce the visible confirmation of infection as phage numbers are amplified following infection of a susceptible host strain. Phage amplification generally involves four steps:

- 1) Specific infection of target cells by the bacteriophage.
- 2) Inactivation of any remaining exogenous phage particles using a virucide.
- 3) Neutralization of said virucide so that progeny phage are not inactivated.
- 4) Addition of host cells for the newly formed phage particles to infect and produce a visible signal (plaques).

The addition of virucide to inactivate any phage that have not infected a host cell is necessary for guaranteeing that any plaques formed after the incubation period are the result of reproduction of phage within the target cells in the sample. The virusol agent used in for this work was Ferrous ammonium sulphate (FAS) ($\text{FeH}_8\text{N}_2\text{O}_8\text{S}_2$) but other virusol agents have been reported including tea and other plant extracts (de Siqueira *et al.*, 2006). Each plaque formed is evidence of a single infection and replication event. It is therefore possible to determine the number of target cells in the original sample based on the number of plaques visible after the assay (de Siqueira *et al.*, 2006). A schematic illustration of a phage amplification assay is given in figure 1.7.

One example of a phage amplification assay for detecting mycobacteria in clinical samples is the two day phage amplification assay which is the basis for much of the research done in this project and is therefore discussed in more detail.

Figure 1.7. Schematic diagram of a phage amplification assay



1) Target cells in a sample are infected by a suitable phage. 2) Virucide is added to destroy phage particles not infecting mycobacterial cells and only those harboured within target cells survive. 3) Phage replication happens in the infected target cell. 4) The Virucide is subsequently neutralized with fresh media. Sensor cells and agar are added and plated. Phage released from lysed target cells infect sensor cells. 5) After incubation sensor cells infected with phage originally harboured inside target cells are lysed and from visible plaques. Every plaque represents one mycobacterial cell infected by phage D29 during the assay. 6) No plaques will be visible in a negative sample. Adapted from Botsaris et al., 2013

1.4.2. The two day phage amplification assay for the detection of *M. tuberculosis*

Developed by PBD Biotech LTD, the two day phage assay for the detection of mycobacterial pathogens is a phage amplification assay that can detect viable mycobacterial cells in a variety of samples such as sputum, blood or milk and produces results in 48 hours. The overall limit of detection (LOD) of the PBD Biotech one day assay was stated, to be ≥ 20 cells in a sample and therefore was more sensitive and considerably faster than culture-based detection methods (Swift *et al.*, 2013; Grant, 2021). The basis of the assay is mycobacteriophage D29 which has a relatively broad host range infecting a wide range of both fast and slow growing members of the genus mycobacterium (Ford *et al.*, 1998; Rybniker *et al.*, 2006). This makes D29 especially useful for the detection of slow growing mycobacteria since it allows the use of the non-pathogenic and fast-growing mycobacterium *M. smegmatis* as the sensor cells in the last stage of the amplification assay. In addition, since *M. smegmatis* is non-pathogenic it also only requires an ACDP 1 laboratory environment for the testing of samples (Advisory Committee on Dangerous Pathogens, HSE, 2023). An interesting aspect of phage D29 biology is that it is closely related to mycobacteriophage L5 which is a temperate phage able to use both the lysogenic and the lytic pathway of replication. D29 however has lost the ability to replicate lysogenically during its evolution and is therefore an obligate lytic phage (Ford *et al.*, 1998). As with other diagnostic assays pre-treatment and concentration steps may be required, depending on the origin of the sample, to ensure the highest possible concentration of potential target cells and the absence of inhibitors to phage adsorption, infection, and replication. The phage are then allowed to infect the target cells, usually *M. tuberculosis* or *M. bovis*, during a 50-60 minute incubation period at 37 °C. All free phage particles not currently harboured inside a target cells are then destroyed using the virusol Ferrous ammonium sulphate (FAS). The advantages of this method are first of all the specificity of the phage that ensure that only mycobacteria will produce a positive result and

the ability to produce a positive or negative result after only 48 hours is a huge advantage over culture-based methods. However, one of the greatest advantages of the two day phage assay is the extremely low cost, the low requirement in regard to laboratory equipment and the low skill level required to perform the assay. This is especially relevant in the context of developing countries where money, well equipped laboratories with nucleic acid amplification technology and skilled personnel are in short supply and where tuberculosis and bovine tuberculosis are still major public health concerns (Foster *et al.*, 2015). The two day phage assay has been used successfully for the detection of pulmonary tuberculosis in sputum samples with a specificity of around 98% and a sensitivity of 87% (Muzaffar *et al.*, 2002; Butt *et al.*, 2004). The major weakness of this assay is that since mycobacteriophage D29 has a very broad host range for a bacteriophage and has the ability to infect many species of the genus mycobacterium it cannot differentiate between pathogenic mycobacteria (such as *M. tuberculosis* or *M. bovis*) and non-pathogenic environmental mycobacteria frequently present in stool, soil, etc. This means that only samples with a low natural background flora of mycobacteria can be effectively used in this form of the assay (Botsaris *et al.*, 2013).

Multi drug resistant *M. tuberculosis* strains have become an ever-increasing problem for global public health especially so in developing countries. In a survey of 35 countries up to 22.1 % of *M. tuberculosis* isolates were found to be resistant against the two main antimycobacterial antibiotics rifampicin and isoniazid. A variation of the two day phage assay, the Fast Plaque TB-RIF assay, can be used for the detection of antibiotic resistant *M. tuberculosis* since only cells that are resistant to the antibiotic can be productively infected by the phage and release phage progeny. Therefore, any plaques produced by the assay are indicative of rifampicin resistant *M. tuberculosis* which allows rapid detection (< 48 hours) of antibiotic resistances and facilitates better health care decisions and more effective treatment of the patient (Kisa *et al.*, 2003; Butt *et al.*, 2004).

1.4.3. The two day phage amplification assay for the detection of MAP

As discussed in section 1.1.4, *M. avium subsp. paratuberculosis* is an economically relevant pathogen causing Johne's disease, an inflammatory bowel disease in ruminant animals like cattle, sheep and deer causing chronic wasting and death (Sweeney *et al.*, 2012). It has also been linked to Crohn's disease which is an inflammatory bowel disease in humans (McNees *et al.*, 2015). With this in mind it is easy to see why rapid detection of this pathogen is desirable in a farm setting. Due to its broad host range phage D29 is able to infect MAP cells and the two day assay can in theory be used directly for the detection of MAP in veterinary samples. However, a veterinary sample taken on a farm is rarely as clean as one obtained from patients in a clinical setting and therefore there is a high risk of contamination of the sample (like milk or faeces) by non-tuberculous environmental mycobacteria (NTMs; Covert *et al.*, 1999) and (Falkinham, 2009). As mentioned before, the two day assay in its basic form cannot differentiate between MAP cells and cells of NTMs that may have contaminated the sample due the presence of NTMs in or on the animal or in its environment (Botsaris *et al.*, 2013). It is therefore required to combine the two day assay with a further method to provide increased specificity. One method of doing this is the use of Immuno-magnetic separation (IMS) which is based on MAP cells in a suspended sample being specifically bound by polyclonal antibodies linked to small paramagnetic beads (Foddai *et al.*, 2010). When exposed to a magnetic field the particles will be attracted by the field and migrate towards its origin pulling the MAP cells attached to the particles with them. The remainder of the sample can then be discarded and only the immunologically bound MAP cells should remain and can be used in the two day phage assay (Foddai and Grant, 2011). This is beneficial since it allows the specific retention of the MAP target cells while other non-target mycobacteria are discarded together with any possible inhibitors to the phage or the infection process which may be present in faecal or milk samples (Foddai and Grant, 2011). The other possibility to increase specificity of the essay is to add a PCR identification step after

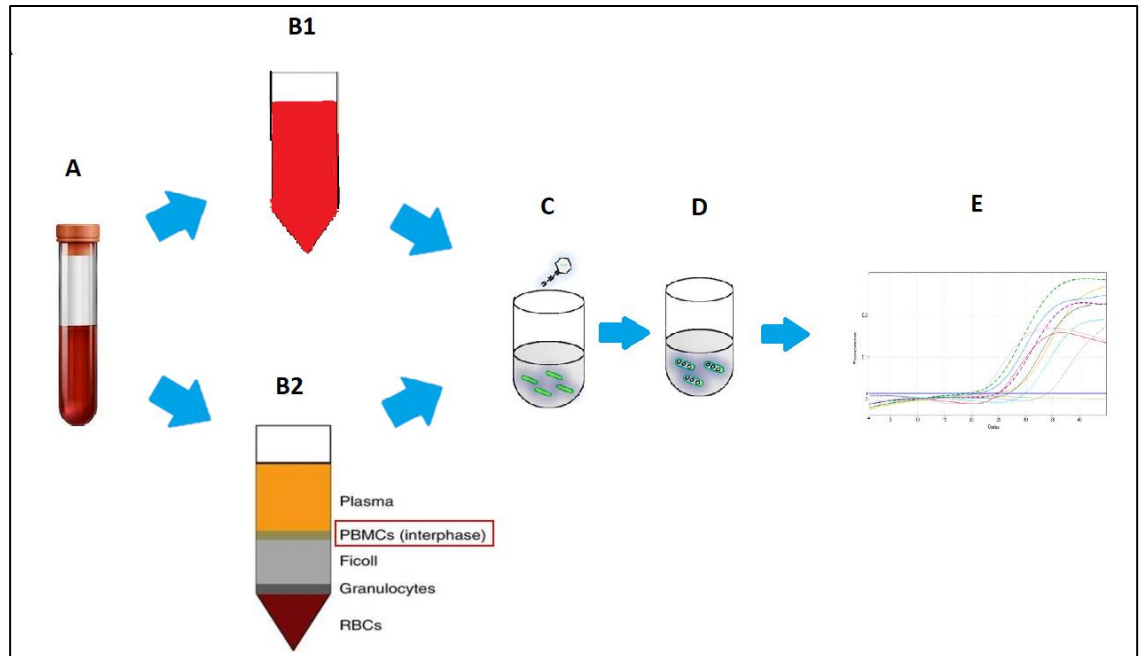
performing the assay. This was described by *Stanley et al.* (2007) who described a PCR based on a plaque picked from the bacterial lawn. Since a plaque originated from a single lysed mycobacterial cell, that cells nucleic acid content should still be present in the middle of the plaque. This DNA in the centre of the plaque can be extracted, purified, and subjected to PCR. Based on the PCR results a conclusion can be drawn whether the organism that originated the plaque was a MAP cell or a non-related environmental mycobacterium (*Stanley et al.*, 2007; *Botsaris et al.*, 2016). This allows rapid (< 48 hours) and specific detection and enumeration of *M. avium subsp. paratuberculosis* in a variety of veterinary and clinical samples including milk and blood (*Botsaris et al.*, 2013; *Swift and Rees*, 2013; *Foddai et al.*, 2017). It was also found that the two day phage amplification assay combined with a PCR step was able to detect viable MAP cells in cattle blood with a higher sensitivity than direct PCR and was also able to detect low numbers of cells in the early stage of the infection (*Swift and Rees*, 2013). This could make the two day phage assay a valuable tool for the detection and control of MAP shedders in cattle herds with the aim of reducing the horizontal spread of MAP throughout the herd. In light of MAP being a suspected cause for Crohn's disease it can also be a useful tool for preventing the dissemination of MAP through the food chain by allowing rapid and specific testing of milk, cheese and other dairy products (*Botsaris et al.*, 2010).

A variation of the phage amplification assay, the phage enumeration assay, was developed to allow a more accurate estimate of the number of cells present in the sample (see 2.6.2). Briefly, the phage amplification assay is performed as normal (see 2.6) however, after the addition of virusol and the 5 minutes of agitated incubation 100 µl of sample is removed and added to 900 µl of Media Plus (MP, see 2.1.1.) thus diluting the sample 1:10. Additional 1:10 dilution steps can be performed as required. The resulting sample dilutions can then be mixed with sensor cells, MP and warm agar to form bacterial lawn plates like in the classic phage amplification assay.

1.4.4. The Actiphage® Blood assay for the detection of Mycobacteria

The Actiphage® blood assay for the detection of mycobacteria was developed to allow faster more specific and more sensitive detection of pathogenic mycobacteria in clinical and veterinary samples. It uses mycobacteriophage D29 as a lysis agent to specifically infect and destroy mycobacterial cells thus releasing the host cells nucleic acids. The DNA is subsequently concentrated and purified before being amplified using PCR (Swift *et al.*, 2020). By forgoing the need to grow a bacterial lawn and the plaque extraction steps the Actiphage assay is able to produce a definitive PCR result after only 6 hours. In a study carried out by Swift *et al.* (2020) the limit of detection (LOD) of the Actiphage assay was found to be ≥ 10 cells ml⁻¹ while the one day phage amplification assay can only reliably detect 20 or more cells in a sample. In a group of 41 cows suspected of *M. bovis* infection the sensitivity and specificity of the Actiphage blood assay for the detection of mycobacteria was found to be 95% and 100% respectively (Swift *et al.*, 2020). In a 2021 review by Grant on phage based diagnostic tools for the detection of MAP it was noted that phage-based diagnostics, including the Actiphage assay, would repeatedly yield higher numbers of MAP positive samples when compared with culture-based diagnostics (Grant, 2021). The Actiphage blood assay for the detection of mycobacteria therefore provides a fast, sensitive, highly specific detection assay for MAP and other mycobacteria. Due to its high sample throughput, comparably low cost (Swift *et al.*, 2020) and good scalability the Actiphage Rapid blood assay has the potential to be a major factor in the global efforts to monitor and control JD and *M. bovis* infection in livestock (Jones *et al.*, 2020; Grant, 2021). A schematic diagram of the Actiphage blood assay is given in figure 1.8.

Figure 1.8. Schematic diagram of the Actiphage blood assay



Blood in a vacutainer tube (A) underwent a method for the isolation of PBMCs potentially harbouring MAP cells: either the ACK lysis buffer – (B1; see section 2.7.2) or Ficoll buoyancy gradient (B2; see 2.7.1) depending on experimental design. Putative MAP cells were subsequently lysed using phage D29 (C and D). DNA from lysed MAP cells were then purified, amplified and analysed using qPCR (E).

1.5. Aims of this thesis

Phage based diagnostics has immense potential in the global effort to control mycobacterial pathogens such as *M. tuberculosis*, *M. bovis* or MAP which cause great suffering as well as economic loss worldwide, especially in developing nations. However, in order to fully use the potential of phage based diagnostic kits they have to be deployable in regions with potentially very warm and humid climates as well as poor infrastructure. Therefore, the requirement for constant refrigeration limits the usefulness of the current generation of phage based diagnostic kits that deliver phage in liquid culture. Thus, the primary aim of this work was to develop a dry and room temperature shelf stable preparation of mycobacteriophage D29. This could then be used as the detection agent in the PBD Biotech® two day phage amplification assay as well as the Actiphage® assay for the detection of pathogenic mycobacteria. This novel phage preparation required subsequent validation and optimization to ensure that the dried phage preparation could be used as a replacement for liquid phage culture in both the two day phage amplification assay as well as the Actiphage assay without loss in sensitivity or specificity. The second aim of this thesis was to produce a room temperature and shelf stable form of *M. bovis* and MAP culture that could be used as a positive control in the assays. These had to then be optimized in order to give a reliable and quantifiable positive control for the phage amplification assay and the Actiphage assay.

Chapter 2

Materials and Methods

2.1. Growth Media

2.1.1. Middlebrook 7H9 medium and Media Plus

BD Difco™ Dehydrated Culture Media: Middlebrook 7H9 broth was used to grow all liquid cultures of Mycobacterial species used in this study, including *M. smegmatis*, *M. avium* ssp. *paratuberculosis* (MAP) and *M. bovis* (BCG). Media was prepared according to manufacturer's instructions and autoclaved at 121 °C for 15 minutes. After cooling, Oleic Albumin Dextrose Catalase (OADC) supplement (Abtek, UK) was added aseptically up to a concentration of 10% (v/v). The Abtek OADC supplement contains 2 mmol l⁻¹ CaCl₂. 7H9 media supplemented with OADC was referred to as Media Plus (MP) and these terms may be used interchangeably.

Due to the notorious slow growth and fastidiousness of MAP, culture media intended for growing MAP was additionally supplemented with 4 µg ml⁻¹ (final concentration) Mycobactin J (Synbiotic Corporation, France).

2.1.2. Middlebrook 7H10 agar

BD Difco™ Dehydrated Culture Media: Middlebrook 7H10 Agar was used for growth on solid media of all mycobacterial species used in this study. Media was prepared according to the manufacturer's instructions followed by autoclaving for 15 minutes at 121 °C.

2.1.3. Nutrient agar and broth

Nutrient agar and broth were sourced from Oxoid (UK) and prepared according to manufacturer's instructions followed by autoclaving at 121 °C for 15 minutes.

Half strength nutrient agar and broth for the cultivation of *L. monocytogenes* was produced by using 50 % of the amount of media base advised by the manufacturer per litre of water.

2.1.4. BHI agar and broth

BHI agar and broth were sourced from Oxoid (UK) and prepared according to manufacturer's instruction followed by autoclaving at 121 °C for 15 minutes.

2.2. Solutions and buffers

2.2.1. Lambda buffer

Lambda buffer was produced by dissolving (per 100 ml buffer) 0.6 ml 1M Tris HCl, 0.2 g of MgSO_4 and 0.5 ml of a 1 % gelatine solution (Sigma Aldrich, USA) in 98.7 ml water. This was then sterilized by autoclaving at 121 °C for 15 minutes.

2.2.2. Calcium chloride

Calcium chloride solution was prepared by dissolving 0.022 g of Calcium chloride in 100 ml of RO water to obtain a final concentration of 2 mM and sterilised by autoclaving at 121 °C for 15 minutes.

2.2.3. Gelatine

20 % gelatine stock solution was prepared by adding 10 g of gelatine powder (bovine skin) to 40 ml of water followed by sterilization at 121 °C for 15 minutes. Autoclaving also helped to dissolve the gelatine.

2.2.4. ACK buffer

ACK lysing buffer was prepared by dissolving (in 1 l of RO water) 8.02 g Ammonium chloride, 1.0 g Potassium bicarbonate and 0.034 g EDTA di sodium salt. ACK lysis buffer was subsequently filter sterilized using a 0.45 μm Milisart® filter sterilization unit (Sartorius, Germany).

2.2.5. Virusol

Virusol was prepared by aseptically dissolving one virusol tablet (Ferrous ammonium sulphate; PBD Biotech, UK) in 4.5 ml of sterile RO water (10 mM final concentration).

2.2.6. Mycobactin J

2 mg of dried Mycobactin J (Synbiotic Corporation, France) was reconstituted in 1 ml 95% ethanol to yield a final concentration of 2 mg ml⁻¹.

2.3. Standard procedures

2.3.1. Phage plaque assay

Single isolated plaques for picking/harvesting were produced by combining 0.1 ml of diluted phage lysate with a titre of $\sim 2 \times 10^3$ pfu ml⁻¹ (equivalent to ~ 200 plaques) with 6 ml of Middlebrook 7H9 media (Difco) supplemented with 10% (v/v) OADC (Abtek, UK) and 0.5 ml of an overnight *Mycobacterium smegmatis* culture ($>1 \times 10^8$ cfu ml⁻¹ section 2.5.1) to serve as the host for phage replication. The mixture was solidified by adding 5 ml of molten Middlebrook 7H10 agar (Difco) before pouring into standard petri dishes. The petri dishes were subsequently incubated at 37 °C for 24 hours. The final agar concentration in the lawn was approximately 0.8 %.

2.3.2. Picking of plaques

In the early stages of this project, plaques were picked by mechanically cutting of the agar around the plaque with a spatula and then removing the plaque. This method was found to not only be laborious and time consuming but also introduced a large degree of variability as excised plaques were not uniform in size. To reduce variability of manual excision of plaques this was subsequently performed using a biopsy punch needle with a diameter of 3mm (Kai Medical, Japan) resulting in uniform plaques. After picking, 5 plaques were transferred into 1.5 ml Eppendorf tubes and stored at 4 °C.

2.3.3. Eluting phage from agar plaques

Fresh, frozen or refrigerated plaques (see section 2.3.2) were immersed in 100 µl of lambda buffer and allowed to stand for approximately 5 minutes to allow the phage particles to elute from the plaque into the buffer.

2.3.4. Production of *Mycobacterium smegmatis* lawn plates for determining phage titre

Lawn plates were produced by mixing 120 ml of 7H9 broth containing OADC supplement (Abtek, UK), 15 ml of *M. smegmatis* culture with a titre $> 1 \times 10^8$ cfu ml⁻¹ (section 2.5.1.) and 100 ml of molten 7H10 agar. The resulting mixture was poured into 20 standard petri dishes and allowed to solidify. The plates were then stored at 4 °C until needed. It is important to note that the agar should only be warm to touch (30-40 °C) and not hot when added to the mixture since the agar being too hot may damage the bacteria and hamper lawn development.

2.3.5. Production of non-mycobacterial bacterial lawn plates (*E. coli*, *S. enterica* and *L. monocytogenes*)

Production of bacterial lawn plates for the propagation of bacteriophage PRD1, Felix, BP and B1 is essentially the same procedure for each species and thus amalgamated into one section. Per one lawn plate 6 ml of liquid media (Depending on host bacteria used, see table 2.2.) was combined with 1 ml of bacterial culture with a titre $> 1 \times 10^8$ cfu ml⁻¹ (section 2.5.3. - 2.5.5.) and solidified using 6 ml of molten, warm not hot, agar (see table 2.1). Lawn plates were stored at 4 °C until needed. Note, *L. monocytogenes* is able to actively grow at refrigeration conditions (Omac, Moreira and Castell-Perez, 2018) and storage of *L. monocytogenes* lawn plates at 4 °C beyond a few days is not recommended.

2.3.6. Production of Semi Confluent Lysis Plates (SCLPs)

Semi confluent lysis plates (SCLP) were prepared by adding (per SCLP) ~600-700 phage D29 particles (600-700 μl of 1×10^3 pfu ml^{-1} phage suspension) to 6 ml of MP (*aka.* Middlebrook 7H9 broth containing OADC supplement (Abtek, UK)), 0.75 ml of *M. smegmatis* culture ($>1 \times 10^8$ cfu ml^{-1} section 2.5.1) and 5 ml of molten Middlebrook 7H10 agar. The resulting mixture is poured into standard petri dishes and allowed to solidify. Plates were then incubated at 37 °C for 12-24 h.

2.3.7. Gram stain

Gram staining was performed to determine the presence/absence of peptidoglycan for the identification of the bacteria. A colony or 10-20 μl of bacterial culture was heat fixed onto a microscope slide and stained using crystal violet for 1 minute. Iodine solution would then be added for 30 seconds followed by an ethanol wash decolourizing the stain. Safranin would then be added for 1 minute to serve as a counterstain. Gram positive bacteria would appear purple/blue while gram negative bacteria would appear red when viewed through a microscope.

2.3.8. Ziehl-Neelsen Stain

Ziehl-Neelsen staining technique is used for the identification of Mycobacteria which are notoriously resistant to staining by gram staining, due to their thick lipid rich cell wall. Bacterial culture or a colony is suspended in Carbol fuchsin and heat fixed to a microscope slide. Bacteria are then decolourized using acid alcohol (2.0 ml concentrated hydrochloric acid and 98.0 ml 95% ethyl alcohol). A 1% methylene blue solution is then applied for 30 seconds. Excess methylene blue is washed away using water and stained slide can be examined using oil immersion microscopy (1000 x magnification). Acid fast bacteria will appear red while non-acid fast bacteria will appear blue (See figure 1.1).

2.3.9. Production of artificial plaques

“Artificial plaques” were produced by adding 5 µl of high titre bacteriophage D29 or bacteriophage TM4 suspension to 5 µl of warm, liquid agar (either, depending on experimental design, Nutrient agar (Oxoid, UK) or Middlebrook 7H10 agar (Difco). This was allowed to solidify. Gelatine artificial plaques were produced in the same way but using 5 µl of warm, liquid 20 % (w/v) gelatine solution to replace the agar.

2.3.10. Freeze-drying

Freeze-drying of bacteriophage and mycobacteria was performed by aliquoting between 0.1 and 1 ml of sample (depending on experimental design) into freeze-drying vials with a fitting rubber stopper loosely covering the vials while allowing air exchange to persist. Freeze-drying vials were subsequently transferred to a Christ Alpha 1-2 LDplus freeze-drying unit (Christ, Germany). Samples were freeze-dried for up to 12 hours or until the temperature and pressure in the drying chamber was registered to be below -60 °C and below 0.02 mBar respectively. During freeze-drying the temperature is reduced from room temperature to < -10 °C within 10 – 20 minutes and is held at a temperature of < -60 °C for the majority of the freeze-drying process. When the freeze-drying process was deemed complete, the vials were sealed before being removed from the freeze-drying unit. The rubber stoppers were additionally secured by screw caps and freeze-dried samples were stored in a dark place at room temperature unless otherwise specified.

2.3.11. Enumerating phage using the Miles and Misra technique

The number of viable phage in a sample was determined using a method adapted from Miles and Misra (1938). Phage suspension was serially diluted 1:10 in sterile MP (for mycobacteriophage) or lambda buffer for non-mycobacteriophage. 5 replicates (10 µl each) of each dilution were spotted onto a *M. smegmatis* lawn plate (section 2.3.4). Spots of liquid on bacterial lawn were allowed to dry fully before the plate was incubated at 37 °C overnight.

(~12 hours). Spots with a number of plaques between 3 and 30 were considered to be in a countable range. The average of 5 spots was used to calculate the phage concentration in the original sample using the formula:

$$\text{pfu ml}^{-1} = \frac{\text{average number of plaques per spot} \times \text{dilution factor}}{\text{volume plated in ml}}$$

2.3.12. Extracting genomic DNA from MAP/BCG using boiled lysate method

A 1.5 ml plastic microfuge tube was filled with 1 ml of bacterial culture with a titre $> 1 \times 10^6$ cfu ml⁻¹ and heated in a heating block at 100 °C for 20 minutes. The resulting boiled lysate was subsequently centrifuged at 13000 x g for one minute to remove cell debris. The remaining supernatant was removed and either used directly or stored frozen at -20 °C until needed.

2.3.13. Washing of culture to remove eDNA

Washing of MAP culture was utilized in an attempt to remove eDNA from MAP culture intended for freeze drying and use as a positive control. Excessive amounts of eDNA produced during growth was found to result in significant background signal when used in the Actiphage assay + qPCR (section 5.2.8). MAP culture was washed by centrifuging at 4000 x g for 5 min at room temperature before re-suspending the cell pellet in original volume of fresh MP. This washing step was performed three times in total.

2.4. Microorganisms

2.4.1. Bacteriophage

Table 2.1 lists the bacteriophage used in experiments carried out as part of this work as well as the host strains used for phage propagation and the media used for culturing the host cells.

Table 2.1. List of bacteriophage

<i>Phage name</i>	<i>Phage morphology</i>	<i>Host strain for propagating phage</i>	<i>Media used for culture</i>	<i>Source Reference for phage</i>
<i>D29</i>	<i>Siphoviridae</i>	<i>Mycobacterium smegmatis</i> mc ² 155	Middlebrook 7H9 broth* and 7H10 agar (Difco)	(Rybniker et al., 2006)
<i>TM4</i>	<i>Siphoviridae</i>	<i>Mycobacterium smegmatis</i> mc ² 155	Middlebrook 7H9 broth* and 7H10 agar (Difco)	(Rybniker et al., 2006)
<i>PRD1</i>	<i>Tectiviridae</i>	<i>E. coli</i> S17-1	Nutrient broth and agar (Oxoid, UK)	(Bamford and Bamford, 1990)
<i>BP</i>	<i>Siphoviridae</i>	<i>Salmonella enterica</i> serovar Kedougou	BHI broth and agar (Oxoid, UK)	Moeiniyan Bagheri, PhD thesis UoN 2013
<i>B1</i>	<i>Siphoviridae</i>	<i>Listeria monocytogenes</i> ATCC 23074	Nutrient broth and agar (Oxoid, UK)	ATCC 23074-B1
<i>Felix 01</i>	<i>Myoviridae</i>	<i>Salmonella enterica</i> , serovar Typhimurium DB7156	BHI broth and agar	Felix and Callow, 1943

* *Mycobacterial growth supplement OADC (Abtek, UK) was added up to a final concentration of 10 % (v/v) (see 2.1.1).*

2.4.2. Bacterial strains

Table 2.2 lists the bacterial species and strains used as part of the work discussed in this thesis as well as the media used for cultivation.

Table 2.2. List of bacteria and growth media

<i>Species name</i>	<i>Strains used</i>	<i>Media used for culture</i>	<i>Source reference</i>
<i>M. bovis</i>	BCG (Pasteur)	Middlebrook 7H9 broth* and 7H10 agar (Difco)	ATCC 35734 Karlson and Lessel
<i>M. avium ssp. paratuberculosis</i>	ATCC 19851	Middlebrook 7H9 broth* and 7H10 agar (Difco)	ATCC 19851 Bergey <i>et al.</i> , 1923
<i>M. avium ssp. paratuberculosis</i>	K10	Middlebrook 7H9 broth* and 7H10 agar (Difco)	Genome sequence published Li <i>et al.</i> , 2005
<i>M. smegmatis</i>	mc ² 155	Middlebrook 7H9 broth* and 7H10 agar (Difco)	Genome sequence published Mohan <i>et al.</i> , 2015
<i>E. coli</i>	S17-1	Nutrient broth and agar (Oxoid, UK)	ATCC S17-1λpir Castellani and Chalmers
<i>Salmonella enterica</i>	Typhimurium DB7156	BHI broth and agar	Winston <i>et al.</i> , 1979
<i>Salmonella enterica</i>	Kedougou	BHI broth and agar	Silva de Siqueira, PhD thesis UoN 2003
<i>Listeria monocytogenes</i>	ATCC 23074	Nutrient broth and agar, half strength see 2.1.3., (Oxoid, UK)	ATCC 23074 Murray <i>et al.</i> , 1926

**** Mycobacterial growth supplement OADC (Abtek, UK) was added up to a final concentration of 10 % (v/v).**

2.5. Growth of bacterial cultures

2.5.1. Growing *M. smegmatis* culture

M. smegmatis culture was prepared by aseptically inoculating 100 ml of MP containing the OADC supplement (Abtek, UK) with 2-3 colonies of *M. smegmatis* (mc²155) from a Middlebrook 7H10 culture plate. Liquid cultures were grown in a shaking incubator (150 RPM) at 37 °C for 24-36 hours.

2.5.2. Growing BCG and MAP culture

To avoid contamination and thus loss of the culture BCG cultures were prepared in two stages:

A) Initial seed cultures from colonies

Liquid MAP cultures were produced by inoculating multiple (5 or more) 20 ml volumes of sterile MP with colony material from 7H10 agar plates or Lowenstein-Jensen slopes (Beckton Dickinson, USA). Inoculated cultures were incubated, shaking 150 rpm at 37 °C for 4 to 8 weeks. Regular inspections of cultures were carried out to monitor any contaminant growth. Liquid cultures of slow growing mycobacteria are at increased risk of developing contaminant growth due to the slow growing organisms being outcompeted by faster growing contaminants such as bacteria and fungi. The importance of avoiding contamination cannot be overstated as cultures that are found to be contaminated need to be discarded and cannot be easily replaced due to the long time required for growth to appear. First signs of visible growth usually appear after ~3-4 weeks of incubation and typically presents itself as clumps or flakes in liquid culture. These small volume cultures of BCG would be kept at 37 °C shaking incubation for months, regularly visually checked for contaminant growth, and would serve as seed stock for future BCG cultures.

B) Large batch culture from seed cultures

Larger volumes of BCG culture were produced by aseptically inoculating 100 ml volumes of sterile MP with 1 ml of MAP seed culture produced in step A.

Cultures would again be incubated at 37 °C for 4-8 weeks, shaking 150 rpm, at 37 °C with regular inspection to monitor contamination.

This method of producing BCG culture was also used for growing MAP culture. However, for growing MAP mycobactin J (Allied Monitor, USA; see section 2.2.6.) was added at a concentration of 4 µg ml⁻¹ (v/v) to improve growth of MAP as it appears to be vital for good growth of fastidious slow growing mycobacteria such as MAP (Yoshimura and Graham, 1988; Janagama *et al.*, 2010). Apart from this everything else was done identically as for growing BCG culture.

The method for the production of MAP culture described in section 2.5.2 is applicable for both K10 and ATCC strains of MAP used during this project. Note that mycobacterial culture could be stored at 37 °C shaking incubation for many months and be successfully used for the inoculation of fresh media to produce fresh BCG/MAP culture. In liquid culture MAP, as well as BCG, tends to aggregate into clumps which made accurately estimating viability of cultures challenging and usually required mechanical homogenization of the culture using a vortexer and glass beads necessary before the culture could be used. Number of cells in a culture was determined by performing the two day phage amplification assay for the enumeration of mycobacteria.

2.5.3. Growing *E. coli* culture

Escherichia coli S17-1 culture used for the propagation of phage PRD1 was grown by aseptically inoculating 100 ml of Nutrient broth (Oxoid, UK) with 1-2 colonies of *E. coli* S17-1 followed and incubating at 37 °C, shaking 150 rpm for 18-24h

2.5.4. Growing *S. enterica* culture

Salmonella enterica cultures were required for the propagation of bacteriophage Felix (serovar Typhimurium DB7156) and BP (serovar Kedougou). Cultures of both serovars were produced by aseptically inoculating 100 ml of BHI broth (Oxoid, UK) with 1-2 colonies of the respective serovars followed by 18-24 hours of shaking (150 RPM) incubating at 37 °C.

2.5.5. Growing *L. monocytogenes* culture

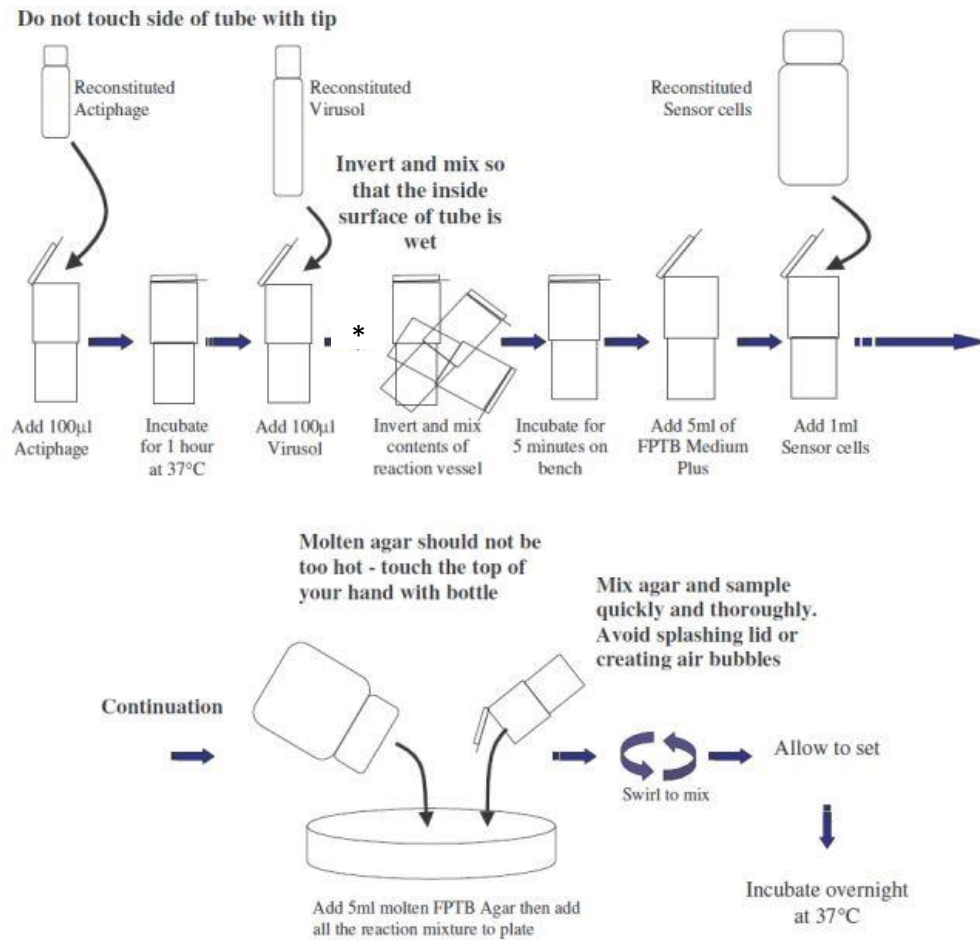
Listeria monocytogenes (ATCC 23074) culture required for propagating phage B1 was prepared by aseptically inoculating 100 ml of half strength Nutrient broth (Oxoid, UK) with 1-2 colonies of *L. monocytogenes* (ATCC 23074) before incubating at 30 °C in a shaking incubator for 24 to 48 hours.

2.6. The two-day phage amplification assay

When performing the two-day phage amplification assay, one millilitre of test sample and 100 µl of phage D29 suspension (titre of $> 10^8$ pfu ml⁻¹) was added to a reaction vessel. The same procedure was applied to the positive and negative controls consisting of 1 ml of sterile MP (-ve) or 1 ml of *M. smegmatis* culture with a titre $\sim 1 \times 10^2$ cfu ml⁻¹ (+ve). All samples with added phage were then incubated for 60 minutes at 37 °C. During this time, the phage will infect mycobacterial cells in the sample but will not have sufficient time to complete the lytic amplification cycle (Swift *et al.*, 2020). After incubation 150 µl of virusol was added and the reaction vessel was shaken for 5 minutes ensuring all inner surfaces had sufficient contact with the virusol and all external free phage not infecting mycobacteria had been destroyed (Swift *et al.*, 2013; Swift *et al.*, 2020). After 5 minutes, 5 ml of sterile MP was added diluting the virusol below cytotoxic levels to not damage the host cells. The mixture was then poured into pre-labelled petri dishes followed by 1 ml of *Mycobacterium smegmatis* sensor cells with a titre of $> 1 \times 10^7$ cfu ml⁻¹ providing fresh hosts for phage that are currently infecting mycobacteria in the sample. Finally, 5 ml of molten (40 – 50 °C) Middlebrook 7H10 agar (Difco, USA) was added to solidify the mixture and form a bacterial lawn. After solidifying the petri dishes are incubated at 37 °C for 24 hours. A schematic overview of the two-day phage amplification assay is presented in figure 2.1. Every plaque observable on the bacterial lawn is theoretically equivalent to one mycobacterial cell in the original sample. Results of 20 plaques or more

were considered positive while 20 plaques or less was considered a negative result as per the manufacturers guidelines (See figure 2.2.).

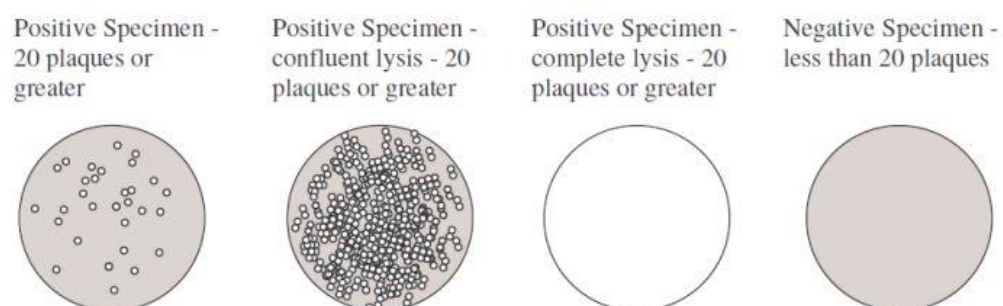
Figure 2.1. Schematic overview of the two-day phage amplification assay



Schematic overview of the two-day phage amplification assay sourced from FastPlaqueTB™ user manual.

* Volume of virusol added to assay was increased from 100 µl to 150 µl.

Figure 2.2. Interpreting results of the two-day assay



Schematic illustration of the possible results of the two day assay sourced from FastPlaqueTB™ user manual. More than 20 plaques would constitute a positive result while 20 plaques or less was considered a negative result.

2.6.1. Two-day phage amplification assay controls

M. smegmatis culture with a known titre was serially diluted to achieve around 100 cells ml⁻¹. One ml of this culture was used as a positive control for the two-day phage amplification assay. 1 ml of sterile MP served as the negative control. The assay is deemed successful if the negative control produces less than 10 plaques while the positive control produces more than 20 plaques as illustrated in figure 2.3.

Figure 2.3. Examples of positive and negative results of the two-day assay

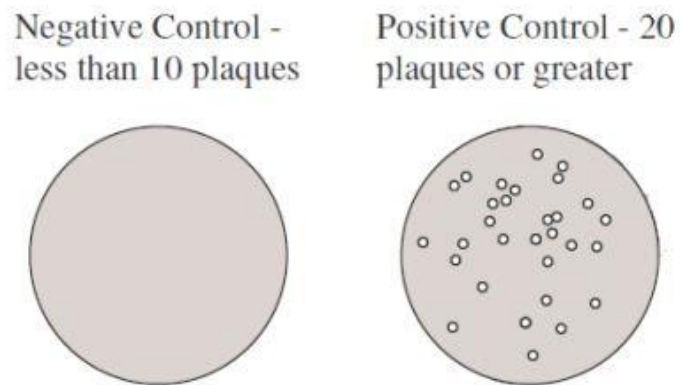


Illustration of the expected results of the positive and negative controls of the two day assay sourced from sourced from FastPlaqueTB™ user manual.

2.6.2. Two-day phage amplification assay for the enumeration of mycobacteria

The two day phage amplification assay can be modified to allow a more accurate estimation of the number of mycobacteria in the original sample. This is done by incorporating a series of 10-fold dilution steps in the assay. This was done by performing the two-day assay as normal (Figure 2.1.) until the addition of the 150 µl virusol (FAS) and 5 minutes of agitated incubation at room temperature to destroy extracellular phage. After the 5 minutes of incubation 100 µl of reaction mix was removed and added to 900 µl of sterile MP thus creating a 10⁻¹ dilution. The 10⁻¹ dilution was in turn diluted 1:10 in sterile MP to create 10⁻² dilution. The dilution series can be continued as much as necessary. Diluted samples were plated by combining 900 µl of diluted sample with 5 ml sterile MP, 1 ml of *M. smegmatis* culture (> 1 x 10⁷ cfu ml⁻¹) and 5 ml warm (40 – 50 °C) Middlebrook 7H10 agar (Difco) before pouring into standard petri-dishes. The petri dishes were subsequently incubated at 37 °C for 24 hours. During incubation, phage will lyse the cell they have infected during the assay and phage progeny will form plaques on *M. smegmatis* lawn. One plaque is counted as one infection event and thus one detected mycobacterial cell. The countable range of plaques for this method is 20-100 plaques per plate. Calculation of the titre was carried out using the formula:

$$\text{cfu ml}^{-1} = \frac{\text{number of plaques} \times \text{dilution factor}}{\text{volume plated in ml}}$$

The result obtained by this method and calculation must be treated as an estimation since it assumes that each plaque counted represents a single infection and amplification event. Mycobacteria however tend to clump together and form complexes thus one plaque may be the result of multiple infection and replication events of individual cells in close proximity.

2.6.3. Extraction and purification of DNA from agar plaques

A modified version of the method of described by Stanley *et al.* (2007) for extraction and purification of mycobacterial DNA from plaques was adapted to allow more specific identification of mycobacterial cells detected using the two-day assay. The centre of the plaque (containing the DNA of the original target cell) was excised using a 2mm biopsy punch tool (Kai Medial, Japan) and placed in a 1.5 ml microfuge tube. This was then either frozen for storage or DNA was extracted immediately using the Zymo™ Gel extraction kit (Zymo, USA), for later specific PCR based identification of the organism causing the plaque.

2.6.4. Extraction of DNA from agar plaques using the Zymo™ Gel extraction kit

Mycobacterial DNA was extracted from agar plaques using the Zymo™ Gel extraction kit (Zymo, USA) according to manufacturer's instructions. Five plaques were picked from a two day assay lawn plate and placed in a 1.5 ml microcentrifuge tube. They were immersed in approximately three times the volume of agarose dissolving buffer (eg. for 300 µl of buffer for 100 µl of agar material). This was briefly vortexed before being incubated at 45-50 °C until all agar had dissolved (approx. 10 minutes). The dissolved agar and buffer mixture was then transferred into a Zymo-Spin™ column which in turn was placed inside a collection tube (both provided as part of the kit). This was then centrifuged at 10000 x g for one minute and the flow through was discarded. DNA in the original plaque was retained on the filter of the spin column and washed using 200 µl of DNA wash buffer followed by another centrifugation step at 10000 x g for one minute. This wash step was repeated one more time. The flow through was again discarded. In order to increase the quality of the DNA obtained and remove any ethanol that may still be present, a "dry" centrifugation run at 10,000 x g for 30 seconds was performed. The collection tube was replaced with a sterile 1.5 ml microfuge tube and 10 µl of heated (50-60 °C) nuclease free water was carefully placed on the filter of the spin

column followed by a final centrifugation step at 10000 x g for one minute. DNA of the original mycobacterial cells detected by the phage amplification assay has now migrated to the water and can be used directly as a template for the IS900 or other PCR reactions.

2.7. Blood processing methods

This section describes the different methods used for the isolation of peripheral blood mononucleocytes (PBMCs) from cow and deer blood. All blood samples were obtained in sodium heparin vacutainers (Beckton Dickinson, USA).

2.7.1. Processing of whole blood using Ficoll buoyancy gradient

Ficoll is a specialized density gradient media for separation of blood components. It contains highly branched polymers to achieve a density of 1.077 g ml⁻¹ at 20 °C. It requires the use of a specialized centrifugation tube (Leucosep™ tube; Greiner, Austria) that contains a plastic divider called a frit to improve layer separation during gradient centrifugation.

A 10 ml Leucosep™ tube (Greiner, Austria) was filled with 2.5 ml PBS (under the frit) and 3 ml Ficoll density gradient medium (GE Healthcare, USA) above the frit. 2 ml of whole deer blood was added to the density gradient medium, and the Leucosep tube was centrifuged at 800 x g for 20 minutes in a spin out centrifuge with no deceleration. After the centrifugation, different blood fractions separate out with the plasma on the top, the erythrocytes at the bottom and the blood mononucleocytes forming a characteristic “buffy coat” layer above the frit. The plasma fraction was carefully removed and discarded. Care was taken not to disturb the buffy coat layer. Then, the buffy coat layer was removed and added to 6 ml of PBS in a 15 ml centrifuge tube. Buffy coat in PBS was then centrifuged again at 800 x g for 10 minutes with the brakes on. After centrifugation the supernatant was discarded and the pellet containing the blood mononucleocytes was resuspended in 1 ml of MP

(Abtek, UK) and left over night at room temperature. This final resuspension and room temperature incubation step in MP serves to lyse blood mononucleocytes through osmotic shock and release MAP cells harboured inside. After this step, the sample can be stored at -20°C or directly used for the two day phage amplification and Actiphage assays (section 2.6. and 2.8. respectively).

2.7.2. Processing of whole blood using ACK lysis buffer

3 ml of whole blood collected in Sodium heparin tubes (Beckton Dickinson, UK), was added to a 50 ml conical based tube followed by 40 ml of ACK buffer. When the buffer is first added, the solution will appear cloudy. The cloudiness dissipates over the course of ~5 minutes of agitated incubation at room temperature until the solution is crystal clear (see 6.2.3.2). When the solution is clear, it was centrifuged at $300 \times g$ for 5 minutes. The supernatant was then poured off and the pellet was resuspended in 5 ml of phosphate buffered saline (PBS). A second centrifugation step was performed at $300 \times g$ for 5 minutes. After centrifugation the supernatant was discarded, and the pellet resuspended in 1 ml of fresh MP and incubated at room temperature overnight in order to lyse the peripheral blood mononucleocytes harbouring the mycobacteria through osmotic shock. The sample can now be stored at room temperature for ≤ 2 days, stored at -20°C or used immediately in the two day assay or Actiphage assay. In order to be used in the Actiphage assay, the resuspended pellet was transferred into a 1.5 ml Eppendorf tube and centrifuged at $13,000 \times g$ for 3 minutes. The supernatant was removed, and the pellet resuspended in 110 μl of phage D29 suspension and transferred into Actiphage assay spin columns.

2.8. The Actiphage assay for the detection of Mycobacteria

The suspended buffy coat in media plus obtained after blood mononucleocyte separation (section 2.7) was pelleted at 13,000 x g for 3 minutes and the supernatant was discarded. The pellet was resuspended in 110 µl of phage D29 suspension with a titre of $> 1 \times 10^8$ cfu ml⁻¹ and transferred into a 1 – day assay filter column placed in a collection tube. This differs slightly from the usual procedure employed by using a 10 fold higher phage concentration than what the Actiphage assay kit instructions suggest (10^8 instead of 10^7 pfu ml⁻¹). This was done deliberately to ensure all putative target cells in the sample get in contact with at least one phage particle. The issue of lysis from without due to multiple phage infecting one cell was considered irrelevant since the phage only serve as a lysing agent and no productive infection is required for this assay. The sample was subsequently incubated at 37 °C for 3.5 hours to allow phage adsorption, infection, and lysis of all mycobacterial cells. After the incubation, columns were centrifuged at 13000 x g for 3 minutes to allow liquid containing DNA from lysed mycobacteria to drain into the collection tube. Raw DNA can be frozen for storage at this stage. Before being used as template for a PCR reaction the raw DNA was cleaned using Zymo™ clean & concentrator kit (Zymo, USA).

2.8.1. Zymo™ clean and concentrator kit for the purification of mycobacterial DNA

Sample DNA obtained from the Actiphage assay was purified using the Zymo™ clean and concentrator kit (Zymo, USA) according to manufacturer's instructions. DNA samples obtained during the assay were mixed with DNA binding buffer at a ratio of 1:2 before being transferred into a Zymo-Spin™ column and centrifuged at 13000 x g for 30 seconds. Flow through was discarded and the DNA now bound to the filter of the column was washed twice by adding 200 µl Zymo™ DNA wash buffer and centrifuging at 13,000 x g for 30 seconds. As with the Gel extraction kit procedure (section 2.6.4) a

“dry” centrifugation run at 13,000 x g for 30 seconds was performed to remove any excess solvent still present. Cleaned DNA was eluted using 10 µl of warm (50-60 °C) nuclease free water and collected in a sterile microfuge tube. Purified DNA could be amplified using PCR or qPCR immediately or stored at –20 °C until needed.

2.9. PCR reactions

2.9.1. IS900 end point PCR

IS900 PCR uses the method described by Whittington *et al.* (1998) involving the P90 forward and P91 reverse primer (see table 2.3). IS900 PCR was developed for the detection of the specific insertion sequence 900 in *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Cleaned and purified DNA obtained by either purifying DNA from the Actiphage assay using the Zymo™ clean and concentrator kit or by extracting DNA from plaques obtained through the two day assay (sections 2.6.3. and 2.6.4. respectively) was used as a template for the PCR reaction.

The PCR mixture consisted of 1 µl P90 forward and 1 µl P91 reverse primer (250 ng of primer per µl), 10 µl HotStar Taq Plus Master Mix (Qiagen, Germany), 2 µl coral loading dye and 5 µl of template DNA. One µl of RNase free water was used to reach a final volume of 20 µl reaction mixture. Negative controls were produced by using 5 µl of nuclease free water instead of the template DNA. The PCR reaction involved an initial denaturation of the template DNA at 95 °C for 15 minutes followed by 37 amplification cycles of 94 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 1 minute. After 37 cycles there was a final 4-minute extension at 72 °C. PCR products were run at 75V on a 1.5% TAE agarose gel containing ethidium bromide at a final concentration of ~125 µM. The DNA visualized using UV light and imaged using a Bio-Rad gel doc system (Bio-Rad, USA). The expected size of the IS900 PCR product is 394 bp (Foddai and Grant, 2020).

Table 2.3. P90 and P91 Primer sequence and DNA target

Primer Name	Primer Sequence	Target DNA
(F) P90	5'-GAAGGGTGTTCGGGGCCGTCGCTTAG-3'	MAP IS900 Sequence
(R) P91	5'-GGCGTTGAGGTCGATCGCCACGTGAC-3'	MAP IS900 Sequence

Sequence of P90 (Forward) and P91 (Reverse) primers used for the amplification of MAP specific sequence IS900.

2.9.2. Detection of MAP using qPCR

a) Ingenetix BactoReal kit

The PCR master mix was produced by combining (per sample) 10 µl DNA reaction mix, 3 µl nuclease free water and 1 µl each of MAP assay mix and CR assay mix (all included in kit). Five microliters of cleaned template DNA was added to 15 µl of master mix and amplified using a Rotor-Gene qPCR machine (Qiagen, Germany). The PCR conditions consisted of two minutes of initial denaturation at 50 °C followed by 20 seconds at 95 °C and 45 cycles of 5 seconds denaturation at 95 °C and one minute of extension at 60 °C.

Fluorescence of the fluorescent probe, indicating DNA amplification, was recorded during elongation at 60 °C. The reporter probes used were either FAM (red light emission) or Cy5 (green light emission) and recorded using the red and green detection channels. Results were recorded and analysed using Qiagens QREx software.

b) Biosellal BioTK050 qPCR kit

The Biosellal BioTK050 kit is manufactured by Biosellal (France) and optimized for the detection of MAP from faecal samples. The master mix was premade and included in the kit. For the detection of *M. bovis* DNA 10 µl of master mix was combined with 5 µl cleaned and concentrated template DNA and amplified using a Rotor-Gene qPCR machine (Qiagen, Germany). Parameters for the qPCR reaction were set according to manufacturer's instructions and included an initial 5 minutes denaturation at 95 °C followed by 40 cycles of 15 seconds denaturation at 95 °C and 30 seconds elongation at 60 °C. Data acquisition was performed during the 60 °C elongation step by measuring light emission of the fluorescent probe. The reporter probes used were either FAM (red light emission) or Cy5 (green light emission) and fluorescence was recorded using the red and green detection channels. Results were recorded using Qiagens QREx software.

2.9.3. Detection of *M. bovis* using the Biosellal BioTK088 qPCR kit for the detection of MTBC organisms

The Biosellal BioTK088 kit is manufactured by Biosellal (France) and optimized for the detection of *M. bovis* from faecal samples. The master mix was premade and included in the kit. For the detection of *M. bovis* DNA 10 µl of master mix was combined with 5 µl cleaned and concentrated template DNA and amplified using a Rotor-Gene qPCR machine (Qiagen, Germany). Parameters for the qPCR reaction were set according to manufacturer's instructions and included an initial 5 minute denaturation at 95 °C followed by 40 cycles of 10 seconds denaturation at 95 °C and 60 seconds elongation at 60 °C. Data acquisition was performed during the 60 °C elongation step by measuring light emission of the fluorescent probe. The reporter probes used were either FAM (red light emission) or Cy5 (green light emission) and fluorescence was recorded using the red and green detection channels. Results were recorded using Qiagens QREx software.

2.9.4. Analysis and interpretation of qPCR results

qPCR results were analysed using the QREx software supplied by Qiagen with its Rotor-Gene qPCR machine. Interpretation of results was performed using the C_q value which represents how many amplification cycles were required before the fluorescence emitted by the PCR probe crossed the detection threshold. Note that a sample simply crossing the detection threshold did not imply a positive assay result. Amplification of non-specific PCR products has been noticed especially during the last 10 cycles of the qPCR and usually all samples eventually crossed the detection threshold. This was expected and is in line with what the literature reports (Ruiz-Villalba, 2017; Park *et al.*, 2020; Jaeger *et al.*, 2021). Instead, samples were analysed using the ΔC_q method where C_q values were interpreted in relation to the C_q value produced by the negative assay control. A sample would be considered positive if it crossed the detection threshold at least 2 cycles before the negative control (expressed as $\Delta C_q \geq 2$). Samples detectable $\Delta C_q \geq 1.5$ were considered weakly positive.

Chapter 3

Freezing of plaques as a method for preserving bacteriophage

3.1. Introduction

The challenge of rising levels of antibiotic and disinfectant resistance in bacteria has led to an increased interest over the last 15 years in isolation of bacteriophage and the establishment of collections of phage isolates (Gibson *et al.*, 2019; Singh *et al.*, 2023). Maintaining genetically stable and viable stocks of bacteriophage for prolonged periods of time has always been challenging. Compared to, for example, bacterial or eukaryotic cell lines or protozoa, bacteriophage are relatively easy to store as virions and can be kept as a liquid suspension at 4 °C for years. However, storage properties of phage at 4 °C appear to be highly variable between phage type with virions of some phage maintaining viability for years (Lobocka *et al.*, 2018) while Gonzalez-Menedez *et al.* (2018) found that different *Staphylococcus* phage (philPLA88, philPLA35, philPLA-RODI and philPLA-C1C) would lose between 1 and 3 log₁₀ in phage viability over a period of up to 24 months when phage virions were stored as liquid suspension at 4 °C. Furthermore, liquid stocks are prone to contamination by bacteria or fungi, potentially leading to loss of samples due to protease production by the contaminants. Therefore, in order to maintain a high titre phage stock, continuous sub-culturing is often necessary, but this is detrimental to the genetic stability of the phage as each sub-culture has the potential to introduce mutations into the population.

Freezing would solve these problems as samples stored below 0 °C would remove the potential risk of titre loss due to growth of contaminants and could therefore be kept for many years, removing the need for sub-culturing. However, freezing of phage in suspension results in a high - and sometimes almost complete - loss of phage viability and is therefore not an ideal solution (Clark and Geary, 1973; Marton *et al.*, 2021). The main cause of damage to the bacteriophage during freezing is believed to be the crystallisation of water and the resulting increase in volume damaging and destroying the phage capsid and or disruption of tail protein structure and, thus, rendering the phage non-infective (Puapermpoonsiri *et al.*, 2010; Vandenheuvel *et al.*, 2014). Indeed, the complexity of the tail apparatus of bacteriophage has been

suggested as the major factor determining the likelihood of bacteriophage survival during freezing (Warren and Hatch, 1969; Puapermpoonsiri *et al.*, 2010).

Bacteriophage intended for long term storage are commonly freeze-dried, but this process can also be accompanied by substantial loss in phage titre due to exposing the phage particles to both, freezing and drying stress (Clark and Geary, 1973). Again, loss of viability during lyophilization and subsequent storage appears to be highly variable and dependent on the species of phage being used with reports ranging from close to no loss in viability after years of storage (Merabishvili *et al.*, 2013; Gonzalez-Menedez *et al.*, 2018) to almost complete loss in viability during the lyophilization process (Croyle *et al.*, 2001; Merabishvili *et al.*, 2013).

Different attempts have been made in the past to add stabilizing agents (excipients) to the phage suspension to protect the phage from damage during the freeze-drying process. Mono- and polysaccharides, such as trehalose, mannitol and sucrose and glycerol and biodegradable polyester microspheres have all been evaluated as potential excipients to protect phage during the freeze-drying process (Puapermpoonsiri *et al.*, 2009) with carbohydrate based excipients such as trehalose and sucrose producing the most promising results (Merabishvili *et al.*, 2013). Carbohydrate excipients such as sucrose and trehalose are thought to stabilize phage during drying by binding to phage proteins and providing structural support thus preventing misshaping and allowing the protein to remain functional (Golovina *et al.*, 2009). Vandenheuvel *et al.* (2014) also found that phage particles in a trehalose solution could survive freeze drying in much higher numbers as the trehalose provided structural support to the phage particles by “mimicking” the presence of water. However, it is noted that freeze-dried phage also tend to lose viability over time (Ackermann *et al.*, 2004) and it appears that residual water content in the freeze dried product is a critical factor in determining the long terms stability of freeze-dried phage (Puapermpoonsiri *et al.*, 2010)

suggesting that protein structure can change during storage of these products.

Bacteriophage D29 and TM4 are both double stranded DNA, tailed, lytic mycobacteriophage from the family *Siphoviridae*. They have been shown to infect a wide range of mycobacteria including (but not limited to) the slow growing mycobacteria *M. tuberculosis*, *M. avium* ssp. as well as the fast growing *M. smegmatis* (Froman *et al.*, 1954; Swift *et al.*, 2014). Their broad host range, ease of culture and quick infection and lysis cycle has made phage D29 the focus of anti-mycobacteria phage based research since at least the 1990s (Wilson *et al.*, 1997; Park *et al.*, 2003; Grant, 2021). Phage TM4 was found to be less infective than phage D29 (Swift *et al.*, 2014) however it has been postulated that phage TM4 has the ability to infect host cells in a stationary growth phase due to a motif within the tape measure protein (*tmap*) gene present in TM4 conferring peptidoglycan-hydrolysing activity and allowing infection of stationary phase cells (Piuri and Hatfull, 2006). Phage D29 lacks this ability and thus requires cells to be actively growing in order to infect productively. This is a clear disadvantage when using phage D29 in a detection assay as all viable but not actively growing cells would remain undetected and thus produce a false negative result. The ideal solution would be a combined Phage D29 and TM4 preparation that could detect mycobacterial cells in a sample efficiently and regardless of growth state.

3.1.1. Aims and objectives

In this study, the ability of agar to act as the stabilising agent by freezing bacteriophage *in situ* in plaques extracted from standard phage propagation plates was investigated. As plaques result from multiple rounds of host cell lysis, they contain high numbers of bacteriophage embedded in agar. Agar is a carbohydrate polymer with a molecular structure similar to that of known cryoprotectants such as trehalose or sucrose (see figure 3.7), therefore if agar could protect phage from structural damage during freezing, it would provide a simple and attractive method to preserve phage stocks with a relatively high titre.

3.2. Results

3.2.1. Freezing of plaques for storing bacteriophage

3.2.1.1. Freezing of mycobacteriophage D29 and TM4 plaques and suspension

To determine the effect of freezing on a liquid suspension of phage in lambda buffer, a sample of either phage D29 or TM4 phage suspension (approx. 10^9 pfu ml⁻¹) was stored at 4 °C overnight or was frozen at -20 °C and stored overnight. The frozen samples were thawed and both sample groups were brought to room temperature before being re-titred. After storage at 4 °C, as expected, there was no significant ($p > 0.1$) difference in the titre of either phage suspension. However, after freezing, the titre recovered from the frozen D29 suspension was 5.5×10^7 pfu ml⁻¹ indicating that freezing the phage in lambda buffer resulted in a $> 1 \log_{10}$ drop in phage titre ($p < 0.05$). A similar pattern of results was found when a liquid suspension of phage TM4 was frozen at -20 °C which resulted in a $2 \log_{10}$ drop in phage titre (2.6×10^6 pfu ml⁻¹).

To investigate whether agar could protect phage during freezing, plaques containing each type of phage were prepared by plating samples on a lawn of *M. smegmatis*. Individual plaques were then excised from the lawn for each phage and these were stored in microcentrifuge tubes without the addition of lambda buffer. The excised plaques containing phage were stored at either 4 °C or at -20 °C overnight. To provide a base line figure, immediately after excision phage were eluted from 20 individual phage D29 plaques by adding 100 µl of lambda buffer and incubating for 5 min (section 2.3.3). This method produced an average titre of 1.51×10^9 pfu ml⁻¹ (SD ± 0.38).

For these experiments, plaques were harvested or “picked” using a 3 mm biopsy punch needle (Kai medical, Japan). Phage replication and plaque formation are complicated processes where factors such as number and growth rate of the host bacteria, agar concentration, ion concentration and temperature can introduce a considerable amount of variability on the number of phage present in a plaque. It was thus deemed necessary to

confirm consistency in the number of phage that could be recovered from single 3 mm plaque extractions. To this end phage D29 plaques were grown on *M. smegmatis* lawn plates and two sets of plaques (2 x n=20) were excised, eluted and titred as described in materials and methods (2.3.2. and 2.3.3). The average titre of the two sets was recorded as 1.84×10^9 and 2.24×10^9 pfu ml⁻¹ with intra-set standard deviations (log₁₀) of 0,39 and 0,38 respectively. These results indicate that there is a low degree of variability in the number of phage particles recoverable from individual 3 mm excised D29 plaques which indicates that eluting phage from plaques was a reproducible process that allowed these results to be compared with results gained using liquid suspension, which is by its nature a more uniform sample.

To determine the effect of the different storage conditions on phage titre, number of phage particles released from the plaques stored under different conditions was then determined. The average titre of phage D29 recovered from plaques stored at 4 °C (n=5) was found to be 2.4×10^9 pfu ml⁻¹ (SD ± 0.28) indicating that no significant change in the number of phage recovered from the plaques occurred. When the titre of plaques stored at -20 °C was determined it was also seen that only 1 log₁₀ reduction in titre had occurred (average titre of 5 plaques = 3.76×10^8 pfu ml⁻¹; SD ± 0.3). In parallel, when another liquid phage suspension was frozen at -20 °C in a parallel experiment the average phage titre recorded was only 3.28×10^7 pfu ml⁻¹ (SD ± 0.24) which confirmed the 2 log₁₀ reduction seen in the earlier experiment. It was found that the difference in the reduction in phage titre between the two samples (liquid suspension and phage embedded in agar) stored at -20 °C was significant (p < 0.01). Similarly, when plaques containing phage TM4 were stored at 4°C, the average titre of phage recovered from the agar was 2.34×10^9 pfu ml⁻¹ (SD ± 0.11), and the titre of phage recovered from plaques frozen at -20 °C (2.42×10^9 pfu ml⁻¹; SD ± 0.13) so in this case no significant decrease in phage titre (p = 0,85) was detected. This data is summarized in table 3.1 and visualized in Figures 3.1 (D29) and 3.2 (TM4). In summary, for both D29 and TM4, the drop in titre on freezing in agar was significantly less (p < 0.05)

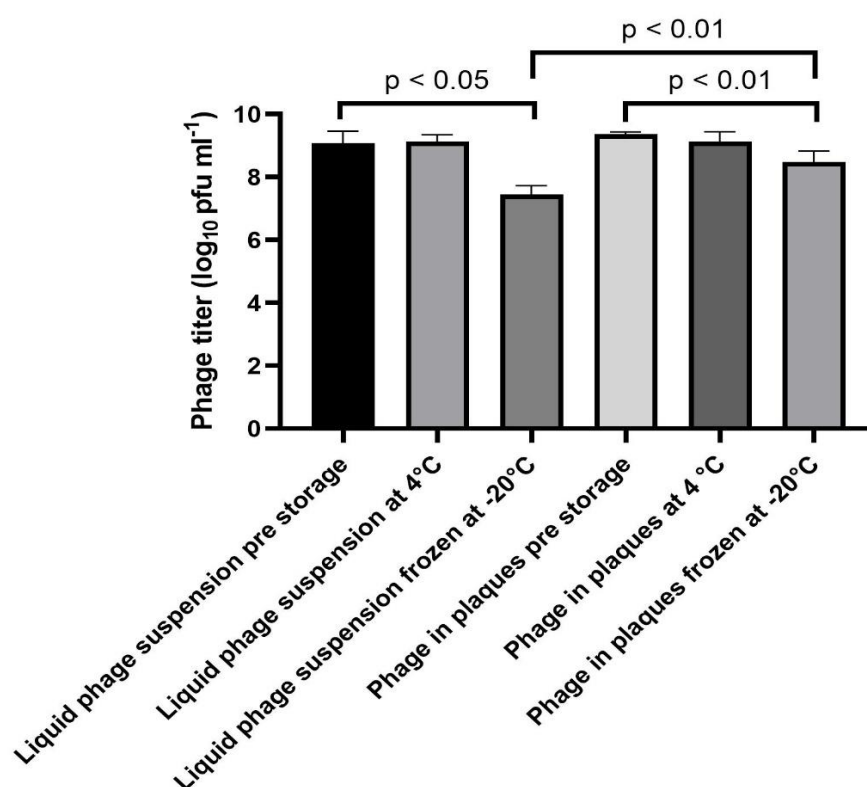
than that seen when liquid suspensions of the phage were frozen at the same temperature and suggested that plaque material was acting as a cryoprotectant.

Table 3.1. Comparing phage D29 survival in plaques or suspension at refrigeration and freezing conditions

STORAGE CONDITIONS	TITRE PHAGE D29 (PFU ML ⁻¹)	SD± (LOG ₁₀)	TITRE PHAGE TM4 (PFU ML ⁻¹)	SD± (LOG ₁₀)
LIQUID PHAGE SUSPENSION PRE STORAGE	1.52 x 10 ⁹	0.38	2.54 x 10 ⁹	0.05
LIQUID PHAGE SUSPENSION STORED AT 4 °C	1.48 x 10 ⁹	0.19	2.34 x 10 ⁹	0.06
LIQUID PHAGE SUSPENSION STORED AT -20 °C	3.28 x 10 ⁷	0.24	2.44 x 10 ⁶	0.04
PHAGE PLAQUES PRE STORAGE	2.38 x 10 ⁹	0.06	2.54 x 10 ⁹	0.04
PHAGE PLAQUES STORED AT 4 °C	1.60 x 10 ⁹	0.28	2.34 x 10 ⁹	0.11
PHAGE PLAQUES STORED AT -20 °C	3.76 x 10 ⁸	0.30	2.42 x 10 ⁹	0.13

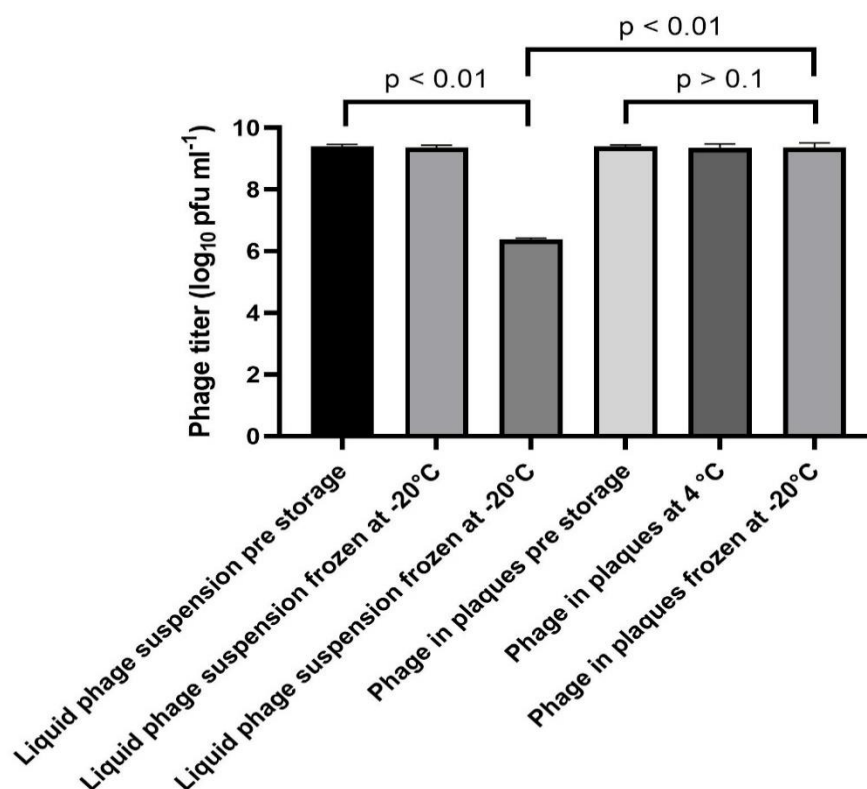
Table showing the average number of phage D29 and TM4 particles recovered from liquid samples and plaques pre storage, at 4 °C and at -20 °C as well as sample group standard deviation (log₁₀). For both liquid suspensions and plaque samples, 5 replicates were tested to generate each data set.

Figure 3.1. Comparison of phage D29 survival in liquid suspension or embedded in plaques when storing at refrigeration or frozen at -20 °C



Comparison of effect of different storage methods on phage D29. Samples used were either liquid suspensions of phage D29 or samples of the same phage embedded in agar (excised plaques). To determine the effect of storage conditions on phage viability, both types of sample were titred and then either stored at 4°C for 24 h and then re-titred or were frozen at -20 °C and then thawed and re-titred. For samples where phage were embedded in agar, prior to phage titration phage were eluted from the agar by immersing in λ buffer.

Figure 3.2. Comparison of phage TM4 survival in liquid suspension or embedded in plaques when storing at refrigeration or frozen at -20 °C

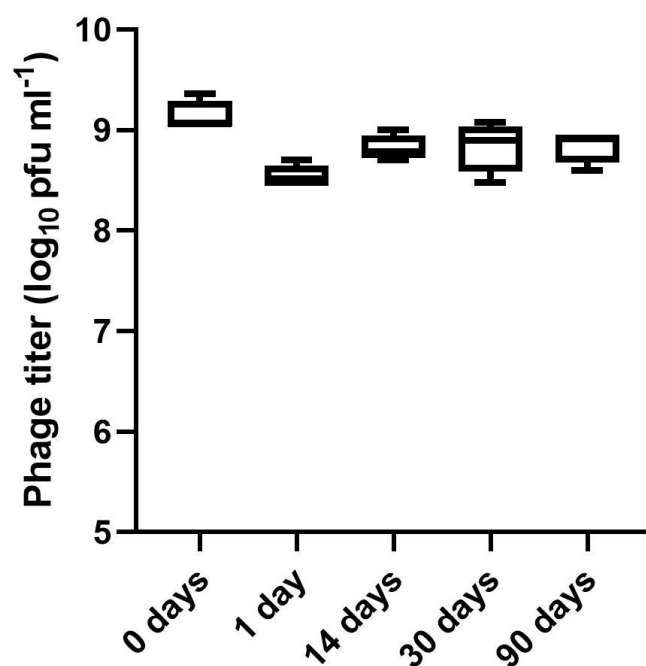


Comparison of effect of different storage methods on phage TM4. Samples used were either liquid suspensions of phage TM4 or samples of the same phage embedded in agar (excised plaques). To determine the effect of storage conditions on phage viability, both types of sample were titred and then either stored at 4°C for 24 h and then re-titred or were frozen at -20 °C and then thawed and re-titred. For samples where phage were embedded in agar, prior to phage titration phage were eluted from the agar by immersing in λ buffer.

3.2.1.2. Viability over time of phage stored frozen in plaques

After establishing that the number of viable phage particles is maintained when freezing embedded in agar compared to when phage were frozen in liquid suspension, it was decided to investigate whether continued storage at -20 °C would result in any loss in phage viability. To do this, phage D29 plaques were excised and then transferred to individual microcentrifuge tubes. As before, a baseline the number of viable phage in plaques was determined by immediately eluting phage from some plaques by immersing in lambda buffer. Sets of individual plaques (n=4) were stored at -20 °C for 1, 14, 30 or 90 days before samples were thawed and phage recovered from the plaques by elution. Before freezing the average number of phage recovered from plaques stored at 4 °C (n=4) was 1.43×10^9 pfu ml⁻¹ while after 1, 14, 30 and 90 days storage at -20 °C the average titre of the phage recovered from the plaques was determined to be 3.50×10^8 ; 6.75×10^8 ; 7.75×10^8 and 7.50×10^8 pfu ml⁻¹, respectively (Fig. 3.3). The standard deviation for all sets of samples was determined to be $< 0.3 \log_{10}$ indicating that the results were very reproducible. Comparing the titre of phage samples stored at -20 °C for 1 day with those stored at -20 °C for up to 3 months, there was no significant drop in phage titre ($p < 0.05$) (Fig. 3.3) indicating low or no decay of phage titre during frozen storage.

Figure 3.3. Effect of long term frozen storage on stability of phage D29



Sets of three phage D29 plaques were excised and phage were either immediately eluted from the agar or plaques were stored frozen at -20 °C for 1, 14, 30 and 90 days before phage were eluted from the agar. The average number of viable phage particles recoverable from each plaque sample was then determined.

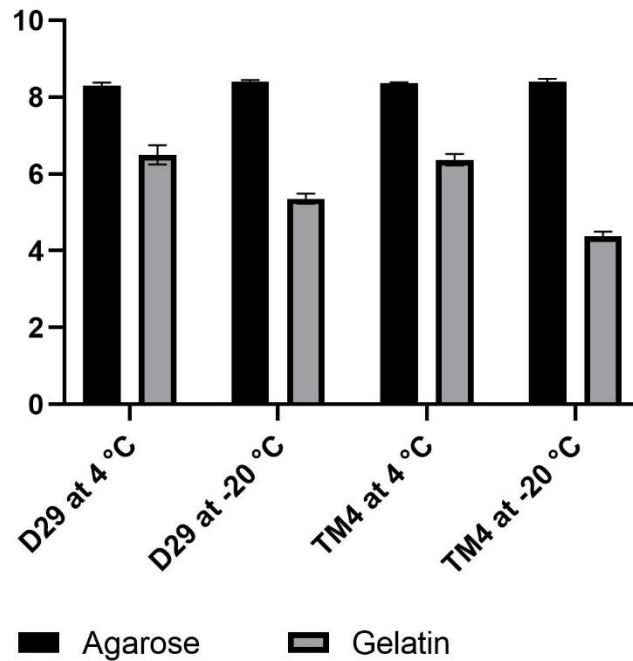
3.2.1.3. Investigation of critical components of plaques for cryoprotection

The initial bacteriophage used in this study were those specific for mycobacteria. These bacteria are routinely grown in media supplemented with oleic acid and glycerol, which are also potential cryoprotectants (Beal *et al.*, 2001; Xiao *et al.*, 2022). To test whether this was the case, “artificial plaques” were prepared by adding 5 μ l of a purified D29 phage suspension to 5 μ l of either molten Nutrient agar or molten Middlebrook 7H10 agar which was then allowed to set. Samples of each of these artificial plaques (n=3) were frozen at -20°C for 24 hours and the number of viable phage that could be recovered from the agar after freezing was determined. The nutrient agar plaques produced phage suspensions with a titre of 8×10^8 pfu ml^{-1} while the 7H10 agar yielded 2×10^8 pfu ml^{-1} , and therefore there was no significant difference in the number of phage recovered from the artificial plaques made using these two types of agar. Meanwhile, artificial plaques made from nutrient or 7H10 agar and not frozen produced titres of 2.2×10^9 and 1.95×10^9 pfu ml^{-1} respectively. Hence, the agar itself could confer the cryoprotective properties observed rather than the specific additives present in Middlebrook 7H10 agar.

To investigate whether the cryoprotection was the result of the formation of a gel, or due to the specific chemistry of the carbohydrate components of agar, artificial plaques were created as described above using molten 1.6 % (w/v) agarose or a molten 20 % (w/v) gelatine solutions instead of agar. Agarose was chosen as it is a more purified form of one of the polysaccharides present in agar. Artificial plaques (n=3) were either stored at 4°C or frozen at -20°C and then the number of surviving phage determined after eluting them from the matrix as before. The average titre recovered from D29 plaques made with agarose was 2.00×10^8 pfu ml^{-1} (4°C) and 2.50×10^8 pfu ml^{-1} (-20°C) indicating that the phage were protected by this matrix. In contrast D29 plaques produced using gelatine only yielded titres of 3.35×10^6 pfu ml^{-1} (4°C) and 2.33×10^5 pfu ml^{-1} (-20°C) and in both cases the number of plaques recovered from gelatine was significantly lower than the sample prepared

using agarose ($p < 0.01$; Fig. 3.4). This indicated that the phage did not survive well when mixed with molten gelatine held at the same temperature as the agar. A similar pattern was found using phage TM4 with average titres recovered from agarose plaques of 2.37×10^8 pfu ml⁻¹ (4 °C) and 2.50×10^8 pfu ml⁻¹ (-20 °C). The TM4 plaques created using gelatine were found to contain 2.43×10^6 pfu ml⁻¹ (4°C) and 2.47×10^4 pfu ml⁻¹ (-20 °C) ($p < 0.01$). Even though the number of phage surviving in the formation of the artificial plaques using gelatine was lower, it was still clear that this matrix did not have the same cryoprotective properties as agarose.

Figure 3.4. Comparing the effect of gelatine and agarose on cryoprotection of phage



Phage suspensions of phage D29 and TM4 were solidified using a 1.6 % (w/v) agarose solution or a 20 % (w/v) gelatine solution. These “artificial” plaques were then either frozen at -20 °C or refrigerated at 4 °C for 24 h. To monitor the effect of the different conditions on phage survival, the phage within the “artificial” plaques were eluted and then titred.

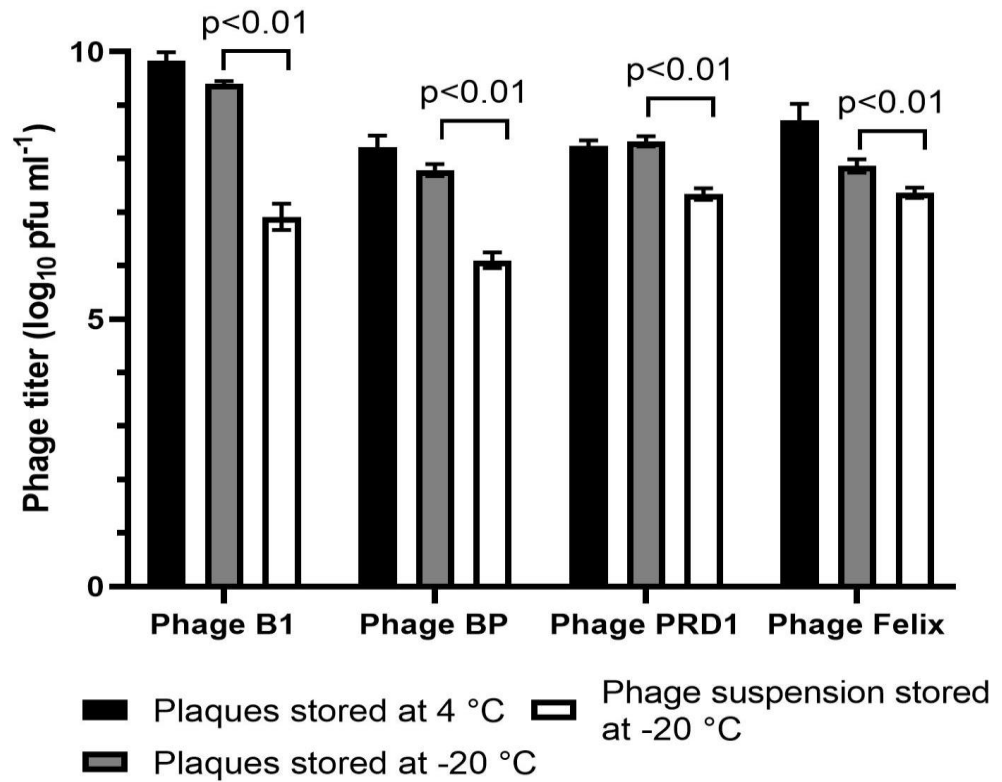
3.2.1.4. Freezing of non-mycobacteriophage plaques and suspension

To determine whether this method of cryoprotection was specific to Mycobacteriophage or whether it could be applied to different phage types and morphologies, a range of other phage were used. Bacteriophage 23074-B1 and BP are both *Siphoviridae* that are specific for *Listeria monocytogenes* and *Salmonella* Kedougou, respectively. Felix 01 is a Myovirus specific for *S. Typhimurium*, and phage PRD1 is a Tectivirus that will infect cells that contain the IncP conjugation system, *E. coli* in this case. For each of these different phage, plaques were prepared using their propagating host strain (see Table 2.1 in Materials and Methods).

To perform this experiment, plaques containing the different type of phage were prepared and excised as before (n=5). In parallel, samples of liquid suspensions of these phage with titres of approximately 10^8 pfu ml⁻¹ were frozen, and a levels of phage recovered after the storage treatment recorded (Fig. 3.5). Phage B1 plaques produced higher titre of phage per plaque compared to the other phage used in this study that consistently produced 10^8 - 10^9 phage per plaque. This was expected as this phage is characterised as having a short replication time (approx. 30 min) and a large burst size (approx. 100) and produces lysates with high phage titres (Moeiniyan-Bagheri, 2013).

In each case, freezing the liquid suspension of phage resulted in a marked reduction in titre (1-3 log₁₀ reduction recorded; see Fig. 3.5 and Table 3.2) compared to the samples recovered from frozen plaques (p < 0.01) and the titre of phage recovered from the frozen plaques was always within 1 log₁₀ value of the sample of the same phage stored at 4 °C showing that significant cryoprotection was being conferred by the agar. From the results it was concluded that this method of preservation was suitable for phage isolated from a range of different bacterial hosts and was also effective for phage from different morphologies groups.

Figure 3.5. Evaluation of plaque storage in different phage morphologies



Plaques of bacteriophage BP, B1, PRD1 and Felix were mechanically excised from bacterial lawn plates and either frozen at -20 °C or refrigerated at 4 °C. After overnight storage the phage with the “artificial” plaques were eluted and then titred. High titre phage suspensions of these phage were frozen at -20 °C for 24 h before being thawed and titred. N=5.

Table 3.2. Titres of different phage recovered from plaques stored at 4 °C or –20 °C

	PHAGE STORED AT 4 °C (PFU ML ⁻¹)	PHAGE STORED AT -20 °C (PFU ML ⁻¹)
B1	1.82 x 10⁸	6.32 x 10⁷
BP	7.2 x 10⁹	2.56 x 10⁹
PRD1	1.78 x 10⁸	2.16 x 10⁸
FELIX	6.12 x 10⁸	7.6 x 10⁷

Table 3.3. shows the data that is visualized in figure 3.5. Each data point represents the average of 5 repeats.

3.2.2. Drying of plaques for storing bacteriophage

3.2.2.1. Drying of plaques: initial experiments

For the purposes of the sponsors of the project, frozen samples of phage D29 were not ideal as the long term aim was to produce room temperature-stable phage D29 samples that could be incorporated into a commercially produced phage-based detection kit. Therefore, alternative methods of preserving phage were investigated. Being able to simply pick, dry and rehydrate plaques without major loss in titre would be a very cheap and convenient solution of the problem.

To investigate whether this would be successful, two sets of five phage D29 plaques ($2 \times n=5$) were picked (see section 2.3.2 for method) and transferred into individual microfuge tubes with perforated lids to allow moisture to escape. These were then either dried at room temperature ($\sim 20^\circ\text{C}$) under vacuum or in an incubator at 37°C without vacuum overnight. Visual inspection of the samples after drying revealed that the plaques had lost most of their mass and were reduced to thin flakes. To extract phage from the plaques after drying, dried plaques were rehydrated in $100\ \mu\text{l}$ of lambda buffer and phage released into the buffer was then titred (section 2.3.11). As before, a control group of undried plaques were collected and stored at 4°C and these produced an average titre of $2.7 \times 10^9\ \text{pfu ml}^{-1}$. The results showed that for plaques dried at 37°C , very few viable phage were recovered, producing a titre of $< 1 \times 10^4\ \text{pfu ml}^{-1}$. In contrast, plaques dried at 21°C yielded a phage titre of $1.6 \times 10^7\ \text{pfu ml}^{-1}$ which indicates that the low titre recorded did not seem to be due to entrapment of phage in the dried agar matrix. This result indicated that simply picking and drying naturally prepared plaques was not a viable option for producing a dry and stable form of phage D29 with sufficient titre to perform the two-day phage amplification assay.

Since the plaques dried at $\sim 20^\circ\text{C}$ retained a higher number of viable phage particles than the plaques dried at 37°C , it was possible that drying the plaques at a lower temperature could reduce the loss in phage titre that was

recorded during the drying process at higher temperatures. To investigate this, plaques (n=5) were picked and placed into individual 1.5 ml microfuge tubes with perforated lids. These were then placed into a closed container with a layer of silica gel at the bottom which in turn was placed into a 4 °C fridge. The hygroscopic properties of the silica gel allow it to absorb moisture from the air inside the tubes as the water evaporated from the plaques. Due to the slower rate of evaporation at cooler temperatures, the plaques were allowed to dry in the fridge for 5 days. After drying the plaques had the same thin flaky appearance as the plaques dried at higher temperature and were rehydrated and titred as described above. After rehydration the average phage titre recorded was 2.7×10^8 pfu ml⁻¹ which was a 1 log₁₀ increase in the number of phage recovered per sample compared to the plaques dried at 20 °C, and a 4 log₁₀ increase compared to the plaques dried at 37°C, suggesting that phage survival during this drying process is temperature dependent. This was attributed to structural contraction of the agar polymer encountered during drying damaging the phage particles or its delicate phage apparatus. Shrinkage and build-up of kinetic stresses resulting in cracks and shape changes is a common occurrence during drying of aqueous gels like agar (Mahiuddin *et al.*, 2018). It has also previously been reported that temperature can affect the long term stability of spray dried bacteriophage powders (Leung *et al.*, 2018), but the short time frame of these experiments argue against this observation being due to phage instability after drying. Rather these results are similar to that reported by Richter *et al.* (2021), who also noted that lower temperatures improved the number of viable phage particles detected after attaching them to polypropylene in an attempt to stabilise phage for use in phage therapy. The fact that phage seem to be more stable during these different processes that will clearly exert forces on the phage structure may simply reflect the well known effect of temperature on protein structure, such that lower temperatures lead protein compaction, due to subtle repacking of the molecule, resulting in increased protein stability (Tilton *et al.*, 1993; Jaenicke, 1990)

3.2.2.2. Stability of cold dried plaques

To determine how well the dried plaques would retain their viability during storage, two sets of five individual cold dried plaques were produced with one set being stored at 25 °C and the other being stored at 4 °C for 8 weeks. After the storage time, the plaques were rehydrated, and the number of phage released determined as above. The plaques stored at 4 °C produced an average phage titre of 2.2×10^8 pfu ml⁻¹, only marginally lower than the 2.7×10^8 pfu ml⁻¹ recorded for freshly dried plaques indicating very little loss in phage titre during storage. The plaques stored at 25 °C however did not produce any plaques at any of the dilutions tested indicating near complete loss in phage viability. To determine how quickly the plaques lost viability at 25 °C, further sets of cold dried plaques were prepared and stored at 25 °C for 48 h, 7 days, 2 weeks or one month before the phage were titred. Surprisingly, even after only 48 h of storage at 25 °C the phage titre in all samples of cold dried plaques had dropped to levels of less than 10^1 pfu per ml suggesting that the conditions within these cold dried samples were not suitable to produce a reagent that could be stored at room temperature.

Phage are notoriously difficult to dry due to their fragile structure and usually require added excipients such as sugars or proteins to stabilize phage capsid and tail proteins during drying (Prestrelski *et al.*, 1993; Merabishvili *et al.*, 2013; Wdowiak *et al.*, 2022). However, adding sugars or proteins directly to bacterial lawns was seen as a suboptimal solution due to the risk of bacteria utilizing the added compounds for their growth and thus potentially reducing their stabilizing effect as well as making it hard to precisely determine excipient concentration in any given plaque. It was therefore decided to attempt solidifying liquid phage suspension using agar and creating “artificial plaques” which would provide the cryoprotection seen when phage were embedded in agar, but to which excipients could be added to the molten agar before adding to the phage suspension.

Artificial plaques were produced without excipients as described in section 2.3.9. and the number of phage that could be recovered immediately after the artificial plaques had been prepared was determined. To do this, phage were eluted from the samples in 100 μl of lambda buffer. The freshly made artificial plaques ($n=5$) produced an average titre of 2.20×10^8 pfu ml^{-1} which was $\sim 1 \log_{10}$ lower than the titre achieved from natural plaques ($\sim 1 \times 10^9$ pfu ml^{-1}) and was consistent with the levels recorded when producing artificial plaques in in section 3.2.1.3 but was high enough to warrant further investigation. Five artificial plaques were then cold dried at 4°C as described in section 3.2.2.1. and then the phage recovered from the plaques and titred as before. However, when the control samples were tested, it was found that the artificial plaques did not stabilise phage titre during cold drying and less than 10^1 pfu could be recovered from each plaque once dried. This result was unexpected as it was previously found that natural plaques dried at 4°C would retain most of their phage titre. One possible explanation for this observation was that natural plaques harvested from bacterial lawn contain a wide range of proteins, lipids and lipopolysaccharides as well as DNA released by the cells during growth and later lysis (Turnbull *et al.*, 2016; Shehadul Islam *et al.*, 2017). These compounds may act as natural excipients aiding the phage particles retain shape and stability during drying (Wdowiak *et al.*, 2022). Proteins have been previously described to aide phage survival during drying for example when Vonasek *et al.* (2013) used whey protein to stabilize phage T4 during storage when used in an antimicrobial packaging. Prestrelski *et al.* (1993) also found significant success in stabilizing freeze dried proteins using sugar based excipients such as sucrose, glucose, mannose, and others. It was thus decided to attempt drying phage in artificial plaques with the addition of different excipients to improve phage survival.

3.2.2.3. Use of excipients to improve phage survival in artificial plaques

3.2.2.3.1. Trehalose

Large protein complexes, like bacteriophage, are notoriously difficult to preserve in a dry form due to their molecular complexity and tendency to misfold when water availability is reduced (Prestrelski *et al.*, 1993; Vandenheuvel *et al.*, 2014). This misfolding often leads to the individual protein no longer being able to function properly as part of the complex, and in this case, this would cause the phage to become non-infective. Excipients such as glycerol, sucrose or trehalose are commonly employed when trying to preserve biological material, for example during freeze-drying, due to their ability to provide structural support to protein molecules and “mimicking” the presence of water (Prestrelski *et al.*, 1993; Vandenheuvel *et al.*, 2014). These excipients could not be used in the drying of plaques experiments earlier since these natural plaques were harvested directly from a bacterial lawn and the host bacteria would consume sugars such as trehalose and sucrose for their own growth thus potentially reducing the stabilizing effect as well as introducing an unknown into the equation since assessing how much of the excipient in any given plaque had been lost to bacterial growth. By preparing artificial plaques, excipients could be added directly to the agar used for making the artificial plaques.

The first compound to be investigated was trehalose which was chosen due to it being a commonly employed sugar based excipient, its availability and its known ability to enhance bacteriophage survival during freeze-drying (Merabashvili *et al.*, 2013). To do this 5 µl of high titre phage D29 suspension was solidified by adding 5 µl of Middlebrook 7H10 agar containing 8% trehalose (w/v) bringing the final concentration of trehalose in the artificial plaque to 4% (w/v). These artificial plaques were then cold dried as described before and when fully dried they were rehydrated in 100 µl of lambda buffer and the number of phage released from the sample determined by titration. In this case the average phage titre (n=5) was 2.54×10^7 pfu ml⁻¹ (SD Log₁₀ ± 0.03) marking a vast improvement in retained phage viability compared to the

results gained when the artificial plaques dried without excipients. This result lent credibility to the hypothesis that the reason for the high number of active phage in dried natural plaques is due to some unknown compound in the natural plaque acting as an excipient that is missing in artificial plaques.

To determine the impact of increased or decreased trehalose concentration on phage survival in dried artificial plaques artificial plaques were produced containing varying final concentrations of trehalose (2%, 4% and 6% (w/v)). Sets of these (n=2) were then cold dried and the number of surviving phage determined as before. The results produced average titres (n=2) of, 9.0×10^7 , 2.2×10^8 and 2.7×10^8 pfu ml⁻¹ respectively indicating that an increase in trehalose concentration could, albeit not significantly, further increase phage survival in dried plaques (see table 3.3).

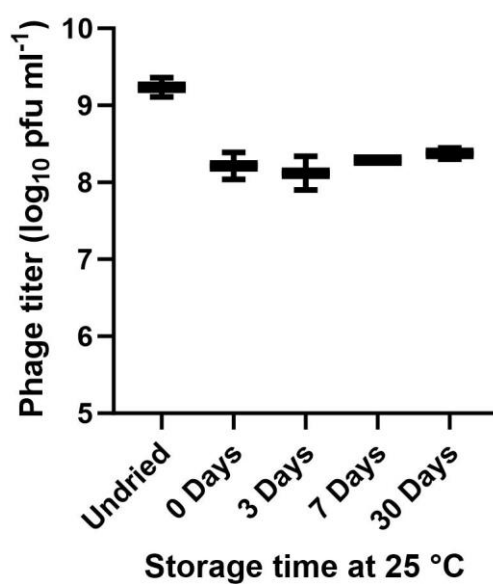
Table 3.3. Effect of varying trehalose concentration on phage survival

FINAL CONCENTRATION OF TREHALOSE (W/V)	TITRE OF PHAGE ELUTED FROM SAMPLES (PFU ML⁻¹)
2%	9.0 x 10⁷
4%	2.2 x 10⁸
6%	2.7 x 10⁸

Each result represents the average of 2 samples tested.

Next it was decided to determine stability of cold dried artificial plaques containing trehalose at 25 °C for up to 30 days. Artificial plaques were produced using nutrient agar as described in section 2.3.9 containing 10% trehalose (w/v) before being dried at 4 °C. 10% Trehalose was chosen due to Merabishvili *et al.* (2013) as well as Ly *et al.* (2019) reporting good results with similar concentrations when freeze drying phage ISP and D29 respectively. Before drying, phage were eluted from artificial plaques (n=2) and produced an average phage titre of 1.8×10^9 pfu ml⁻¹. After drying completely for 5 days, the average number of phage recovered dropped to 1.78×10^8 pfu ml⁻¹ (n=2) indicating a 1 log₁₀ loss of viable phage as a result of the drying process. The remaining plaques (n=2 for each sampling point) were transferred to a 25 °C incubator and stored for 3, 7 and 30 days before being rehydrated in 100 µl of lambda buffer and the number of viable phage remaining in the sample was determined. In this experiment dried artificial plaques containing trehalose were shown to be able to maintain a stable phage titre $>1.0 \times 10^8$ pfu ml⁻¹ for up to 30 days (see fig. 3.6). These results indicate that the addition of trehalose is of considerable benefit to the survival of phage throughout the drying process and also during storage at room temperature for prolonged periods of time. These results are also in agreement with those reported in the literature where trehalose has been widely used to stabilize dried phage preparations. For example, Ly *et al.* (2019) successfully used trehalose as an excipient for spray freeze drying of phage D29 while Zhang *et al.* (2020) used a mixture of trehalose and either PBS or SM buffer to thin film freeze dry phage T7 and limit loss in viability to $< 2 \log_{10}$ pfu ml⁻¹.

Figure 3.6. Stability of dried artificial plaques containing 10% trehalose at room temperature



Artificial plaques containing 10% w/v trehalose were cold dried and stored at room temperature. Plaques were titred before drying, immediately after drying and after 3, 7 and 30 days of 25° C storage. Undried artificial plaques produced 1.8×10^9 pfu ml⁻¹ while dried plaques retained a titre of $> 1.0 \times 10^8$ pfu ml⁻¹ for up to 30 days of storage (n = 2).

3.2.2.3.2. Glycerol

Since the addition of more trehalose only yielded a relatively small increase in the number of retained bacteriophage, it was decided to investigate whether the addition of a further excipient would enhance phage survival during drying. Glycerol was chosen as the next excipient to test due to its availability, cost and wide use as an excipient and cryoprotectant especially in freeze-drying of sensitive biological material (Starciuc *et al.*, 2017; Horn *et al.*, 2018). In order to gain an understanding if, and at what concentration, glycerol could enhance phage survival during drying of plaques it was decided to produce artificial plaques containing a final concentration of glycerol of 5%, 10% or 15% (w/v). In addition, artificial plaques containing a final concentration of 6% trehalose (w/v) were also prepared as this concentration was found to produce the highest level of phage recovery in the previous experiments (Table 3.4). Sterile glycerol was aseptically added to the molten nutrient agar and mixed well before being used to create the artificial plaques. Once solidified the artificial plaques were cold dried at 4 °C and subsequently rehydrated and the phage released from duplicate samples was determined. The results are shown in Table 3.4 and it is clear that there is a reduction in phage titre recovered from the dried artificial plaques compared to the results gained when 6% (w/v) trehalose was used. This was not surprising since glycerol's cryoprotective properties are mainly derived from its ability to reduce and delay ice crystal formation during freezing (Zhang *et al.*, 2022) and this was unlikely to happen when drying was achieved above 0 °C. However, Zhang *et al.* (2022) also reported that glycerol had the ability to mitigate osmotic stresses when used for the stabilization of eukaryotic cells and osmotic stress is known to be damaging to phage structure (Cordova *et al.*, 2003). However, no evidence of protection was found when including glycerol in the matrix when drying phage, suggesting that osmotic stress is not a critical factor during the drying process.

Table 3.4. Effect of varying glycerol concentration on phage survival

FINAL CONCENTRATION OF CRYOPROTECTANT (W/V)	TITRE OF PHAGE ELUTED FROM SAMPLES (PFU ML⁻¹)
5% GLYCEROL	4.0 x 10⁶
10% GLYCEROL	2.5 x 10⁶
15% GLYCEROL	2.0 x 10⁶

Each result represents the average of 2 samples tested.

3.2.2.3.3. Gelatine

Since glycerol did not improve phage survival during cold drying of artificial plaques it was decided to attempt using gelatine as an excipient when cold drying artificial plaques again due to its availability, low cost and known cryoprotective properties (Karnjanapratum and Benjakul, 2014). Additionally, gelatine is a protein and an increased survival of phage in dried artificial plaques containing gelatine would lend additional support to the theory of unknown (possibly protein) components present in natural plaques but absent in artificial plaques which act as an excipient. Albeit we found in previous experiments that gelatine does not aide phage survival during freezing (section 3.2.1.3) it was decided to investigate whether it would improve phage survival during drying. To this end, artificial plaques were produced containing a final concentration (w/v) of 6% trehalose and either 1%, 5% or 10% gelatine by adding appropriate amounts of a 40% gelatine solution to nutrient agar before autoclaving. The gelatine containing agars were then fortified with 12% (w/v) trehalose to reach a final concentration of 6% (w/v) in the artificial plaque. These artificial plaques were again cold dried until visibly dry and rehydrated in 100 µl of lambda buffer before the number of phage released from the sample was determined. The results gained are shown in Table 3.5 and indicate that the addition of gelatine does not improve phage survival during drying in artificial plaques and that phage survival is in fact hampered by the addition of the gelatine.

Table 3.5. Effect of varying gelatine concentration on phage survival

FINAL CONCENTRATION OF CRYOPROTECTANT (W/V)	TITRE OF PHAGE ELUTED FROM SAMPLES (PFU ML⁻¹)
1% GELATINE	1.15 x 10³
5% GELATINE	2.21 x 10⁵
10% GELATINE	3.05 x 10⁵

Each result represents the average of 2 samples tested.

These results corroborate the observations made in section 3.2.1.3 indicating that gelatine lacks the phage stabilizing properties found in carbohydrates such as trehalose or agar. Despite these findings, since this data was generated in 2017 research by other groups has produced significant evidence suggesting gelatine does improve phage stability during drying. For example, Manohar and Ramesh (2019) found that a 1% solution of gelatine could be used as the sole excipient when freeze drying three different phage (ECP311, KPP235, ELP140) and produce titres $> 5 \times 10^7$ pfu ml⁻¹ after lyophilization. However, they also found that increasing gelatine concentration to 2 % (w/v) would result in a lower rate of phage survival in all three phage studied when compared to the results gained using a concentration of only using 1 %. In our experiments gelatine was used at final concentrations of 5 and 10% (w/v) which might have been the wrong concentration for the cryoprotecting properties of gelatine to be noticeable. Although freeze drying and the cold drying method employed here are different processes that impose different stresses upon phage particles it is an interesting observation that is supported by findings of Tovkach *et al.* (2012) who observed that a gelatine concentration of 600-1000 µg ml⁻¹ (= 0.6-1 % (w/v)) was the ideal range for improving phage survival at 57 °C while concentrations above or below that would result in rapid reduction of phage survival. This indicates that gelatine concentration is an important factor to phage survival at a wide range of temperatures.

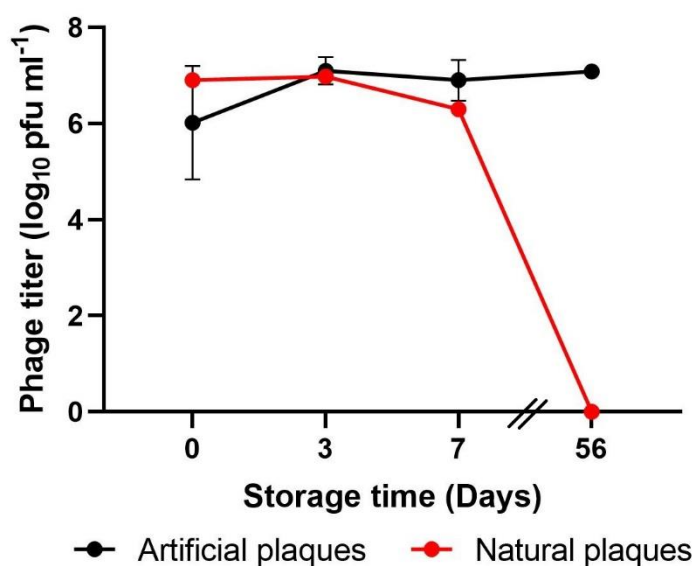
3.2.2.4. Freeze-drying of plaques and artificial plaques

Cold drying of plaques and artificial plaques produced mixed results when initially used for the long-term preservation of mycobacteriophage D29. Cold dried natural plaques would retain most of their phage viability throughout the drying process but would lose viability very quickly when stored at room temperature. Cold dried natural plaques on the contrary cannot be dried without the addition of excipients like trehalose but are relatively stable at room temperature for many weeks (See 3.2.2.2). It was decided to determine whether freeze-drying natural and artificial plaques would produce different

results to cold dried plaques and artificial plaques produced in section 3.2.2. In order to test this, individual plaques were picked and transferred into a freeze-drying vial with a stopper. Artificial plaques were produced as described in materials and methods 2.3.9 using high titre phage suspension and nutrient agar. Trehalose was added to the artificial plaques at a final concentration of 6% (w/v). These artificial plaques were then transferred into individual freeze-drying vials with stoppers and freeze dried as described in materials and methods section 2.3.10. Once freeze-dried, the plaques and artificial plaques were stored at ambient temperature (20-23 °C) before being rehydrated in 100 µl of lambda buffer and titred using the Miles and Misra technique (see 2.3.11.). Freeze-dried plaques and artificial plaques were rehydrated and titred (n=2). Titration was performed immediately after freeze-drying, 3 days of storage, 7 days of storage and 8 weeks of storage at ambient temperatures. It was found that the natural and artificial plaques titred immediately after freeze-drying yielded average titres of 8.0×10^6 and 3.58×10^6 pfu ml⁻¹ respectively. When titred after 3 days of storage, the average titres for freeze-dried natural and artificial plaques were found to be 9.5×10^6 and 1.4×10^7 pfu ml⁻¹ respectively while 7 days of storage produced average titres of 2.0×10^6 and 1.0×10^7 pfu ml⁻¹ respectively. After 8 weeks of storage at room temperature the average titre of freeze-dried natural plaques had decreased so significantly that no phage were detectable at a 10^{-3} dilution for the natural plaques while freeze-dried artificial plaques containing trehalose produced an average titre of 1.2×10^7 pfu ml⁻¹. These results are visualized in figure 3.7. These results are very similar to those obtained in section 3.2.2.2 in that dried natural plaques would lose their viability over time while dried plaques containing trehalose as an excipient would retain a stable phage titre for prolonged periods of time. In earlier experiments on drying plaques however it was noted that cold dried natural plaques would lose most of their viability within 48h of being moved to 25 °C while freeze dried natural plaques would retain phage titres above 10^6 pfu ml⁻¹ for at least 7 days. This improvement in phage survival was attributed to freeze drying being a much “gentler” way of drying biological material. Freeze drying rapidly

cools the sample to below the triple point of water where water will sublime out of a sample as a gas and leave biological structures intact while during cold drying water is simply evaporated and sequestered from the sample. This causes profound changes to the structure and the proteins involved (Tilton *et al.*, 1993; Prestrelski *et al.*, 1993) and results in significant kinetic stresses and conformational changes not only to proteins but also to the surrounding agar polymer matrix. Indeed, cracking and changes in polymer arrangement are a common problem when drying aqueous gels like agar (Mahiuddin *et al.*, 2018) and has the potential to damage phage particles. Similarly, trehalose was found to considerably improve phage survival both in cold dried and freeze dried plaques which is not surprising since trehalose is a known stabilizing agent able to confer structural support to dried proteins by mimicking the presence of water (Vandenheuvel *et al.*, 2014) while also allowing the proteins a degree of flexibility to reshape (Prestrelski *et al.*, 1993) and still retain functionality.

Figure 3.7. Comparing phage viability in freeze-dried natural and artificial plaques during room temperature storage



Natural plaques not containing excipient and artificial plaques containing 6% w/v trehalose were freeze-dried and stored at room temperature for up to 8 weeks. Both sets of plaques were found to contain $> 1 \times 10^6$ pfu ml⁻¹ after freeze-drying but the natural plaques lost viability rapidly during storage while artificial plaques maintained most of their viability.

3.3. Discussion

3.3.1. Freezing of plaques

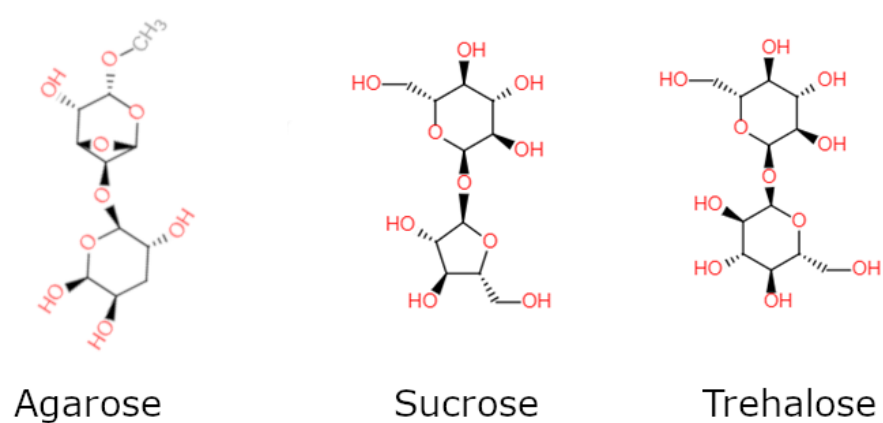
Embedding bacteriophage in agar resulted in great recovery of viable particles after freezing at -20 °C compared to phage in suspension. The main cause of damage to the bacteriophage during freezing is the crystallisation of the water molecules and the increase in volume damaging and destroying the phage capsid and or tail proteins - rendering the phage non-infective (Vandenheuvel *et al.*, 2014). As previously mentioned, the complexity of the tail apparatus of bacteriophage has been suggested as one factor determining the survival of phage during freezing. *Myoviridae* such as phage Felix used here have complex tail fibres (Calendar, 2006) and are thus expected to suffer the most from freezing stress. However, it was found that all phage tested, including the Myovirus (phage Felix), were able to survive freezing without resulting in a significant drop in phage viability when embedded in agar.

In studies of phage preservation, different approaches have been made to add stabilizing agents to the phage to reduce or neutralize the effect of the ice crystallization on the phage particles or to reduce crystal formation. Glycerol based stabilizing agents have been evaluated in the past (Clark, 1962; Ackermann *et al.*, 2004), not only for freezing but also for freeze drying and for long term storage at 4 °C. As has been shown with the freezing of artificial plaques, and the use of nutrient agar for non-mycobacteriophage, the cryopreservation effect seen was not due to oleic acid or glycerol which are included in the 7H10 agar. In earlier studies disaccharide and polysaccharide sugars, such as sucrose, trehalose and mannitol, have been shown to protect phage particles from damage during freeze drying (Merabishvili *et al.*, 2013; Vandenheuvel *et al.*, 2014). This effect was attributed to these sugars giving the capsid and other structural proteins of the phage structural support and imitating the presence of water. Agar polysaccharide polymers are made up of galactose subunits and therefore its structure is not dissimilar to sucrose and trehalose molecules (Fig. 3.8). It is hypothesized that the molecules of the

agar perform a similar role when a plaque is frozen. This is supported by observation that gelatine which consists of polypeptides but no saccharides (Rawdkuen *et al.*, 2013) did not confer any cryoprotective properties to the phage during freezing.

While this method works well with natural plaques extracted from phage isolation or titre plates, it must be acknowledged that these samples will always also contain viable host bacteria which may survive freezing and can potentially then be transferred into any culture derived from the plaque when the phage are eluted from it after storage. However, the production of artificial plaques for the storage of bacteriophage isolates provides a simple method for the freezing of purified phage stocks so that the frozen samples are not contaminated with host bacteria. “Artificial” plaques can simply be produced by eluting phage from a primary plaque, filtering to remove any bacteria and then solidifying a purified phage suspension.

Figure 3.8. Schematic diagram comparing the molecular structure of Agarose, Sucrose and Trehalose



2- dimensional image of an agar polymer molecule and molecules of Sucrose and Trehalose. Note the structural similarity to Sucrose and Trehalose.

Adapted from Li et al., 2024.

Although freezing of plaques does not provide a suitable solution for the storage of bacteriophage in the context of the FastPlaqueTB and Actiphage assays, due to requiring constant cooling, it provides a viable very low cost solution for the long term storage of bacteriophage in a laboratory setting. Due to the rising interest in bacteriophage caused by the challenge of antibiotic and disinfectant resistance in bacteria, strain and isolate collections have increased significantly over the last decade (Calendar, 2006). Maintaining genetically stable and viable stocks of bacteriophage for a prolonged period of time has been a challenge, especially for strain and culture collections which need to maintain stable stocks of organisms without relying on repeated sub-culture like strain banks and culture collections. Freezing of plaques could prove to be a useful tool for storing large numbers of phage isolates for long periods of time without the risk of contaminants growing or the phage culture losing viability over time.

3.3.2. Drying of plaques

It was discovered that simply picking and drying natural plaques is not a suitable option for storage of bacteriophage. Although plaques dried at 4 °C were found to contain $>10^8$ pfu ml⁻¹ and maintain that titre for weeks when stored at refrigeration conditions, they would lose most or all of their viability within a few hours of being stored at room temperature. The exact mechanism of how this sudden loss in phage viability occurs are still unclear however one possible explanation is that it is simply the result of protein compaction and/or misfolding due to changes in temperature as described by Jaenicke (1990) or Dong *et al.* (2018) with Dong *et al.* finding in a study on malate dehydrogenases of marine molluscs that proteins, especially enzymes, require a large degree of structural flexibility to work properly at a variety of different temperatures. This protein structural flexibility is however highly dependent on the presence of water (Prestrelski *et al.*, 1993) which is problematic in the context of a dried protein preparation such as dried bacteriophage. It seems therefore likely that the sudden loss in phage viability

encountered when storing cold dried plaques at room temperature is due to protein misfolding or even breaking of phage proteins when cold dried plaques are moved from a 4 °C environment to a > 20 °C room temperature environment and the phage proteins don't have the flexibility necessary to conform to a new shape and retain their functionality due to the absence of water. When producing and drying artificial plaques it was found that trehalose would significantly improve phage stability within the dried plaque. Indeed, dried artificial plaques containing 10% w/v trehalose and stored at room temperature would retain a viability of $> 1 \times 10^8$ pfu ml⁻¹ for at least 30 days. Prestrelski *et al.* also observed in their experiments on freeze drying different proteins including IFN- γ , Casein, bFGF and α Lactalbumin that the addition of excipients such as sucrose, glucose, mannose, mannitol and others could significantly improve protein flexibility in dried preparations while Vandenheuvel *et al.* (2014) found trehalose to exert structural support to phage proteins in a dry state by mimicking the presence of water. Other excipients used for the production of artificial plaques did not produce results as good as the trehalose with artificial plaques containing gelatine only being able to retain $\sim 10^5$ pfu ml⁻¹. However, as discussed in section 3.2.2.3.3 gelatine appears to require a "sweet spot" concentration of around 1% w/v to have a noticeable positive effect on the stability of bacteriophage preparations (Tovkach *et al.*, 2012; Manohar and Ramesh, 2019) and the concentrations used in our experiments were 6 – 10 times higher than the optimal reported in the literature. Glycerol, which is frequently used as an excipient for frozen storage of bacteria and phage (Clark, 1962; Wdowiak *et al.*, 2022) was not found to offer significant protection to phage during drying. This was not surprising since glycerol's cryoprotective properties are mainly derived from its ability to reduce and delay ice crystal formation during freezing (Zhang *et al.*, 2022) which didn't happen here since all drying was done > 0 °C. However, Zhang *et al.* also reported that glycerol had the ability to mitigate osmotic stresses when used for the stabilization of eukaryotic cells. However, no evidence of this could be found when including glycerol in the matrix when drying phage.

In terms of real world applicability dried artificial plaques could find use as a ultra-low tech and low cost method for storing bacteriophage in a no infrastructure environment where neither continuous refrigeration or freezing can be guaranteed and where use of a freeze driers are not possible. Possible fields of application that come to mind include field work in remote areas or strain libraries in countries or regions where the continuous power supply needed to run fridges and freezers is a challenge. Further research needs to be done on this method before it can be used reliably. This further research must first and foremost establish that solidifying phage suspension with agar and subsequently drying it in the presence of excipients works for many different phage and not just phage D29. Additional work should also be carried out on long term stability of dried artificial plaques past the 30 day period as well as the potential of replacing trehalose with cheaper and more accessible sugar based excipients such as sucrose or glucose that have previously been demonstrated to possess phage stabilizing properties (Prestrelski *et al.*, 1993; Merabishvili *et al.*, 2013; Zhang *et al.*, 2020). However, drying of artificial plaques was not seen as a suitable method for producing a dry and stable form of bacteriophage D29 to be included in the phage based detection assays produced by PBD Biotech and it was decided to redirect research effort towards freeze drying. This was mainly due to the time and labour intensive production method required for making and drying artificial plaques as well as the general ease of use, ease of quality control and shortened production time that freeze drying can offer in addition to it being a tried and tested method for producing dry stable phage preparations.

3.4. Conclusion

From the work described in this chapter it can be concluded that bacteriophage frozen in agar survived freezing in significantly higher numbers than bacteriophage frozen in liquid suspension. This makes freezing of plaques an easy cost-effective way of storing large numbers of phage isolates. It is not expected that there will be any further drop in phage titre after the initial drop seen during freezing since all structural changes, including water

crystallization have already occurred, and any enzymatic activity, such as protease or nuclease activity, will be inhibited in samples stored at -20°C (Sambu, 2015). The cryo-preservative properties of agar are likely derived from its molecular similarity to sucrose and trehalose which are known to act as stabilizing agents during freezing and drying of phage. This makes the method very attractive for programs that wish to store large number of new phage isolates before they are characterised for applications such as phage therapy. Since there is no need for continuous propagation to maintain stocks, the risk of introducing mutations to the laboratory phage isolate is greatly reduced. In addition, there is no risk of bacterial or fungal contaminants growing in and degrading the sample. The presence of viable host bacteria in the sample can be avoided by producing artificial plaques by using a pre-purified phage suspension. It must be noted that continuous freezing and thawing could prove detrimental to phage viability (Rivers, 1926) and should be avoided, and therefore it would be recommended that multiple samples are stored, so that individual tubes can be thawed out for initiating new growth of a phage. However frozen plaques require continuous refrigeration and are therefore not a suitable replacement for liquid phage suspension in the FastPlaque and Actiphage assay kits.

Producing and drying of artificial plaques containing trehalose as an excipient has the potential to be an effective ultra-low tech and low cost method for storing bacteriophage in a dry form that doesn't require an expensive freeze drying unit. However, the process was found to be too time and labour intensive to be an effective method of producing a dry and stable form of bacteriophage D29 for use in phage based detection kits. It was thus decided to redirect efforts towards developing a freeze dried preparation of bacteriophage D29 for use in the assay kits.

Chapter 4

Freeze drying of Bacteriophage D29

4.1. Introduction

The Actiphage® Rapid Blood assay, described in more detail in section 1.4.4, has great potential for improving the diagnosis of a range of mycobacterial diseases which are a recognised threat around the world, whether as a clinically important human pathogen (TB) in both developed and developing countries or as an economically relevant pathogen in animals (Johne's disease) in the developed countries with the associated issues of zoonotic transmission (Pai *et al.*, 2016; Ghielmetti and Giger, 2020).

For the Actiphage assay to be produced as a commercial kit, mycobacteriophage D29 needs to be provided as a stable, active phage reagent. One of the main challenges associated with making phage-based diagnostics, or phage-based therapeutics, more widely available is that the most common, and most readily available, form of bacteriophage that can be produced is a liquid lysate. These are not ideal for incorporating into commercially produced test kits for a variety of reasons. First of all, liquid phage suspensions require chilled storage since phage lysate tends to lose viability at room temperature making a cold chain an essential consideration for sale and deployment of products containing bacteriophage in liquid form (Gorski *et al.*, 2019). Additionally, storage properties of phage at 4 °C appear to be highly variable between different phage, with virions of some phage maintaining viability for years as liquid suspension (Lobocka *et al.*, 2018) while others rapidly lose titre. For instance, Gonzalez-Menedez *et al.* (2018) describe how different phage specific for *Staphylococcus* (phiPLA88, phiPLA35, phiPLA-RODI and phiPLA-C1C) would lose between 1 and 3 log₁₀ in phage titre levels over a period of up to 24 months when phage virions were stored as liquid suspension at 4 °C. Furthermore, liquid stocks are prone to contamination by bacteria or fungi, potentially leading to loss of samples due to protease production by the contaminants, and the only way to reduce the risk of this occurring is by storing the samples at low temperature, again requiring the use of a cold chain for a commercial product.

Pathogens such as *M. tuberculosis*, MAP and *M. bovis* are a significant economic and public health concern worldwide but especially so in developing countries of sub-Saharan Africa, southeast Asia and Latin America. These regions are characterized as having warm and possibly humid climates while also frequently lacking transport and electricity infrastructure to maintain constant refrigeration of assay kits. Thus, the requirement for constant temperature control for storage of liquid phage suspensions was a significant limitation on the potential impact of the commercial development of the Actiphage assay in the global fight against mycobacterial pathogens.

The issues around using simple freezing of phage have been discussed in Chapter 3, and clearly this is also not a viable option for producing samples of preserved phage for inclusion in a commercial kit, especially as this would then require storage of kit components at -20°C , which is also not desirable. However bacteriophage intended for long term storage are commonly freeze-dried, and these products can often be stored at room temperature. However, this process can also be accompanied by substantial loss in phage titre due to exposing the phage particles to both, freezing and drying stress (Clark and Geary, 1973). Again, loss of viability during lyophilization and subsequent storage appears to be highly variable and dependent on the specific phage being used, with reports ranging from close to no loss in viability after years of storage (Merabishvili *et al.*, 2013; Gonzalez-Menedez *et al.*, 2018) to almost complete loss in viability during the lyophilization process (Croyle *et al.*, 2001; Merabishvili *et al.*, 2013). It has also been noted that freeze-dried phage also tend to lose viability over time (Ackermann *et al.*, 2004), indicating that the phage are not in a completely stable state in the freeze dried product. Different attempts have been made in the past to add stabilizing agents (excipients) to the phage suspension to protect the phage from damage. Mono- and polysaccharides, such as trehalose, mannitol and sucrose and glycerol and biodegradable polyester microspheres have all been evaluated as potential excipients to protect phage during the freeze-drying process

(Puapermpoonsiri *et al.*, 2009), with carbohydrate-based excipients such as trehalose and sucrose producing the most promising results (Merabishvili *et al.*, 2013). Carbohydrate excipients such as sucrose and trehalose are thought to stabilize phage during drying by binding to phage proteins and providing structural support thus preventing misshaping and allowing the protein to remain functional (Golovina *et al.*, 2009; Vandenheuvel *et al.*, 2014).

Up to this point in the project, phage had been produced by the standard double layer agar phage propagation method which involves adding a dilute phage suspension to a lawn of the host strain to produce a semi-confluent lysis plate (Kropinski *et al.*, 2009). The progeny phage resulting from this phage infection and amplification event are harvested by scraping off the soft agar layer and eluting the phage from the mixture into a buffer (e.g. lambda buffer). This process is very labour intensive and the efficiency of phage particle recovery can vary (Fortier and Moineau, 2009) because there are losses of phage at various points during the process required to remove the agar and residual bacteria from the resulting phage suspension.

Another approach is to prepare high titre phage stocks in a liquid lysate by adding phage at a low m.o.i. (Multiplicity of Infection) to an actively growing culture and then allowing multiple rounds of infection to occur before the culture is totally lysed. Although simpler as a methodology, not all phage will propagate well like this and D29 in particular does not produce a very high burst size when grown in liquid cultures, probably due to the need for the host strains to be in a particular physiological state for productive infection (Swift *et al.*, 2014). Hence, before work could begin on developing a method to freeze dry phage D29, a simple and reproducible method for the production of large quantities (> 10 ml) of liquid phage D29 suspension with a consistent high titre of $> 1 \times 10^9$ pfu ml⁻¹ was required.

4.1.1. Aims and objectives.

The ability to produce dried preparations of bacteriophage that were stable at higher temperatures would solve many of these practical considerations and make shipping, storage and deployment of the Actiphage phage assay kits for the detection of mycobacteria easier and cheaper. Therefore, the objectives of this work was to produce a bacteriophage preparation that a) was stable at room temperature for at least 6 months and, b) had a phage titre larger than 1×10^8 pfu ml⁻¹ after drying. It was also important to confirm that phage stabilised in this way were able to infect Mycobacterial cells as efficiently and specifically as a fresh, liquid preparation of bacteriophage D29 which had been used to develop the Actiphage assay and this would serve as a gold standard for determining whether a new phage preparation was met the acceptance criteria for the Actiphage kit reagents.

4.2. Results

4.2.1. Production of a high titre D29 phage suspension

4.2.1.1. Introduction

Both the one and the two-day phage-assays require a minimum threshold of bacteriophage in a sample (final concentration of approximately 1×10^8 pfu ml^{-1}) to ensure reliable detection and infection of the Mycobacteria present in a sample. This can be considered a moderately high titre easily achievable by eluting phage from plaques using the double layer method. However, since we want to produce a dried and (at room temperature) stable form of these phage, our initial titre needs to be significantly higher to accommodate for potential loss of phage viability during drying and storage. Ideally our phage suspension before drying should be $\sim 10^9$ - 10^{10} pfu ml^{-1} to accommodate a 1-2 $\log_{10}$ drop in phage titre during drying and storage. Industrial production processes for high titre phage lysate usually involve continuous culture in bioreactors (Garcia *et al.*, 2019). However, for this project, this was not an option due to the limited budget available and the prohibitive cost of the specialized equipment needed and the requirement for sophisticated growth protocols to ensure continuous and efficient operation of such a system. Other, more feasible, methods for the production of high titre phage lysate in a laboratory setting have been reported, such as the “Phage on tap” method described by Bonilla *et al.* (2016) which is based on producing semi confluent lysis plates followed by picking individual plaques before eluting individual plaques in 1 ml of media. Bonilla *et al.* reports that titres of 10^{10-11} could routinely be achieved but the purification process alone takes 2 days and requires extraction with organic solvents. This is not ideal for a process that would ultimately be suitable for the industrial production of phage and therefore a new method to produce the phage prior to freeze drying was required.

Plaque development and size is the result of progeny phage diffusing outwards from the site of the initial infection event thus initiating further

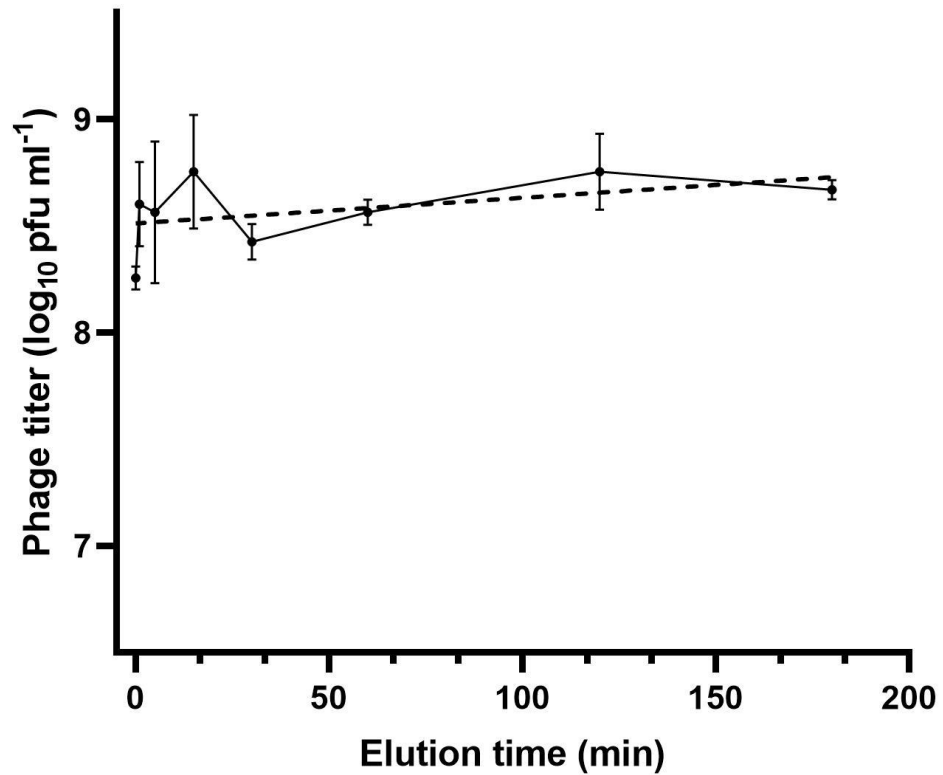
rounds of phage infection and host cell lysis (Abedon and Yin, 2009). As discussed in the previous chapter, the main component of a plaque is agar which is a carbohydrate polymer dissolved in water. The saturated agar solution is liquid at high temperature ($> 50\text{ }^{\circ}\text{C}$) and solidifies as it cools forming a water saturated aqueous gel (sometimes referred to as Hydrogel). Plaques represent localized areas containing large numbers of highly concentrated phage particles dispersed within the hydrogel matrix (Abedon and Yin, 2009; Gun'ko *et al.*, 2017). Plaque development is a very complex procedure as it is dependent on water content of the agar, dispersion/diffusion of the phage after lysis and growth and growth state of the bacterial host (Serwer and Hayes, 1982). Phage can be recovered from plaques by submerging the plaque in an aqueous solvent such as water, buffer, or media. One of the unique properties of hydrogels is that it forms a semi fluid environment with water present in a bound, semi bound, or free state allowing water, other solvents, and even large molecules (including phage) to migrate within and out of the agar matrix (Gun'ko *et al.*, 2017; Johnston *et al.*, 2020). Phage migration out of plaques when suspended in an aqueous solvent is the result of osmosis caused by the difference in phage concentration between the two environments. Osmotic diffusion of phage particles will occur until a concentration equilibrium between the hydrogel (plaque) and the surrounding liquid is reached (Jurczak-Kurek *et al.*, 2016). The point where phage concentration reaches an equilibrium and phage migration into the elution medium stops represents the maximum phage concentration achievable by simple diffusion elution of plaques. The time required for phage particles to migrate out of a plaque and into the surrounding liquid is undetermined and it is likely that agar concentration as well as size and morphology of the phage play a role. Elution times suggested in the literature vary greatly and range from 15 min (Bonilla *et al.*, 2016) to overnight (Basra *et al.*, 2014). Hence developing a method to produce large quantities of high titre phage lysate necessitated an investigation of basic mechanisms of phage elution from plaques, such as determining the time required for phage to migrate from an agar plaque into surrounding liquids, or

how changing the volume of elution medium used would impact phage concentration in the resulting suspension.

4.2.1.2. Initial experiment on the elution time of plaques

In order to optimize phage D29 production procedures, and to eliminate time consuming elution steps, it was decided to determine the time required for the phage titre of the elution medium to reach an equilibrium with the phage titre in the plaque. To do this, plaques were produced and picked as described in sections 2.3.2 and 2.3.3. Individual plaques (n=3 per test) were suspended in 100 µl of lambda buffer and incubated on the bench for < 10 sec (lambda buffer added and immediately removed again) and for a range of longer incubations times (1, 5, 15 and 30 min, 1, 2 and 3 h). After the incubation period, the lambda buffer was removed from contact with the plaque agar, and the number of phage released into the buffer was determined by titration. For all incubation times, the average concentration of phage recorded was (n=27) 2.83×10^8 (SD=0.19) with a lowest value of 1.3×10^8 (<1 min) and a highest value of 4.67×10^8 (15 min and 2 h; see Fig. 4.1). As can be seen, as incubation time increased, there was a general trend towards an increase in phage titre ($\sim 0.25 \log_{10}$ pfu ml⁻¹ over the course of the experiment) but after 1 min, the results fluctuated around the average value for all incubation times and did not significantly change as elution time was increased. The results obtained suggest that osmotic phage diffusion from the agar plaque into the surrounding liquid is a rapid, almost instant, with the process reaching a concentration equilibrium between phage in liquid suspension and phage remaining in the agar plaque (i.e. maximum obtainable phage titre in suspension) within 1-5 min of resuspension in the buffer.

Figure 4.1. Determining elution time of phage D29 plaques



Plaques picked using a 3 mm biopsy punch needle, to ensure uniform size, and then were eluted in 100 μ l lambda buffer for 0, 1, 5, 15, 30, 60, 120 and 180 min. The resulting phage suspension was titred and log₁₀ pfu ml⁻¹ achieved in each sample plotted against elution time. Values represent the average phage titre for triplicate samples; values recorded for each time of elution were: 1.3×10^8 (<1 min), 2.67×10^8 (1 min), 2.33×10^8 (5 min), 4.67×10^8 (15 min), 1.67×10^8 (30 min), 2.33×10^8 (1 h), 4.67×10^8 (2 h) and 3.0×10^8 (3 h) pfu ml⁻¹. Dotted line represents a line of regression with $r^2 = 0.38$.

4.2.1.3. Initial experiments to determine correlation between volume of elution medium and phage titre.

Since reaching high phage titres quickly will mean that the equilibrium point is also quickly established, a second factor to consider when trying to elute phage from the agar efficiently is the volume of the buffer used. To investigate this question, plaques were produced and picked as described in materials and methods (2.3.2 and 2.3.3). After picking, 10 plaques were immediately placed in individual 1.5 ml microfuge tubes and different volumes of lambda buffer (50, 100, 200, 500 and 1000 μl) were added to them ($n=2$). As we were able to previously show that phage migrate out of agar plaques into surrounding liquid rapidly and an equilibrium is reached within a few minutes (see 4.2.1.2), phage containing plaques were allowed to elute for 5 minutes before the resulting phage suspension was titred (section 2.3.11). It was found that the average titre of the phage suspensions produced by eluting one plaque in 100 μl , representing the standard method of obtaining phage lysate employed at the time, was $8.0 \times 10^8 \text{ pfu ml}^{-1}$ ($8.0 \times 10^7 \text{ pfu per plaque}$) which is what was expected. Plaques were then eluted in 50, 200, 500 or 1000 μl of lambda buffer and the phage suspensions were again titred after 5 min incubation. This produced titres of 1.4×10^9 , 8.0×10^8 , 6.5×10^7 , 2.1×10^7 and $2.6 \times 10^7 \text{ pfu ml}^{-1}$, respectively (see Table 4.1). The total number of phage recovered from each plaque was calculated by to be in the range of $10^7 \text{ pfu per plaque}$ (See Table 4.1. and Figure 4.2), which indicated that altering the volume of the elution medium added did not significantly change the total number of phage recovered from the individual plaque, but using a smaller elution buffer volume achieved a higher phage titre.

These results support the observations made by Gun'ko *et al.* (2017) and Johnston *et al.* (2020) that migration of phage out of plaques is the result of osmosis and will cease once an osmotic equilibrium has been established. Based on these results it was decided to use 100 μl of lambda buffer for the elution of phage from one plaque as it was deemed impractical to reduce the volume of elution medium to a level where a significant increase in phage

titre could be expected (i.e. the use of 10 µl would be predicted to increase the phage titre by 1 log₁₀). However, it was clear that in future experiments carried out using larger numbers of plaques and larger volumes of elution media, the media to plaque ratio could be altered to obtain higher concentration phage D29 suspension.

Table 4.1. Effect of volume on total number of phage eluted (n=2)

VOLUME OF BUFFER USED (μL)	PFU ML⁻¹ OF FINAL SUSPENSION *	TOTAL NUMBER OF PHAGE RECOVERED PER PLAQUE **
50	1.4×10^9	7.0×10^7
100	8.0×10^8	8.0×10^7
200	6.5×10^7	1.3×10^7
500	2.1×10^7	1.0×10^7
1000	2.6×10^7	2.6×10^7

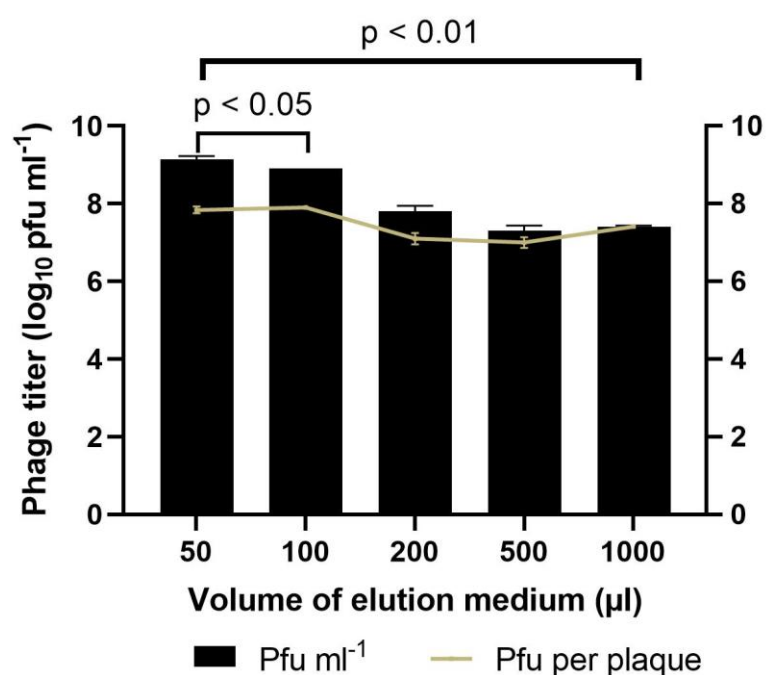
* Represents pfu ml⁻¹ calculated according to the formula:

$$\text{pfu ml}^{-1} = \frac{\text{average number of plaques per spot} \times \text{dilution factor}}{\text{volume plated in ml}}$$

** Represents the total number of phage particles recovered from the plaque, calculated by dividing pfu ml⁻¹ by number of times volume of buffer required to make 1 ml. For example 100 μl of buffer used = 10 (100 x 10 = 1000).

$$\text{pfu (total)} = \frac{8,0 \times 10^8}{10} = 8,0 \times 10^7$$

Figure 4.2. Determining the effect of volume of elution medium on final phage concentration



For data in both Table 4.1 and Figure 4.2, plaques were picked using a 3 mm biopsy needle and were uniform in size and shape. Suspensions were produced by eluting individual plaques in different volumes of lambda buffer and then the number of phage present in the sample was determined by titration. The data represents the geometric mean from duplicate sets of samples, n=2.

4.2.1.4. Production of a high titre phage suspension using semi confluent lysis plates (SCLP)

When a bacteriophage infects a cell in a bacterial lawn and releases progeny phage, these progeny phage will find and infect other suitable host bacteria in the vicinity forming a plaque which originated from the first infection event and acts as a dense concentration of progeny phage particles (Abedon and Yin, 2009). The enlargement of the plaque (and therefore the amplification of the phage) however is limited, and phage reproduction generally stops once the bacteria in the lawn have reached stationary phase and infection and phage reproduction can no longer occur (Serwer and Hayes, 1982).

To achieve a high titre phage preparation it was therefore deemed vital to use the available host cells in a bacterial lawn as efficiently as possible by adding enough phage particles to cause plaque formation on as much of the bacterial lawn as possible (i.e. semi-confluent lysis) while not adding too many phage that would overwhelm the bacterial population resulting in abortive infection and lysis of host cells without replication (Molineux, 2006). This latter effect, sometimes referred to as “lysis-from-without”, is usually the result of a large number of phage particles binding and simultaneously injecting DNA into the same host cell which can cause sufficient damage to the bacterial cell wall that premature cell lysis occurs. This results in the death of the host cell as well as the phage involved without causing productive infection and phage replication (Abedon, 2011).

In order to increase throughput while reducing time and labour involved in the production of high titre phage lysate, it was proposed to produce Semi Confluent lysis Plates (SCLPs) as described by Raya and H`bert (2009). SCLPs are bacterial lawn plates infected with ~500 phage particles such that after incubation individual plaques are barely distinguishable and only small areas of the lawn remain visible (termed “lacing” see Figure 4.3) therefore minimizing the number of bacterial cells not utilized for phage amplification. As described in section 4.2.1.2, migration of phage out of plaques occurs rapidly and, based on this result, a method was devised where instead of

mechanically picking individual plaques or flooding the agar surface with media as described by Bonilla *et al.* the whole agar plate would be cut into small (~5 x 5 mm) pieces before being eluted. Another difference to standard methodology was that, rather than preparing double layer agar plates, plates were prepared using a method developed for the FastPlaque assay (section 1.4.2), in which the cells and phage are diluted in 7H9 media supplemented with OADC (this is referred to as Media Plus or MP in the FastPlaque kits) and then mixed with molten agar. This approach was adopted because it was known that phage D29 forms large plaques in lawns of *M. smegmatis* using this method, which indicated that plaque growth was efficient and not being limited by the growth of the lawn. Accordingly, it was decided to use MP to elute the phage rather than lambda buffer as this media is the basis of the agar plates being made and would limit any difference in osmotic potential between the agar and the elution buffer than may have affected the rate of phage elution.

Five semi confluent lysis plates (SCLP) were prepared (see materials and methods 2.3.6) and after incubation and visual confirmation that >500 plaques were present the agar was cut into ~2.5 mm² (5 x 5 mm) pieces using a sterile scalpel. These agar cubes were then immersed in 7 ml of MP for 5 min. This time was chosen based on results obtained in section 4.2.1.2 and, based on results previously obtained (section 4.2.1.3). 7 ml of media was chosen as the elution volume as this was predicted to increase phage concentration in the final lysate 10-fold since one plate produced an estimated 700 plaques and one plaque would normally be eluted in 100 µl of liquid ($700 \times 100 \mu\text{l} = 70 \text{ ml} \div 10 = 7 \text{ ml}$). After the incubation time, the liquid was recovered using a pipette and the phage lysate was centrifuged at 3500 x g for 10 min at room temperature to remove bacterial cells, debris and agar fragments. The phage titre of the clarified phage lysates were then determined (section 2.3.11.) and it was found that they produced an average titre of 1.66×10^{10} pfu ml⁻¹ with a standard deviation < 0.1 log₁₀.

Figure 4.3. Image of Semi Confluent Lysis Plate.

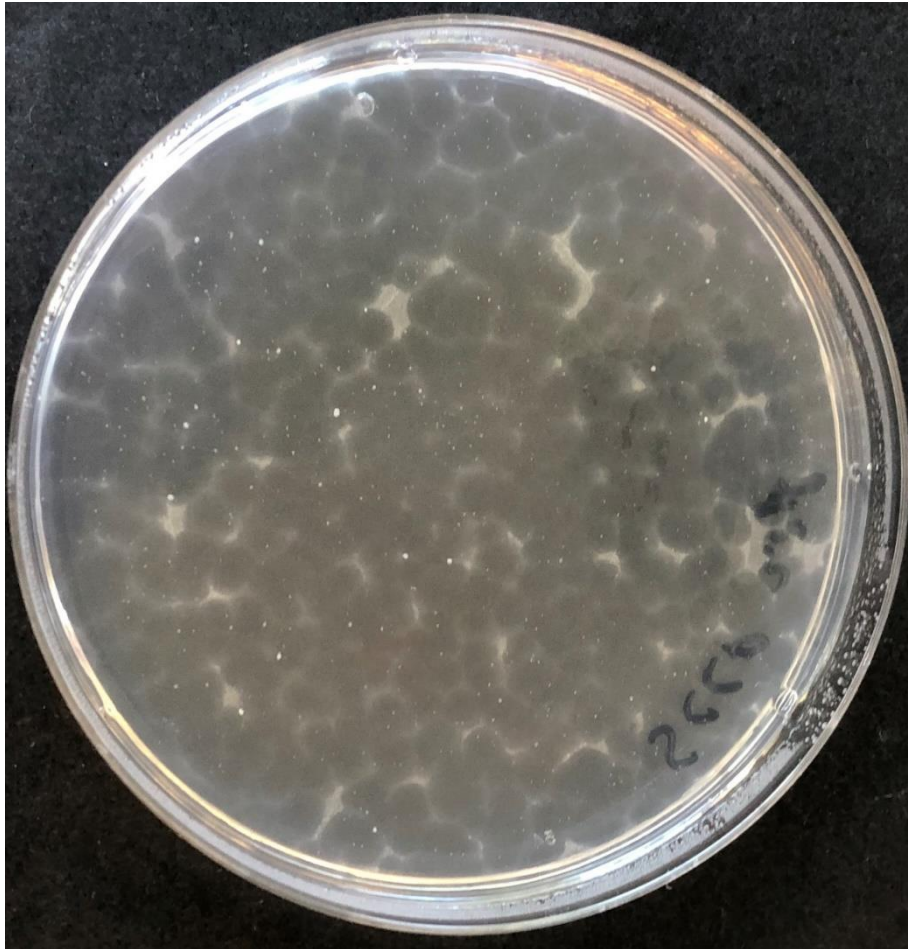
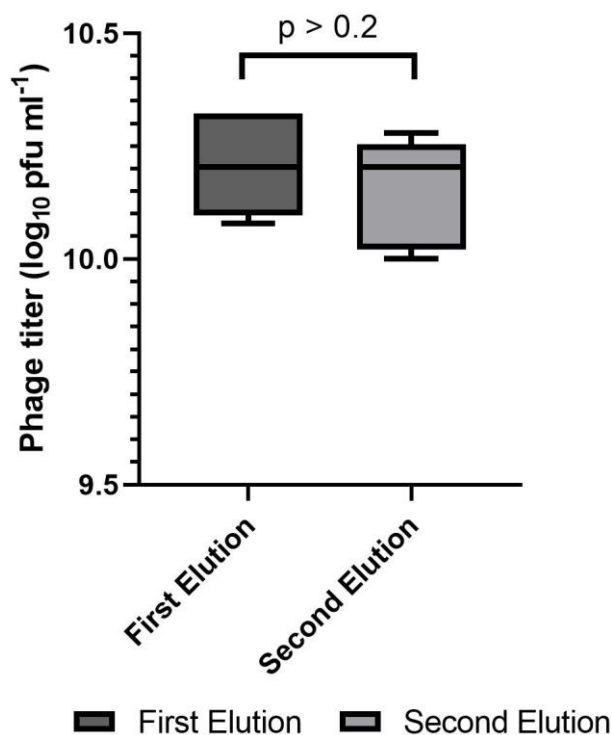


Image of an M. smegmatis Semi Confluent Lysis Plate (SCLP) exhibiting lacing (i.e. individual plaques are barely distinguishable but some evidence of lawn growth remains).

As an agar plaque is a semi fluid environment, and migration of phage out of plaques is driven by osmosis, it was hypothesized that a large number of phage particles are retained in the plaque due to an osmotic equilibrium between plaque and surrounding liquid no longer favouring phage migration out of plaques (Abedon and Yin, 2009; Gun'ko *et al.*, 2017). It was therefore decided to elute the drained agar cubes a second time, in order to obtain further high titre phage suspension. This was done by immersing the drained agar cubes in 5 ml of fresh MP, with a slightly lower volume being used to compensate for the fact that phage had already been eluted from the agar cubes. Following elution, the agar cubes were drained, and the obtained phage lysate was prepared as described above. The average resulting titre of the phage lysate gained from the second elution was 1.46×10^{10} pfu ml⁻¹ with the lowest titre from the second washing being 1.0×10^{10} pfu ml⁻¹. Standard deviation was calculated to be $\sim 0.1 \log_{10}$. There appeared to be no significant ($p > 0.2$) difference in the titres obtained from the first and the second washings of plaques (See figure 4.4). The obtained phage lysates were subsequently stored at 4 °C until further use. In this experiment we were able to show that one semi confluent lysis plate can produce up to 13 ml of phage suspension with a titre of $> 1 \times 10^{10}$ pfu ml⁻¹.

The observation that agar cubes eluted for a second time would still produce a lysate with a titre similar to that of the first elution indicates that phage will only move out of the plaque as long as there is an osmotic gradient between phage particles inside and outside of the aqueous gel. This supports the idea that phage in agar plaques are essentially free floating particles within an aqueous gel whose movements are dependent on Brownian motion as well as osmotic forces.

Figure 4.4. Comparing titres of phage suspensions obtained from 1st and 2nd elution of cubed SCLPs.



SCLPs were mechanically cut into ~5 x 5 mm cubes which were subsequently eluted in 7 ml (first elution) or 5 ml (second elution) of sterile MP (n=2). Titre of phage suspension (in log₁₀ pfu ml⁻¹). There was no significant (p > 0.2) difference between phage titres obtained after the first or second elution.

4.2.1.5. Disinfection of phage lysate obtained from SCLPs using organic solvents

After a few days of storage of the lysates at 4 °C, growth of a contaminant was visible as diffuse clouds of a reddish-orange colour. Due to the use of *M. smegmatis* to form the bacterial lawns and the fact that production of yellow and orange carotenoid pigments is characteristic for mycobacteria and has been observed in *M. smegmatis* under stress conditions (Saviola and Felton, 2011), it was concluded that the most likely contaminant was *M. smegmatis*. Indeed, Shires and Steyn (2001) reported low temperature growth of *M. smegmatis* at 10 °C although at a significantly reduced rate compared to growth at the optimum temperature for this organism (37 °C). Based on culture ATP measurements, the generation time for *M. smegmatis* at 10 °C was estimated to be around 7-8 days (Shires and Steyn, 2001), so if growth reached visible levels within 2-3 weeks, this suggested that the phage lysates still contained high levels of viable bacteria even after the centrifugation step and therefore made sterilisation of the phage lysate a necessity.

Three ways for the removal of contaminating bacteria from phage lysate were explored: addition of chloroform, disinfection by addition of diethyl ether as an alternative to chloroform and filter sterilization. Filter sterilization is a common method for the removal of bacterial contaminants from liquid phage samples by passing the suspension through a 0.45 µm syringe filter which should retain any bacterial cells but will allow the much smaller bacteriophage particles to pass through freely (Rip and Miller, 1997). However, filter sterilizing large volumes of phage lysate was found to be problematic due to filter blockage being a common occurrence. This made pre-filtering of lysate through a larger pore size filter a labour intensive necessity. To avoid this, it was decided to initially explore solvent based methods for disinfecting phage lysates.

Chloroform is a common mean of removing bacterial contaminants from phage cultures. However, chloroform may inactivate certain phage such as filamentous phage or phage with a high lipid content (Bonilla *et al.*, 2016;

Hyman, 2019) and mycobacteriophage tend to be sensitive to organic solvents due to higher lipid contents of the virus compared to other phage classes. This sensitivity however is not uniform among mycobacteriophage and can vary significantly (Schaefer *et al.*, 1977).

Chloroform was added to phage D29 lysate with a titre of $> 1 \times 10^9$ at a concentration of 5 % (v/v) in a chloroform-resistant plastic microcentrifuge tube and incubated for 10 min with agitation to prevent phase separation and ensure sufficient contact between bacterial cells and the solvent. After the incubation period elapsed and the phases separated completely the lower chloroform phase was carefully removed using a pipette and discarded while the remaining aqueous phage lysate layer was incubated at 37 °C with a loose lid for 30 min to allow any remaining chloroform to evaporate. The phage lysate was then titred (as described in 2.3.11.) and it was found that the viability of the phage suspension had dropped to 1.2×10^2 pfu ml⁻¹ and therefore D29 was sensitive to chloroform.

The same procedure was performed using diethyl ether as an alternative to chloroform, in this case the treatment was performed in a glass vessel since ether is known to dissolve various different plastics. In this case residual ether was allowed to evaporate for 1 h at 37 °C before the phage lysate was titred. The initial titre of the phage lysate used was 1.6×10^{10} pfu ml⁻¹ and after treatment with diethyl ether this was still 8.3×10^9 pfu ml⁻¹ which represented a drop in titre of $\sim 0.3 \log_{10}$. The experiment was repeated using phage suspension with a titre of 1.46×10^{10} pfu ml⁻¹ that was again treated with 5% w/v diethyl ether. After ether sterilization the suspension was titred and yielded 4.4×10^9 pfu ml⁻¹ which represents a $\sim 0.6 \log_{10}$ drop in phage titre compared to the non-ether treated sample. The ether-treated phage lysate samples were subsequently refrigerated at 4 °C and after ~ 3 weeks it was discovered that contaminant growth re-appeared in one sample. After ~ 6 weeks 4/10 samples had developed microbial growth identical in appearance to the growth observed in non-ether treated samples. It was concluded that

ether treatment of phage lysates was not sufficient to disinfect phage lysate obtained from SCLPs.

4.2.1.6. Disinfection of phage lysate obtained from SCLPs using filter sterilization

Since diethyl ether failed to completely inactivate bacterial cells present in the phage lysates it was decided to explore filter sterilization as a potential alternative to the use of organic solvents for the sterilization of phage D29 lysate. Although commonly used, there is a risk that filter sterilization could damage the sensitive tail fibres of the bacteriophage, thus reducing the viability of the final phage suspension. To test this 5 ml of a non-sterilized phage D29 suspension with an average titre of 1.3×10^8 pfu ml⁻¹ was passed through a 0.45 µm Minisart syringe filter unit (Sartorius, Germany). This was performed in duplicate and the resulting filtered phage lysates were titred (see 2.3.11.). After this process it was found that both samples of filter sterilized phage D29 suspension contained an average of 1.0×10^8 pfu ml⁻¹ resulting in an average titre loss of 0.3 log₁₀ compared to the titre of the unfiltered suspension. The titre loss associated with filter sterilization is comparable to that encountered when treating phage lysate with ether while filter sterilization avoids most of the health and safety issues encountered with organic solvents like ether or chloroform. The samples of filter sterilized phage suspension were stored at 4 °C with no growth appearing after >8 weeks of storage and therefore this was deemed to be the best method for producing a high titre, sterile phage lysate for freeze-drying.

4.2.2. Freeze-drying of mycobacteriophage D29 suspension

This section discusses the experiments carried out to produce a freeze-dried bacteriophage D29 preparation that would retain a high titre of $> 1 \times 10^8$ pfu ml⁻¹ for up to 6 months without requiring refrigeration.

4.2.2.1. Introduction

Developed in the 1940s as a means to supply blood plasma and penicillin, which both require a cold chain during transport, to troops fighting in the European and Pacific theatres. Freeze-drying - or lyophilisation - has become one of the standard approaches adopted for the preservation of biological materials such as enzymes, other proteins or food items (Ciurzyńska and Lenart, 2011). Lyophilization takes advantage of the tendency of liquids (such as water) to enter the gaseous state from the solid state without entering a liquid state (sublimation) if cooled and pressurized below the triple point of that liquid. The triple point is defined as the point where all three phases of a substance (gas, liquid and solid) can coexist. The triple point is different for each substance with the triple point of water being present at a temperature of 0.01 °C and a pressure of 0.00603659 atm (Wagner and Pruss, 1993). Below this temperature and pressure, the water in the sample will sublime without ever entering the liquid state. This is advantageous since it allows the sample to be dried without having to add heat, which can disrupt the structure of any proteins present. This process also reduces the risk of damaging complex biological structures (such as bacteriophage) during drying since water molecules are significantly more mobile as vapour than as a liquid and tend to leave the sample in a way that retains most microstructures, which makes lyophilization especially useful for the preservation of sensitive biological materials (Puapermpoonsiri *et al.*, 2010).

Bacteriophage intended for long term storage are commonly freeze-dried, however this process can also be accompanied by substantial loss in phage titre due to exposing the phage particles to both, freezing and drying stresses (Clark and Geary, 1973). The main loss in phage titre associated with freeze-

drying occurs during the freeze-drying process with the two main factors responsible for loss in titre during lyophilization being A) Ice crystals forming and physically damaging the phage particles, for example by severing phage heads from tails and B) misfolding of proteins due to the absence of water. Freeze-dried phage also tend to lose viability over time at a rate of $\sim 1 \log_{10}$ per year of storage (Ackermann *et al.*, 2004) which is less relevant for a commercial product but a significant concern for phage banks and reference centres. Different attempts have been made in the past to add stabilizing agents to the phage suspension to protect the phage from damage during lyophilization and storage. Mono- and polysaccharide excipients, such as trehalose, mannitol, or sucrose as well as glycerol and even biodegradable polyester microspheres have all been evaluated as potential agents to protect phage particles during the freeze-drying process with varying success (Puapermpoonsiri *et al.*, 2010; Merabishvili *et al.*, 2013; Malik, 2021).

Trehalose has been reported to improve phage survival considerably (Merabishvili *et al.*, 2013; Vandenheuvel *et al.*, 2014) which is not surprising since it is a molecule commonly found in large amounts in anhydrobiotic organisms. These organisms can survive prolonged periods of time with close to no water, like certain fungal and bacterial spores but also more complex organisms such as plants and microscopic animals such as nematodes. For example, when dried the nematode *Aphelenchus avenae* can convert up to 20% of its weight into trehalose (Elbein *et al.*, 2003) while yeast cultures are known to produce and accumulate trehalose as they enter the stationary phase enabling them to survive severe osmotic stresses including drying (Gadd *et al.*, 1987; Hounsa *et al.*, 1998). The cryo- and desiccation protective properties of trehalose are explained by the water replacement hypothesis which hypothesizes that trehalose confers structural and conformational support to proteins by forming hydrogen bonds with said proteins, limiting its ability to move and mis form thus retaining its functionality (Vandenheuvel *et al.*, 2014). Additionally, trehalose “mimics” the presence of water by forming an insulating surface preventing lipid and proteins from directly contacting

one another in a dried state. This appears to be an important factor in maintaining high phage viability throughout the drying and storage process (Golovina *et al.*, 2009).

4.2.2.2. Freeze-drying of mycobacteriophage D29 suspension without excipient

The results produced from cold drying and freeze-drying artificial plaques containing trehalose described in the previous chapter were promising. However, the phage titre obtained and maintained from artificial plaques was on the lower end of the desired range and the production process (involving production and purification of high titre phage suspension, production of artificial plaques and freeze-drying or alternatively cold drying artificial plaques) was deemed too time and labour intensive to be practical for a commercial product required by the company. It was therefore decided to investigate directly freeze-drying the high titre D29 phage suspension. As a preliminary experiment 400 µl of high titre ($> 1 \times 10^9$ pfu ml⁻¹) phage suspension was aliquoted into freeze-drying vials (n=3) and freeze-dried for up to 24 h (section 2.3.10). The freeze-drying process was deemed complete once the freeze-drying chamber reached a temperature of < -60 °C and a pressure of < 0.02 mbar and the vials were sealed under vacuum before being removed from the freeze dryer. After freeze-drying a small amount of glassy porous solid remained at the bottom of the vial. The freeze-dried phage were rehydrated in its original volume (400 µl) using MP before the number of viable phage particles remaining was determined by titering (see section 2.3.11). It was decided to use MP to resuspend the phage rather than using sterile water because using water as the rehydration fluid has previously been reported to cause damage to the phage due to osmotic shock (Shapira and Kohn, 1974). When this was done, it was found that the phage survived freeze-drying in large numbers even without the use of excipients producing an average titre of 1.73×10^9 pfu ml⁻¹. Three additional samples of freeze dried D29 from the same batch were rehydrated and titred after being stored at room temperature for 1 month. It was found that the titre of the phage

preparation had dropped to 2.55×10^7 pfu ml⁻¹ marking a 2 log₁₀ reduction in phage titre ($p < 0.01$). This indicates that although the phage may be freeze dried without excipients, other than the media they are in, without incurring a major loss in viability, they lose titre rapidly when stored at room temperature.

As it had been shown that the addition of 6% (w/v) trehalose significantly improved the stability of dried and freeze-dried artificial plaques during storage at room temperature it was decided to freeze-dry phage D29 suspension with the addition of trehalose at this concentration. Since trehalose is a common excipient for freeze-drying biological material including phage (Merabishvili *et al.*, 2013; Vandenheuvel *et al.*, 2014) it was not expected that adding trehalose would have a negative impact on phage survival before and during freeze-drying. Therefore, trehalose was added to bacteriophage D29 suspension containing a phage titre of $\sim 1.0 \times 10^{10}$ pfu ml⁻¹ up to a final concentration of 6% (w/v). Then the phage D29 suspension was aliquoted into freeze-drying vials as before ($n=5$) each containing 110 μ l). After freeze-drying the vials containing the freeze-dried phage preparation were rehydrated in 110 μ l of MP before the phage titre was again determined (section 2.3.11). It was found that the five samples containing 110 μ l of rehydrated phage culture produced an average titre of 2.48×10^9 pfu ml⁻¹. The freeze dried samples not containing trehalose produced an average titre of 1.45×10^9 pfu ml⁻¹. These results showed that the addition of trehalose had no negative impact on phage survival during freeze-drying. On average, the samples containing trehalose retained ~ 0.1 log₁₀ more viable phage however the difference in titres obtained from phage freeze dried with and without trehalose was not deemed statistically significant ($p > 0.1$).

4.2.2.3. Freeze-drying different volumes of phage D29 suspension

For the Actiphage assay, each test requires the addition of 10 µl of D29 phage at a titre of more than 1×10^9 pfu per ml, therefore vials which produced 100 µl of phage at this titre after resuspension were suitable to produce kits for this assay. However, the company wished to produce kits for the other assay format (the original FastPlaque assay) which requires 110 µl of rehydrated phage D29 suspension with this titre. It was therefore decided to freeze dry phage D29 in the presence of 6% (w/v) trehalose using 1.1 ml aliquots of phage to equate to ten tests per vial for this assay format. For comparison, vials were prepared using both 110 µl (group A) and 1.1 ml (group B) of D29 phage suspension (n=5 for each volume). After freeze-drying, the phage were rehydrated in their original volume of MP and the number of viable phage determined as before (section 2.3.11). The average phage titre per vial for the 5 freeze-dried samples from group A (110 µl) was found to be 2.5×10^9 pfu ml⁻¹ while the 5 samples from group B (1.1 ml) were found to contain 2.0×10^9 pfu ml⁻¹. The standard deviation for both sets of samples was calculated to be $< 0.1 \log_{10}$. The difference in phage titre between the freeze-dried 110 µl and 1.1 ml samples was $< 0.1 \log_{10}$ and not deemed significant ($p > 0.1$). It was noted that in the samples containing 1.1 ml of freeze drying mixture violent bubbling and foaming would occasionally occur due to gases dissolved in the liquid leaving rapidly when vacuum was applied. This led to spillage and puffed up, foamy cakes instead of the desired white powder cake at the bottom of the vial.

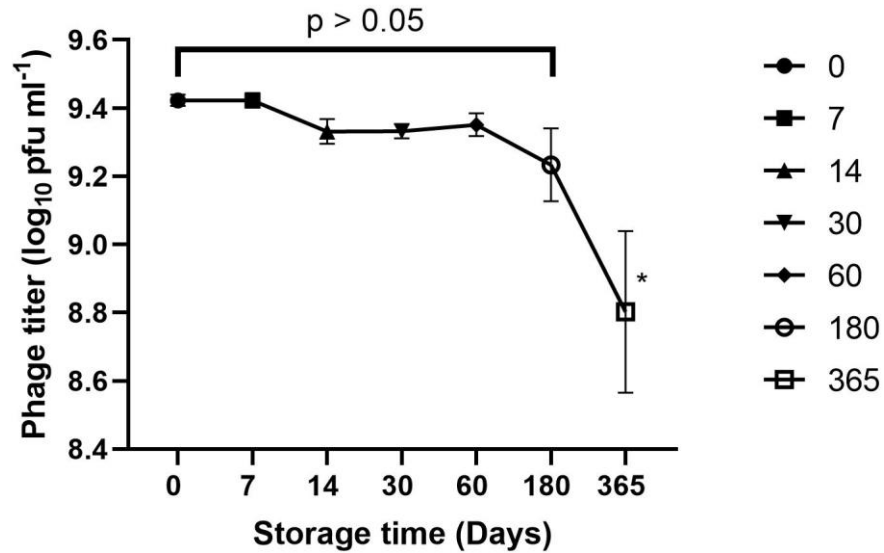
4.2.2.4. Stability of freeze-dried phage D29 suspension stored at room temperature

After determining that the addition of trehalose had no negative consequences on the survival of phage during freeze-drying, it was decided to determine if the addition of trehalose would help phage survival during storage at room temperature. To this end trehalose was added to high titre

phage suspension (8×10^9 pfu ml⁻¹) at a concentration of 6% (w/v) being aseptically aliquoted into freeze-drying vials (500 µl per vial) and freeze-dried as before (section 2.3.10). 500 µl of volume was chosen in this experiment to address issues with bubbling and foaming of the freeze drying mixture observed when vacuum was applied in previous experiments (4.2.2.4). After freeze-drying this volume of phage a porous white “cake” was left at the bottom of the vials. From this batch of freeze dried D29 phage, a set of vials (n=4) were rehydrated using 500 µl of MP immediately after freeze-drying. These samples produced an average phage titre of 2.6×10^9 pfu ml⁻¹ indicating that the loss in viability associated with the freeze-drying process is $<1 \log_{10}$. To determine how stable these samples were, the remaining sealed vials containing the freeze-dried phage preparation were stored at ambient temperature (18-23 °C) for 7, 14, 30 and 60 days as well as for 6 and 12 months before sets of vials were removed (n=4 at each timepoint) the phage were rehydrated using 500 µl MP and then titred as described in section 2.3.11. The results of this experiment are summarised in Figure 4.5. After 7 days of storage at room temperature the freeze-dried phage preparation showed no loss in phage viability. Similarly, no significant change in phage titre was recorded on resuspension after 14, 30 and 60 days of storage at room temperature (average phage titres recorded were 2.1 , 2.1 and 2.4×10^9 pfu ml⁻¹, respectively). After 6 months of storage at room temperature the freeze-dried phage D29 preparation a measurable reduction in phage titre was detected (average phage titre recorded = 1.75×10^9 pfu ml⁻¹), however the standard deviation within the sample groups stored up to 6 months was $< 0.1 \log_{10}$ and therefore this reduction in titre was not found to be significant ($p > 0.05$). After 12 months of storage at ambient temperatures the phage D29 preparation still produced an average phage titre of 6.3×10^8 pfu ml⁻¹. However, it was found that two out of the four vials stored for 12 months had a defective seal allowing moisture from the air to enter the vial and partially re-hydrate the cake. Nevertheless even the vials with defective seals produced titres of 3 and 6×10^8 pfu ml⁻¹, respectively, but the results were much more variable (SD for this set of samples was $0.2 \log_{10}$; see Fig. 4.5). The

results of the overall stability trials indicated that phage prepared using this method met the criteria required by the company for the freeze-dried phage to be used in the prototype Actiphage diagnostic kits.

Figure 4.5. Monitoring stability of freeze-dried phage D29 stored at room temperature



Freeze-dried phage D29 preparations containing 6% (w/v) trehalose were stored at room temperature for 0, 7, 14, 30, 60, 180 and 365 days before being rehydrated and titred. Graph shows average phage titre per vial ($n=4$ vials) on reconstitution. Error bars represent standard deviation within each sample group.

* Two of four samples titred after 12 months suffered from defective seals and entry of moisture during storage. They were nevertheless titred and produced titres $\sim 5 \times 10^8$ pfu ml^{-1} but resulted in more variable results.

4.2.2.5. Evaluating the activity of the freeze-dried phage D29 preparation containing 6% trehalose in phage-based detection assays

After producing a freeze-dried formulation of mycobacteriophage D29 that was able to retain a high phage titre for multiple months without requiring a cold chain for storage, the performance of these samples was compared with fresh liquid lysates of D29 phage when detecting mycobacterial cells using phage-based detection assays. The reason for this was that the addition of 6% (w/v) trehalose as an excipient to the phage sample may have affected phage D29-host cell interactions. In particular it is known that the process of phage adsorption and reversible binding (discussed in section 1.3.2) is susceptible to changes in the environmental conditions, such as changes in osmolarity and pH (Jończyk *et al.*, 2010; Swift *et al.*, 2014). In order to assess if, and to what degree, trehalose interferes with this crucial step of the phage assay it was decided to first use the two-day phage amplification assay due to the fact that it generates numerical results which could be quantified. For this experiment, a culture of *M. smegmatis* was used since this is the standard control sample used when performing the two day assay.

To test whether trehalose affects phage infection of host cells, 4 vials of freeze-dried phage D29 preparation containing 6% (w/v) trehalose were reconstituted in MP. As discussed in section 4.2.2.3, the company required two volumes of phage vial (110 µl and 1.1 ml) to be produced for the different assay formats, so for this experiment, two vials of each size were used, with each vial being resuspended in an appropriate volume of MP. After reconstitution a whole vial of the 110 µl size was required per assay, whereas for the 1.1 ml vials, multiple assays could be performed using the contents of one vial. To perform the two-day phage amplification assay, 1 ml of *M. smegmatis* culture diluted to a calculated titre of $\sim 1 \times 10^2$ cfu ml⁻¹ was prepared and 1 ml of sterile MP was used as a negative control (no cells should be detected). In addition to the freeze-dried samples, one set of assays (*M. smegmatis* and a negative control) were prepared using the liquid D29

phage suspension that had been used to prepare the freeze dried phage samples.

Briefly, to perform this assay 1 ml of sample was spiked with 100 µl of phage culture and incubated. After 1 hour of incubation extracellular phage were destroyed and the reaction mixture solidified by mixing with MP and 7H10 agar before being poured in petri dishes and incubated overnight (see section 2.6). After performing the phage amplification assay, plaques on the assay plates were counted with each individual plaque being interpreted as one infection event and thus being equivalent to the detection of one cell in the original *M. smegmatis* culture and therefore results are recorded as pfu detected. The results from this experiment are summarised in Table 4.2.

Table 4.2. Using freeze-dried D29 in the two-day phage amplification assay to detect *M. smegmatis*

SOURCE OF PHAGE D29	ADDED TO	PFU DETECTED
LIQUID PHAGE LYSATE	1 ml 10^2 cfu ml ⁻¹ <i>M. smegmatis</i> culture	148*
	1 ml Media plus (MP) (negative control)	2
FREEZE-DRIED 1.1 ML	1 ml 10^2 cfu ml ⁻¹ <i>M. smegmatis</i> culture	120, 156
	1 ml Media plus (MP) (negative control)	1, 3
FREEZE-DRIED 110 µL	1 ml 10^2 cfu ml ⁻¹ <i>M. smegmatis</i> culture	204, 196
	1 ml Media plus (MP) (negative control)	0, 0

**For the control sample using the liquid phage lysate, only one sample was prepared. For each of the freeze dried phage samples, tests were performed in duplicate, with a separate vial used for each of the duplicate samples.*

The control experiments performed as expected, with the non-freeze dried liquid phage sample detecting 148 cells, with only two plaques being detected in the no cells negative control sample. Since this assay requires the complete inactivation of 10^8 phage particles to give a 0 plaque results, low level breakthrough is sometimes seen. Due to this, in the original FastPlaque assay a threshold of 20 cells was set as the cut off for a positive test result, although results in excess of 10 plaques for the negative control are rarely seen. In this case, all of the negative control samples recorded were either 0 or less than 3 plaques which indicates that the 6% trehalose used as an excipient in the freeze-dried phage preparation did not interfere with the FAS virucide used during the assay. If this had occurred, inefficient destruction of extracellular phage due to virucide failure is generally accompanied by considerable phage breakthrough and failure of the negative control.

After establishing that the freeze dried D29 formulation containing 6% trehalose could be used interchangeably with liquid phage lysate for the detection of *M. smegmatis* it was decided to confirm that this was also the case for the detection of MAP. To this end 1 ml samples of MAP culture with an estimated titre of 10^2 cfu ml⁻¹ were spiked with 100 µl of either liquid phage lysate with a titre $> 1 \times 10^9$ pfu ml⁻¹ or reconstituted freeze dried phage preparation containing 6% trehalose. Additionally, 1 ml samples of MP were also treated this way to serve as a negative control. The two day phage amplification assay (see section 2.6) was performed on all samples and, similarly to the results obtained from the earlier experiment on *M. smegmatis*, the freeze dried phage D29 preparation produced results similar to those obtained when using liquid phage lysate (see table 4.3). The negative controls generally performed as expected and the variability between the number of detected MAP cells in the different samples was considered to be negligible and could easily be the result of clumping of cells (which MAP is notorious for) in the liquid culture used and thus overlapping of plaques. There also was no indication of the trehalose interfering with the assay indicating that freeze dried phage D29 preparation containing trehalose could

be used interchangeably with liquid phage lysate for the detection of *M. smegmatis* and MAP using the phage amplification assay.

Table 4.3. Using freeze-dried D29 in the two-day phage amplification assay to detect MAP

SOURCE OF PHAGE D29	ADDED TO	CFU DETECTED
LIQUID PHAGE LYSATE	1 ml 10 ² cfu ml ⁻¹ MAP culture	123*
	1 ml Media plus (MP) (negative control)	1
FREEZE-DRIED 1.1 ML	1 ml 10 ² cfu ml ⁻¹ MAP culture	135, 113
	1 ml Media plus (MP) (negative control)	6, 3

**For the control sample using the liquid phage lysate, only one sample was prepared. For each of the freeze dried phage samples, tests were performed in duplicate, with a separate vial used for each of the duplicate samples.*

Overall the freeze-dried phage D29 preparations containing trehalose produced results very similar to the liquid phage lysate used as a positive control. Based on the calculated number of cells added in the experiment ~100 plaques per plate was expected if the phage amplification assay worked at full efficiency. However this will realistically never be the case since the accumulation of dilution errors during sample preparation and transfer, clumping of cells leading to overlapping plaques, as well as the stochastic probability regarding the distribution of a small number of bacterial cells in a comparatively huge volume of liquid introduces a considerable margin of error to the expected results. Thus, realistically, it was expected that between 50 and 250 cells would be detected in each of these assays if they worked as intended. In fact the difference between the lowest and highest number of detected cells is only 84 and notably the freeze-dried phage D29 preparations only detected fewer cells than the liquid phage sample on one occasion, which does not suggest that inhibition of phage binding was occurring due to the presence of trehalose in these samples. Therefore this suggests that freeze-dried phage D29 prepared in this way can be used as a direct replacement to liquid phage lysate in the Actiphage assay kits for the two day assay.

The second stage of this evaluation was to confirm compatibility of the freeze-dried D29 preparation with the one day Actiphage assay format which uses qPCR to detect DNA released from the target mycobacterial cells in the sample. Although it had been established that the amount of trehalose added as an excipient to the freeze dried phage did not interfere with phage-host cell interaction, the main concern when using these phage formulations in the Actiphage assay was possible interference with the qPCR detection event.

Briefly, to perform the Actiphage one day assay MAP cells or lysed blood mononucleocytes potentially containing MAP cells were pelleted and resuspended in phage suspension. The phage will lyse the cells and release its DNA content into the reaction mixture (see section 2.8). DNA obtained from the assays was treated using the ZymoTM clean and concentrator kit (Zymo,

USA) before being amplified in a Rotor Gene Q qPCR machine (Qiagen, Germany) using the Ingenetix BactoReal kit for the detection of MAP (Ingenetix, Austria)

To perform this experiment, three vials of freeze-dried phage D29 preparation were rehydrated in 110 µl of MP and added to 10 µl of MAP culture with a titre of $\sim 1 \times 10^4$ cfu ml⁻¹ (representing ~ 100 MAP cells). Using a viable count method for determining cfu ml⁻¹ of MAP culture was not possible due to MAPs slow growth. Therefore the phage enumeration assay for the detection of mycobacteria (see section 2.6.2) was used to estimate the number of cells in the sample. Three more Actiphage assays were prepared using the same MAP culture but using 110 µl non freeze-dried liquid phage lysate ($\sim 1 \times 10^9$ pfu ml⁻¹) which did not contain any trehalose. Two negative controls were also prepared by adding 110 µl of freeze-dried or non-freeze-dried phage D29 suspension to 10 µl of sterile MP. The Actiphage assay was carried out on all samples and it was found that all MAP samples could reliably be detected with the freeze dried phage D29 preparation containing 6% trehalose. The sterile MP used as a negative control was detected by the freeze-dried and non-freeze-dried phage suspension after ~ 31 amplification cycles while the Ingenetix kit positive and negative controls for the qPCR reaction, MAP DNA and water respectively, behaved as expected and there was no indication of PCR inhibition. The C_q values obtained when challenging MAP culture with freeze-dried phage D29 preparation containing 6% trehalose and non-freeze-dried liquid phage lysate not containing trehalose were found to be very similar (< 1 Ct) and there is no significant difference in results when either type of phage preparation was used ($p > 0.7$). The mean number of amplification cycles required before detection of the MAP culture was calculated to be ~ 23 cycles for both phage cultures used. The difference in C_q value obtained for the negative controls containing freeze-dried and non-freeze-dried phage was also deemed to be negligible (see Table 4.4). As the results obtained from phage with and without trehalose were very similar and there were no signs of PCR inhibition in any of the samples it was concluded

that trehalose has no negative impact on the performance of either the Actiphage assay or the qPCR used to obtain results and were also therefore appropriate for including in the commercial Actiphage kits instead of a liquid phage lysate.

Table 4.4. Comparing freeze dried phage preparation with liquid phage lysate when used in the Actiphage assay

	LIQUID PHAGE LYSATE (CQ VALUE)	FREEZE DRIED PHAGE (CQ VALUE)
SAMPLE I (100 MAP CELLS)	22.8	22.3
SAMPLE II (100 MAP CELLS)	23.5	23.4
SAMPLE III (100 MAP CELLS)	23.7	24.2
-VE CONTROL (MP)	31.1	30.6

In triplicate (I, II, III), either 110 µl of rehydrated freeze-dried phage D29 preparation containing 6% (w/v) trehalose or 110 µl of fresh phage D29 lysate ($> 1 \times 10^9$ pfu ml⁻¹) was used to perform the Actiphage assay on ~100 MAP cells (10 µl of $\sim 1 \times 10^4$ cfu ml⁻¹ culture). As a negative control 10 µl of sterile MP was spiked with 110 µl of each phage (MP -ve). All phage preparations used managed to detect the MAP culture and produced a strong positive result. The mean number of cycles required before a positive result was recorded was 23 for both phage cultures used. The negative control produced Cq values of 30.6 and 31.1 for the freeze dried phage and the lysate respectively.

4.2.3. Freeze-drying mycobacteriophage TM4

4.2.3.1. Introduction

Mycobacteriophage TM4 is a lytic dsDNA phage and part of the family of Siphoviridae. TM4s broad host range and ability to infect both slow and fast growing mycobacteria including the clinically and economically relevant species of *M. tuberculosis*, *M. bovis* and *M. avium ssp. paratuberculosis* makes it particularly interesting for phage therapy and phage-based diagnostics (Ford *et al.*, 1998; Swift *et al.*, 2014). Additionally, phage TM4 has been postulated to be able to infect mycobacteria in stationary phase, or those cells that are not actively replicating (Piuri and Hatfull, 2006; Swift *et al.*, 2014). The inability of phage D29 to detect mycobacterial cells that are not actively growing is a potential major shortcoming of the current iteration of the Actiphage® Rapid Blood Test kits due to *M. tuberculosis* and *M. bovis* ability to induce a dormant state *in vivo* when exposed to unfavourable conditions such as depletion of oxygen or nutrients in the environment (Gengenbacher and Kaufmann, 2012; Bacon *et al.*, 2014; Low *et al.*, 2020). It is therefore possible that mycobacterial cells in a clinical sample may not be detected by phage D29 due to them being in a dormant state, although this has not proved to be an issue since it has been shown that D29 is able to successfully infect mycobacterial pathogens recovered from white blood cells (Swift *et al.*, 2013; 2016; 2020). The addition of phage TM4 could solve this problem however it would require the preparation of suitable high titre, freeze-dried TM4 phage preparations that could be included in the Actiphage® Rapid Blood Test kits. Hence the objective of this work was to explore methods for the production of a freeze-dried high titre phage TM4 preparation that would maintain a titre of $> 1 \times 10^8$ pfu ml⁻¹ for at least 6 and up to 12 months without the requirement for refrigeration.

4.2.3.2. Production of high titre TM4 phage lysate using the SCLP method

In order to produce large quantities of mycobacteriophage TM4 lysate for freeze-drying it was decided to employ the semi confluent lysis plate (SCLP) method already successfully used for the production of high titre D29 lysate (section 4.2.1 and 2.3.6). To this end, semi confluent lysis plates were produced as described in 2.3.6 but phage D29 suspension was replaced by 100, 200 or 300 μl of phage TM4 stock with a titre of 1×10^4 pfu ml^{-1} . A higher inoculum was chosen than when producing D29 SCLPs due to the much smaller plaque size of phage TM4 compared to D29. When chopped and eluted these SCLPs produced TM4 phage stocks with titres of 2.75, 2.7 and 2.6×10^9 pfu ml^{-1} respectively. These results demonstrated that the SCLP method of producing high titre phage D29 lysate can also be easily adapted to produce large volumes of high titre TM4 phage lysate.

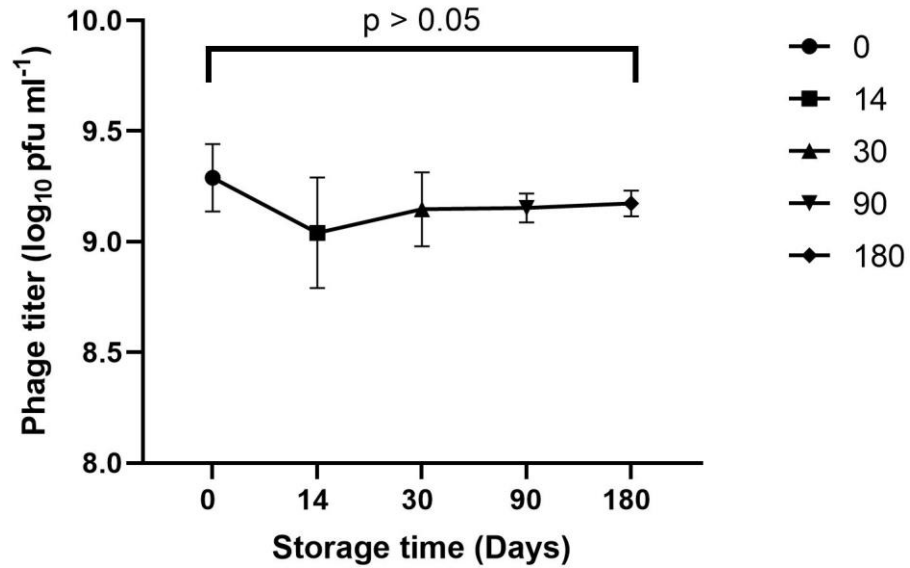
4.2.3.3. Freeze-drying of phage TM4 lysate with trehalose and stability studies

To determine whether the freeze drying protocol developed for D29 was also applicable to TM4, a high titre phage TM4 suspension was supplemented with trehalose up to a final concentration of 6% (w/v). The titre of this mixture before freeze-drying was determined to be 2.8×10^9 pfu ml^{-1} and 1.1 ml aliquots were freeze dried. After reconstituting in original volume of MP, the freeze-dried TM4 samples produced titres of 2.8, 2.8, 2.9 and 3.0×10^8 pfu ml^{-1} indicating that a 1 $\log_{10}$ drop in phage titre occurred during freeze-drying which was comparable to the drop in phage viability encountered when freeze-drying phage D29 (section 4.2.2).

To determine the stability of these freeze-dried TM4 phage, 12 vials containing 1 ml of phage TM4 suspension containing a final concentration of 6% trehalose were freeze-dried as before (2.3.10) and stored at ambient temperatures. The phage titre of the freeze-drying mixture before freeze-drying was determined to be 2.3×10^9 pfu ml^{-1} . Vials containing freeze-dried phage TM4 were titred (n=3), immediately after freeze-drying, after 14 days, 30 days, 3 months and 6 months of storage at ambient temperatures. It was

found that the phage TM4 preparation titred immediately after freeze-drying still retained an average titre of 2.03×10^9 pfu ml⁻¹ and on this occasion the ~ 1 log₁₀ drop in phage titre observed in the initial experiment was not seen. The stability data is shown in figure 4.6 and it is evidence that throughout the storage at ambient temperatures, the phage TM4 preparation was found to be remarkably stable with no significant change ($p > 0.05$) in phage titre detected throughout the 6 months of storage period.

Figure 4.6. Monitoring viability of freeze-dried phage TM4 stored at ambient temperatures.



Freeze-dried phage TM4 vials containing 6% (w/v) trehalose were stored at room temperature for 0, 14, 30, 90 and 180 days before being rehydrated in x ml MP and the number of viable phage determined. Error bars show SD values for 3 independent samples. The p value was determined by performing the Ttest in excel comparing data obtained from samples titred after 0 days and samples titred after 180 day assuming a one tailed distribution.

4.3. Discussion

4.3.1. Using SCLPs for the production of phage lysate

Initial experiments on plaque elution conducted as part of this work found that migration of phage from plaques to a surrounding liquid occurs rapidly and is close to instant and most likely the result of rapid establishment of an osmotic equilibrium between phage within the plaque and phage suspended in the surrounding liquid. Agar, being an aqueous gel, represents a semi fluid environment allowing free passage of liquids and even macromolecules in and out of the gel (Gun'ko *et al.*, 2017, Johnston *et al.*, 2020). Additionally it was found that reducing the ratio of volume of elution medium used for the elution of individual plaques could be employed to increase the concentration of phage in the suspension obtained. The number of total phage recoverable from plaques could however not be changed by altering the volume of elution medium used.

While traditional methods for the production of high titre phage D29 lysate generally rely on propagating the phage in a liquid culture of suitable host bacteria (Bonilla *et al.*, 2016), it was found that producing and eluting semi confluent lysis plates was an effective method for obtaining large quantities of phage lysate. The phage lysate obtained was found to be of higher average titre ($> 1 \times 10^{10}$ pfu ml⁻¹) than that obtained from picking and eluting individual plaques in buffer ($1 \times 10^{8-9}$ pfu ml⁻¹). Additionally, the volume of phage lysate that can be produced from each semi confluent lysis plate is >10 ml compared to 100µl per plaque obtained through eluting individual plaques. This method for the production of high titre phage D29 lysate is also advantageous since it can be scaled for the production of even larger volumes of phage lysate simply by increasing the number of SCLPs produced and processed. In this way litres of phage lysate can be produced in a short period of time.

As reported with many methods for the production of high titre phage lysate (Sargeant and Yeo, 1966; Fortier and Moineau, 2009; Bonilla *et al.*, 2016) a disinfection and purification step of the obtained phage lysate is necessary to

remove host bacteria and debris from the phage culture before storage and use to avoid contamination and spoilage of the culture. Indeed, growth of a mycobacterial contaminant determined to be *M. smegmatis* was encountered which in turn raised the question how *M. smegmatis* can actively grow in a culture of mycobacteriophage D29 with a titre of $> 1 \times 10^{10}$ pfu ml⁻¹ without being infected and lysed. Experiments carried out by another member of the research group found that mycobacterial cells grown at 4 °C required 1 hour of incubation at 37 °C before infection and detection by phage D29 could occur. Despite actively dividing, albeit at a considerably slower rate (Shires and Steyn, 2001), these mycobacterial cells stored at 4 °C could not be infected by phage D29 suggesting an intermediary growth state bridging dormancy and full active growth. To address this problem, three commonly employed methods for the purification of phage lysate were compared: (1) Centrifugation to remove large debris particles, (2) the addition of either chloroform or di ethyl ether to inactivate bacterial contaminants and (3) filter sterilization for removal of smaller debris and bacteria. Centrifugation was found to be a sufficient method for the removal of large pieces of debris such as agar particles but does not remove host bacteria carried over from the bacterial lawn in sufficient numbers resulting in bacterial growth upon storage. It was found that chloroform treatment was detrimental to phage D29 survival which was surprising considering mycobacteriophage I3 which is a structurally similar to mycobacteriophage D29 (Schaefer, 1977) only suffers a 1 log₁₀ loss in titre when exposed to chloroform and ether (Gope *et al.*, 1981). Phage D29 did tolerate the use of diethyl ether considerably better than that of chloroform only suffering a < 0.5 log₁₀ reduction in phage titre. However di ethyl ether failed to completely remove all bacterial cells from the phage suspension. The best method for the sterilization of phage lysate was found to be filter sterilization using a 0.45 µm filter (Sartorius, Germany) which resulted in an average titre loss smaller than 0.5 log₁₀ while avoiding the potential risks involved with using highly flammable organic solvents. Filter sterilization also controlled bacterial growth in phage lysate much better than ether. This method for the production of phage lysate was also tested on

mycobacteriophage TM4 and we were able to produce large volumes (> 10 ml per SCLP) of TM4 phage lysate with a titre of $> 1 \times 10^9$ pfu ml⁻¹ and therefore seems to be applicable to different phage types.

4.3.2. Production of freeze-dried bacteriophage preparations

The ultimate aim of this work was to develop a phage D29 preparation that could be used as the primary reagent in the Actiphage assay kits for the detection of mycobacteria developed by PBD Biotech LTD. Until now phage D29 was supplied in the kit as liquid culture which is far from ideal due to the requirement for constant refrigeration and the increased risk of contaminant growth. In order to be useful in the context of a commercial detection assay the phage preparation had A) be in a dry form to prevent contaminant growth, B) have a titre sufficient to reliably detect mycobacteria in a sample as part of the Actiphage assay ($> 1 \times 10^8$ pfu ml⁻¹), C) be able to retain that titre for over six months without the requirement for refrigeration and D) not reduce sensitivity or specificity of the Actiphage assay. The freeze-dried bacteriophage D29 preparation containing 6% trehalose as an excipient was able to retain a phage titre of $> 1 \times 10^9$ pfu ml⁻¹ for six months while being stored at room temperature. Freeze-dried phage stored for up to one year at room temperature still produced titres $> 1 \times 10^8$ pfu ml⁻¹ despite not being stored in a temperature control environment and temperatures within the lab recorded as ranging from ~ 15 °C to ~ 30 °C during this period. The freeze-dried phage D29 preparation was then successfully used in the two-day phage amplification assay and the Actiphage assay for the detection of mycobacteria. It was found that the freeze-dried phage D29 preparation could be used as a replacement for liquid phage culture with no loss in sensitivity or specificity. Additionally, a freeze dried phage TM4 preparation containing 6% (w/v) trehalose was produced and found to retain a titre of $> 1 \times 10^9$ pfu ml⁻¹ after 6 months of non-temperature controlled storage. The ability of the freeze-dried phage preparations to retain a stable high titre over multiple months despite no temperature control and significant fluctuations in

temperature is of great significance since it will make application and distribution of the Actiphage kit easier. Developing countries that have a high prevalence of mycobacterial disease also commonly suffer from lacking infrastructure thus eliminating the need for constant refrigeration allows easier deployment of the Actiphage assay for the detection of mycobacteria in rural areas that are oftentimes particularly hard hit by mycobacterial pathogens in humans and livestock (Carruth *et al.*, 2016).

4.3.3. Conclusion

In this study we have shown that mycobacteriophage D29 lysate can be freeze-dried and stored at room temperature for multiple months while retaining a high viability. The freeze-dried preparation was found to perform on par with, or better than, liquid high titre phage lysate when used in the two-day phage amplification as well as the Actiphage assay. This freeze-dried D29 preparation containing 6 % trehalose was later incorporated into the prototype Actiphage Blood Test kits produced by PBD Biotech LTD and provided a foundation for future work within a commercial GMP production facility for large scale production of kits. Now that a method has been devised to also produce freeze-dried mycobacteriophage TM4 it should be possible to explore its potential to infect mycobacterial cells in stationary phase thus increasing the sensitivity of the phage based detection assays if clinical samples contained such cells, or even if clinical samples were not stored under optimal conditions which in turn reduced the infectivity of D29 (Swift *et al.*, 2014).

Ultimately a combined freeze-dried D29 and TM4 preparation that is able to detect stationary and actively growing mycobacterial pathogens with a high degree of sensitivity and reliability would be ideal. However additional research is required to determine if any interference in the efficiency of phage infection occurs if both phage are present. Unfortunately, the receptor on the mycobacterial cell surface for these two broad spectrum phage is unknown,

so competition for binding sites cannot be ruled out. Indeed it is notable that a D29-resistant strain of *M. smegmatis* isolated in this lab has also been found to be resistant to phage TM4 (A. Hunyh, PhD student, University of Nottingham, personal communication). It should also be noted that a combined phage D29 and TM4 reagent could only be used in the Actiphage one day assay since, unlike D29, TM4 is not inactivated by the FAS virusol used in the current version of the two day assay. A green tea extract has been reported to kill phage TM4 (Swift *et al.*, 2014) and could thus theoretically be used as a virucidal agent in the two-day phage amplification assay. However, its compatibility with the two-day phage amplification assay and especially with the FAS virusol used for destroying phage D29 has not been sufficiently studied.

Chapter 5

Freeze-drying of Mycobacteria

5.1. Introduction

The Actiphage® Rapid Blood Test kit for the detection of MTC and MAP was developed by PBD biotech LTD for the rapid detection of *M. bovis* or MAP in clinical and veterinary blood samples. Currently the kit does not provide a viable cell culture to be used as a positive control. Therefore, a positive control to accompany these kits using viable cells to verify the accuracy of the assay results would be advantageous. This positive control needs to be delivered as viable MAP or *M. bovis* (BCG) cells which can then be tested alongside clinical or veterinary blood samples to confirm that the phage are active and able to lyse mycobacterial cells. However, the addition of live mycobacterial cells, in the correct growth phase to allow infection by phage D29, required the development of a method for the preservation and storage of said live cells. The aim would be to provide a positive control, where the number of viable cells was above the limit of detection of the assay (10^1 cfu; Swift *et al.*, 2013, 2020).

As discussed in section 4.2.2.1, cryopreservation - and especially freeze-drying is one of the most common methods for long term preservation and storage of biological samples (Ciurzyńska and Lenart, 2011). This is also true for the preservation and storage of bacterial species where bacterial culture collections routinely freeze-dry their samples (Prakash *et al.*, 2013) as are commercial products like freeze-dried yoghurt and wine starter cultures (Dimitrellou *et al.*, 2019; Ale *et al.*, 2015). The main arguments for preferring freeze-drying over cryopreservation is that cryopreservation requires maintaining a constant cold climate around the sample which is dependent on continuous supply of electricity or a coolant such as liquid nitrogen. The requirement for constant refrigeration is a major disadvantage when it comes to storage and distribution of any kits designed for routine use in different laboratories. It not only makes shipping and storage of the kits more expensive but also makes them more susceptible to spoilage as a result of power outages or malfunctioning cooling equipment (Peiren *et al.*, 2015). This is of particular concern in regions such as Sub-Saharan Africa, rural China,

India or South and Central America with potentially lacking infrastructure where maintaining a constant cooling chain is often difficult and sometimes impossible (Wilson *et al.*, 2018). As these regions also suffer the burden of mycobacterial infections in human and animals (Olea-Popelka *et al.*, 2017), it was deemed important to develop a positive control for the Actiphage kit that would not need constant refrigeration. As freeze-drying was previously used successfully to develop a room temperature stable preparation of phage D29 (Chapter 4), it was decided to explore ways to develop a freeze-dried preparation of MAP and *M. bovis* (BCG) cultures for use as a positive control in the Actiphage assay.

Freeze-drying bacteria usually incurs a significant loss in viability as a result of the freeze-drying process (Prakash *et al.*, 2013) because of bacteria experiencing both freezing and drying stresses simultaneously (Arellano-Ayala *et al.*, 2021). The main cause for loss of viability when freeze-drying bacterial culture is the formation of ice crystals and the stresses that are exerted on the cell membrane as the surrounding liquid crystalizes. Expanding ice crystals can rupture the bacterial cell wall as well as damaging cell organelles. The second common cause for loss in viability during freeze-drying of bacteria is protein denaturation as a result of desiccation which can also cause cell membrane lipid oxidation and subsequent rupturing of the cell wall. DNA and RNA denaturation as a result of absence of water can also cause a problem leading to reduced viability (Arellano-Ayala *et al.*, 2021). Survival of bacteria during freeze-drying and subsequent storage can be improved by the addition of cryoprotective excipients. Mono- and polysaccharide excipients, such as trehalose, mannitol or sucrose, as well as glycerol have all been evaluated as potential agents to protect bacteria during the freeze-drying process with varying success (Prakash *et al.*, 2013; Peiren *et al.*, 2015; Arellano-Ayala *et al.*, 2021).

In particular trehalose has been reported to improve bacterial survival considerably during and after freeze-drying (Leslie *et al.*, 1995; Vaessen *et al.*, 2019). The cryo- and desiccation protective properties of trehalose are well

known and have been discussed in detail in the section on freeze-drying mycobacteriophage D29 (4.2.2.1). These properties are explained by the water replacement hypothesis which suggests that trehalose confers structural and conformational support to proteins by forming hydrogen bonds with said proteins, limiting its ability to move and misfold thus allowing them to retain functionality (Vandenheuvel *et al.*, 2014). Additionally, trehalose “mimics” the presence of water and forms an insulating surface preventing lipids and proteins of freeze-dried cells from directly contacting one another when in a dried state. This appears to be an important factor in maintaining high viability throughout the drying and storage process (Golovina *et al.*, 2009).

One of the few studies specifically on this topic of freeze-drying and preservation of mycobacteria is by Slosarek *et al.* (1976) where the group attempted freeze-drying of a multitude of different mycobacterial species and strains including *M. avium*, *M. bovis*, *M. bovis* (BCG), *M. tuberculosis* and *M. smegmatis* using a plethora of different excipients, such as calf serum and lactose, equine serum, skimmed milk, monosodium glutamate, glycerol, glucose and dextran as well as peptone. It was found that all excipient combinations tried conferred some degree of resistance to freeze-drying stress, and the cells freeze-dried in the presence of calf serum plus lactose, equine serum and monosodium glutamate produced the highest number of viable samples after multiple years of storage. However they did not attempt freeze-drying using trehalose or sucrose. The method used by this group for determining viability was to rehydrate and culture the freeze-dried mycobacterial samples after up to 25 years of storage and record whether the samples were able to resume growth and produce viable cultures or not. This approach would be sufficient for culture and strain collections but did not allow conclusions on the number of viable cells remaining in the sample.

5.1.1. Aims and objectives

The aim of this work was to develop a freeze-dried preparation of MAP and BCG intended to be used as a positive control in the Actiphage® Rapid Blood

Test kit for the detection of MAP or bovine tuberculosis produced by PBD Biotech LTD. The limit of detection of the Actiphage® Rapid Blood Test is 10 cells (Swift *et al.*, 2013) however for a test kit, a positive control should produce a strong positive result which is easily interpreted. Therefore it was decided that an ideal freeze-dried positive control for the Actiphage assay kit should contain $\sim 10^2$ detectable cells which is 10 times higher than the limit of detection of the assay.

The slow growth of MAP, and the tendency to form clumps or flakes in liquid culture, makes an accurate estimation of the viability of the culture almost impossible. Determining viability by plating of culture on solid media is not only time consuming but also inaccurate and overall highly impractical as visible growth may take weeks to months to appear. At this point the original culture will either have increased in viability due to continuous bacterial growth or be nutrient depleted and in stationary phase. As it is known that entering this state makes the cells no longer infectable by phage D29, this would mean they were no longer useful as a positive control sample.

Similarly estimating viability of liquid culture using optical density is also not a viable option due to MAPs tendency to aggregate into clumps rather than evenly dispersing throughout the culture. The issue of clumping also renders the use of the two-day phage amplification assay for the enumeration of MAP (see 2.6.2) a difficult proposition, as a clump containing thousands of MAP cells will only result in one phage plaque and thus be counted as one detectable MAP cell. A standard approach that is taken to overcome this issue when growing mycobacteria is to homogenize cultures by vortexing in the presence of glass beads to mechanically break up clumps (Brennan *et al.*, 2017), therefore this approach was used in this study before performing the phage amplification assay to enumerate the number of phage-detectable cells present in a sample after resuscitation.

5.2. Freeze-drying of *Mycobacterium avium* ssp. *paratuberculosis*

This section discusses the freeze-drying of MAP using different excipients as well as investigating shelf life of freeze-dried MAP preparations and their detectability using the two-day assay as well as the Actiphage® assay.

5.2.1. Initial experiments: Freeze-drying of MAP (ATCC) slope scrape material without excipients

The slow growing nature of MAP means that liquid cultures are difficult to maintain and are prone to contamination. Therefore active cultures are often maintained on agar slopes and so initially experiments were carried out to determine whether resuspended cells recovered from a slope could be freeze-dried to provide suitable positive control material.

Before initiating the experiment, the IS900 PCR method for the detection of the MAP-specific insertion sequence (IS900), described by Whittington *et al.* (1998), was used to confirm that the colonies present on the slopes did contain MAP cells.

To do this, duplicate MAP suspensions were prepared by resuspending a MAP colony from a BBL Lowenstein Jensen Medium slope in 1 ml of MP. A sample (0.1 ml) of this was transferred into 1.5 ml microfuge tube and boiled at 100 °C for ~ 10 min. The crude boiled lysate was then centrifuged at 13000 x g for 5 min to remove cell debris and 5 µl of boiled lysate from was used as a template for the IS900 PCR protocol (section 2.9.1). Both samples produced the expected size of PCR product (394 bp) confirming that the colonies tested were MAP.

Next 0.1 ml samples of MAP suspension without the addition of any excipients were aliquoted into freeze-drying vials and freeze-dried for 12 h (see section 2.3.10). After freeze-drying, the MAP samples were rehydrated in 1 ml of MP before the two-day phage amplification assay was used to determine the number of detectable MAP cells present in the freeze-dried cultures. This method of determining the number of detectable cells was used

as viable count is not a practical method when working with MAP due to the long incubation period (18 weeks) before detectable colonies are formed. Additionally, the MAP suspension used to prepare the freeze-dried samples was also enumerated using this method so the results could be compared.

Results from these initial experiments found that no viable MAP cells could be detected in the MAP suspension either before or after freeze-drying. The *M. smegmatis* used as a positive control for the two-day assay positive produced 41 plaques indicating that the assay was working as expected, but the MAP cells derived from the slope were non-detectable. Since phage D29 used for the amplification assay can only infect and thus detect actively growing mycobacterial cells, it was deduced that the MAP cells were not non-actively growing and thus in a non-detectable state.

5.2.2. Producing MAP culture in an active growth state

To get the MAP cells into an actively growing and reproducing state, MAP was grown in liquid culture to around the mid-exponential phase, when the cell number was $\sim 10^4 - 10^5$ pfu ml⁻¹ as determined using the phage enumeration assay (section 2.6.2). At this point the bacterial growth has not yet progressed into stationary phase meaning that most if not all MAP cells in the culture should be in an actively growing state and thus infectable by the phage. To do this MAP cultures were produced as described in materials and methods 2.5.2. The inoculated cultures were incubated in a shaking incubator at 37 °C for 4 to 8 weeks. First signs of visible growth appeared after ~ 3 -4 weeks. MAP culture grown in this way was found to contain $\sim 3 \times 10^4$ cfu ml⁻¹ detectable MAP cells after ~ 7 weeks of incubation.

5.2.3. Freeze-drying of MAP using excipients initial experiments

After identifying a culture method that would routinely provide a liquid MAP culture containing an appropriate number of cells, it was decided to evaluate different excipients for improving bacterial survival during freeze-drying and storage. The followings excipients were evaluated:

A) 10 % (w/v) trehalose due to its well-known ability to protect from drying and freezing stresses as well as the good results obtained during freeze-drying of mycobacteriophage D29 (Chapter 4).

B) A mixture of 10 % (w/v) sucrose and 4 % (w/v) dextran (40 kDa; w/v) as both sucrose and dextran are common sugar based cryoprotectants used for freeze-drying proteins, bacteria and phage (Obayashi *et al.*, 1960; Slosarek *et al.*, 1976; Merabishvili *et al.*, 2013). Information provided by the company also indicated that a mixture of 10 % (w/v) sucrose and 4 % (w/v) dextran has also previously been used successfully for the freeze-drying of *M. smegmatis* (Dr B Clarke, pers. Communication).

C) A 1 % (w/v) solution of mono sodium glutamate (MSG); this was chosen as it was reported by Slosarek *et al.* (1976) that mycobacteria freeze-dried in the presence of 1 % (w/v) MSG would maintain viability over many years of storage at room temperature.

D) A no excipient control was also prepared as to assess the effects of the excipients tried on bacterial detectability.

To perform these experiments 2.5 ml samples of liquid MAP culture were mixed with the appropriate amount of each excipient and 0.5 ml aliquots of each freeze-drying mix were freeze-dried using the Christ Alpha 1-2 LDplus freeze dryer (Christ, Germany) for 12 h (section 2.3.10). After the freeze-drying process had finished, all samples were retrieved and visually examined. It was noted that relatively thick (~ 0.5 cm) off-white cakes had formed in the freeze-drying vials when 10 % (w/v) trehalose or 10 % (w/v) sucrose and 4 % (w/v) dextran were used as excipients while the samples containing 1 % (w/v) MSG or no excipient resembled a glassy film on the bottom of the freeze-drying vial therefore the addition of the carbohydrate-based excipients clearly altered the overall structure of the cake, which is known to be an important factor in achieving a stabilised product when samples of protein are freeze dried (Holm *et al.* 2021).

The next duplicate samples of each freeze-dried MAP preparation were rehydrated in 0.5 ml of MP for 5 minutes before the number of phage-detectable MAP was determined using the two-day phage amplification assay. The freeze-dried MAP preparation containing 10 % (w/v) trehalose (sample A) produced an average titre of 1.02×10^2 pfu ml⁻¹ while the preparation freeze-dried with 10 % (w/v) sucrose and 4 % (w/v) dextran (B) yielded a titre of 1.16×10^2 pfu ml⁻¹. Samples freeze-dried using 1 % (w/v) MSG (C) or no excipient (D) produced lower detectable titres of 0.89 and 0.2×10^2 pfu ml⁻¹, respectively, representing a $\sim 3 \log_{10}$ difference in detectable MAP cells that were recovered after freeze-drying. These results showed that freeze-dried MAP could be detected using the two-day phage amplification assay and that the addition of excipients increased the number of viable and detectable MAP cells detectable after freeze-drying.

Based on the data obtained, all three carbohydrate-based excipients tested (trehalose, sucrose and dextran) produced similar results in regard to their ability to protect MAP cells during freeze-drying. However, the 1 % (w/v) MSG produced less promising results and therefore was not taken forward to further experiments. One point that was noted was that the overall pattern of data revealed that the number of plaques found when plating a 10^{-1} dilution of the samples containing trehalose or sucrose did not show the expected 10-fold drop in bacterial titre when compared to the values obtained for the 10^{-1} dilution (data not shown). At this point, due to the overall low number of plaques recorded at the 10^{-1} dilution, this observation could just be explained by phage particles surviving the disinfection step which is a common point of failure of the two-day phage amplification assay. However this pattern of results was also seen in later experiments (see Table 5.2)

5.2.4. Freeze-drying of MAP using Sorbitol as an excipient

The chemical sorbitol, commonly used as an artificial sweetener, belongs to the chemical group of the sugar alcohols (polyols) which also includes well known cryoprotectants such as mannitol, xylitol, and glycerol. Polyols are small molecular carbohydrates structurally similar to sugars such as trehalose

and sucrose and are thought to also derive their cryoprotective properties from the number of free hydroxyl groups and the length of the carbon chains present on the individual molecule (Zhang *et al.*, 2019). Indeed, sorbitol has shown significant cryoprotective and stability enhancing properties during freeze-drying of microorganism such as the yeast *Candida sake* (Abadias *et al.*, 2001) or lactic acid bacteria for industrial purposes (Carvalho *et al.*, 2003). However, no data was found in the literature on the effect of sorbitol during freeze-drying of mycobacteria specifically. For this reason it was decided to investigate whether sorbitol could be used alone or in conjunction with other excipients such as trehalose or sucrose and dextran to improve the survival or detectability of freeze-dried MAP cells.

Samples of a MAP culture with a titre of 2.84×10^4 pfu ml⁻¹ were prepared for freeze drying by adding either: A) 5 % (w/v) D-sorbitol , B) a mixture of 5 % (w/v) D-sorbitol, 10 % (w/v) trehalose (w/v) , C) or a mixture of 5 % (w/v) D-sorbitol, 10 % (w/v) sucrose, 4 % (w/v) dextran (w/v). Subsequently, 0.5 ml aliquots of each MAP culture and excipient mixture were freeze-dried as before (section 2.3.10). To test the efficacy of each of these mixtures as an excipient, the freeze-dried samples were subsequently rehydrated in 0.5 ml of MP (n=2) and titred using the two-day phage amplification assay for the enumeration of mycobacteria (2.6.2). The results (Table 5.1) showed that there was no difference in the cryopreservative effect of any of the excipients as the number of detectable cells was approximately the same in all cases ($\sim 1 \times 10^3$ pfu ml⁻¹). The drop in detectable cells after freeze drying was $\sim 1.5 \log_{10}$ in all samples. Therefore it was concluded that when freeze-drying MAP, sorbitol did not provide any better protection than when using the sugar-based additives trehalose, sucrose, and dextran.

However it was noted that the number of plaques recorded at a 10⁻¹ dilution were considerably higher than we would expect after performing a 10 fold dilution of the undiluted sample (ratio of values = 1:13; see legend Table 5.1). This appeared to be the same effect as had already been identified in the previous experiment (5.2.4). Since the negative control behaved as expected,

only producing 3 plaques, this indicated that these results were not due to a failure of the FAS during the phage amplification assay. It was therefore hypothesized that the presence of excipients at high concentrations in the undiluted sample may interfere with the phage enumeration assay thus leading to false low estimates of MAP viability in the undiluted samples, especially since diluting the sample 10 fold appeared to reverse the inhibition (see Table 5.1).

Table 5.1. Two-day phage amplification assay detectability of freeze-dried MAP containing sorbitol

	AVERAGE PLAQUES 10 ⁰ DILUTION	AVERAGE PLAQUES 10 ⁻¹ DILUTION	AVERAGE PLAQUES 10 ⁻² DILUTION	AVERAGE PLAQUES 10 ⁻³ DILUTION	PFU ML ⁻¹	REDUCTION IN DETECTABLE CELLS (LOG ₁₀)
A 5 % (W/V) D-SORBITOL	86*	47*	10	1	9.40 x 10 ²	1.48
B 5 % (W/V) D-SORBITOL, 10 % (W/V) TREHALOSE	51**	33**	3	2	6.60 x 10 ²	1.63
C 5 % (W/V) D-SORBITOL, 10 % (W/V) SUCROSE, 4 % (W/V) DEXTRAN	76***	51***	10	1	1.02 x 10 ³	1.45
+VE	103	-	-	-	-	-
-VE	3	-	-	-	-	-

The two-day assay was carried out on duplicate samples of MAP culture freeze-dried in the presence of A) 5 % (w/v) D-sorbitol, B) 5 % (w/v) D-sorbitol, 10 % (w/v) trehalose and C) 5 % (w/v) D-sorbitol, 10 % (w/v) sucrose, 4 % (w/v) dextran. Dilutions (10⁻¹, 10⁻², 10⁻³) of all samples were prepared and number of detectable MAO cells determined using the phage enumeration method. Results are given in average number of plaques recorded from each sample. Controls behaved as expected (negative control: 3 plaques; positive control: 103 plaques)

* 10 fold dilution of sample did not result in a 10 fold reduction in detectable cells. Instead of being around 10:1 the ratio of plaques at 10⁰ and 10⁻¹ was found to be 1:1.8 for samples A.

** Ratio of plaques at 10⁰ and 10⁻¹ was found to be 1:1.6 for samples B.

*** Ratio of plaques at 10⁰ and 10⁻¹ was found to be 1:1.5 for samples C.

5.2.5. Stability of freeze-dried MAP preparations containing excipients stored at room temperature

After establishing that MAP cells could be successfully freeze-dried at the levels required (approx. 100 cells per vial) and were still in a detectable state for phage-based detection methods, it was decided to determine the shelf stability of the freeze-dried MAP preparations prepared using these different combinations of excipients. Shelf life at room temperature was of primary concern as the freeze-dried MAP preparation is intended to be sold as a positive control in phage based MAP detection kits. In order to determine the loss of viability associated with storage at room temperature 28 ml of MAP culture with a titre of 1.2×10^4 pfu ml⁻¹ was divided into 7 ml aliquots and mixed with: A) 10 % (w/v) trehalose , B) 10 % (w/v) sucrose, 4 % (w/v) dextran , C) 10 % (w/v) trehalose, 5 % (w/v) sorbitol and D) 10 % (w/v) sucrose, 4 % (w/v) dextran, 5 % (w/v) sorbitol (w/v). These excipients were chosen because of their cryoprotective and stabilizing properties reported in the literature. Sugars such as trehalose, sucrose and dextran are all well-known cryoprotectants used for freeze drying bacteria, viruses and proteins (Leslie *et al.*, 1995; Merabishvili *et al.*, 2013; Vandenheuvel *et al.*, 2014; Zhang *et al.*, 2020) while the sugar alcohol sorbitol was reported to improve survival of lactic acid bacteria during freeze drying (Carvalho *et al.*, 2003).

As before, 0.5 ml aliquots of MAP cultures of each of the samples A, B, C and D were freeze-dried as described in section 2.3.10. On this occasion, it was decided that the samples would be rehydrated in double the original culture volume (1 ml rather than 0.5 ml) as this might help alleviate the issues seen with high concentration of excipient and this was also more compatible with the IFU for the commercial test kits that were optimised for a sample volume of 1 ml.

After freeze-drying, 3 samples of each freeze-dried MAP preparation (A, B, C, D) were re-hydrated in 1 ml of sterile MP (double original volume) before undergoing the phage amplification assay for the enumeration of mycobacteria (section 2.6.2). This process was repeated after 7 and 30 days of

storage at room temperature. The titre of detectable MAP cells was calculated using the formula:

$$\text{pfu ml}^{-1} = \left(\frac{\text{number of plaques} \times \text{dilution factor}}{\text{volume plated in ml}} \right) \times 2$$

Therefore the dilution factor was 10^1 while the volume plated was 0.9 ml for each sample. The standard formula was multiplied by two to determine the pfu ml⁻¹ value since 0.5 ml of freeze-dried MAP culture was rehydrated in double volume (1 ml) before being titred. The results for this experiment are summarised in Table 5.2 and Figure 5.1.

5.2.5.1. Results MAP preparations immediately after Freeze Drying

The samples containing trehalose (A) and trehalose and sorbitol (C) were found to have lost $\sim 1 \log_{10}$ in detectable MAP cells compared to the initial culture used for freeze-drying (1.2×10^4 pfu ml⁻¹) while the freeze-dried aliquots of culture freeze-dried in the presence of sucrose and dextran (B) or sucrose, dextran and sorbitol (D) were found to have lost between 1 and 1.5 $\log_{10}$ in detectable MAP cells compared to the initial freeze-dried MAP culture.

5.2.5.2. Calculating dilution efficiency of FD MAP culture dilutions

From the results obtained again it was noticed that the number of plaques counted at the 10^{-1} dilution was usually the same, or even higher, than the valued counted for the undiluted sample. This fitted the pattern of unexpected results already reported, and therefore the increased resuspension volume did not seem to have alleviated this effect.

In order to gain data on this observation the dilution efficiency (Σ) was calculated by dividing the number of plaques counted from each dilution ($10^0 = a$, $10^{-1} = b$, $10^{-2} = c$) by the higher dilution and multiplying by 100 to get a % value ($\Sigma_{b/a} = \left(\frac{b}{a} \right) \times 100$ and $\Sigma_{c/b} = \left(\frac{c}{b} \right) \times 100$). Using this formula, optimal dilution efficiency would be 10%, ($\frac{10}{100} \times 100 = 10\%$), while an increasing divergence from 10% indicates that the dilution process was not linear resulting in over- or under estimation of number of cells detected cells.

Results were grouped in three categories “good efficiency” (Σ between 5 % and 20 %), “moderate efficiency” ($\Sigma \geq 2.5$ % and ≤ 50 %) and “poor efficiency” ($\Sigma < 2.5$ % and > 50 %). The results of this analysis are shown in Table 5.2

It was discovered that the efficiency of all $10^0 \rightarrow 10^{-1}$ dilutions carried out were above 50 % (i.e. results were non-linear) with the average dilution efficiency $\Sigma_{b/a}$ being found to be 137.7% (poor). Meanwhile the second 1 in 10 dilution from 10^{-1} to 10^{-2} was significantly ($p < 0.01$) more efficient with 10/12 dilutions having good efficiency, (average dilution efficiency $\Sigma_{c/b} = 13.4\%$; good) and the remaining two being scored as moderately efficient.

Table 5.2. Number of MAP cells detected after Freeze-drying culture on day 0 and dilution efficiency calculations

Sample number*	pfu Detected in 10 ⁰ dilution (a)	pfu Detected in 10 ⁻¹ dilution (b)	Dilution efficiency (Σ) in % $\Sigma_{b/a} = (\frac{b}{a}) \times 100$	pfu Detected in 10 ⁻² dilution (c)	Dilution efficiency (Σ) in % $\Sigma_{c/b} = (\frac{c}{b}) \times 100$
Sample A1	87	72	82.8	7	9.7
Sample A2	110	122	110.9	18	14.8
Sample A3	78	97	124.7	16	16.5
Sample B1	43	47	109.3	4	8.5
Sample B2	55	41	74.6	9	22.0
Sample B3	62	36	58.1	4	11.1
Sample C1	51	98	192.2	13	13.3
Sample C2	38	108	284.2	20	18.5
Sample C3	170	164	96.5	26	15.9
Sample D1	30	47	156.7	4	8.5
Sample D2	31	60	193.6	3	5.00
Sample D3	32	54	168.8	9	16.7
Average			137.7±61.6**		13.7±4.7**

*Triplicate samples (labelled 1, 2 and 3) of FD MAP cultures A, B, C and D stored for 0 days were rehydrated in 1 ml MP (2 x original volume). Undiluted (a) samples as well as samples diluted 10⁻¹ (b) and 10⁻² (c) underwent the phage enumeration assay.

** Standard deviation of average values

The DE analysis results colour coding: Good –DE between 5.0% and 20.0% (■); Moderate - DE ≥ 2.50% and ≤ 50.0%(■); Poor- Dilution efficiency ≤ 2.50% and ≥ 50.0% (■).

The results obtained support the hypothesis that the excipients added to the culture before freeze-drying interfere with the phage amplification assay in a fashion currently not fully understood. This effect however only appears to be significant when the excipient concentration is at its highest i.e. in the undiluted sample as the number of plaques recorded at the 10^{-2} dilution generally reflect a 10-fold reduction in titre compared to the 10^{-1} diluted sample. This indicates that the unknown interference caused by the addition of excipients is reversible as the excipient concentration was reduced. Additionally it was noted that the plaque morphology of phage D29 plaques on agar plates made using undiluted samples containing excipients was visually different. Plaques grown on lawn plates containing the highest concentration of excipients were considerably smaller (0.5 – 1.5 mm) in size and of irregular shape while D29 plaques produced from 10^{-1} and 10^{-2} diluted samples were found to be of normal size (2.5 – 4 mm) and evenly round shape (data not shown). As the number of plaques recorded for the undiluted samples were likely to be an underrepresentation of the actual number of detectable MAP cells (section 5.2.5.2 and Table 5.2) they were disregarded, and the titre calculations in the following experiment were based on the number of plaques recorded from the 10^{-1} diluted samples.

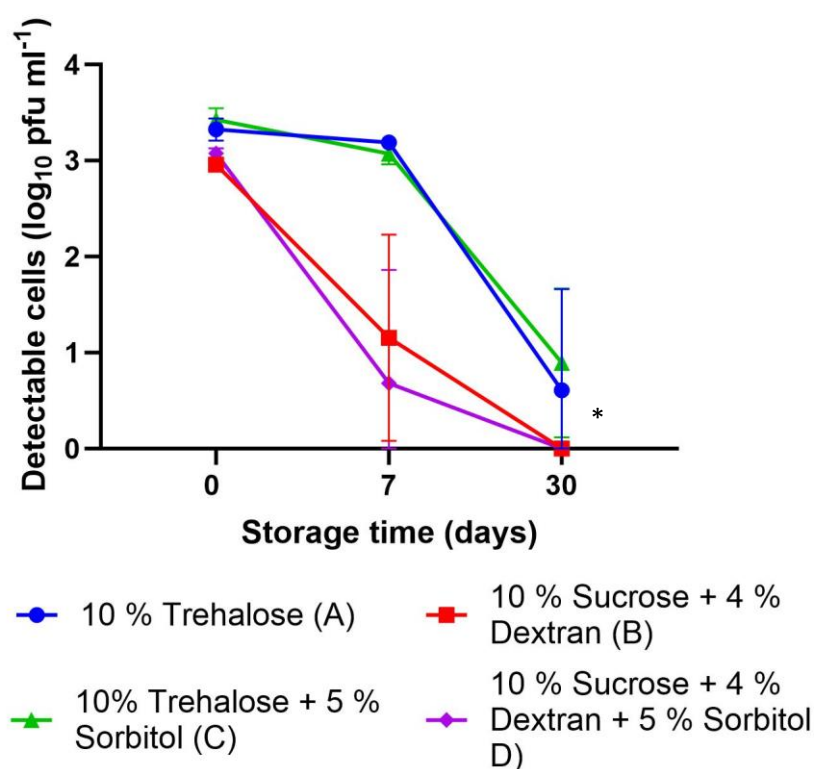
5.2.5.3. Effect of storage at room temperature on Freeze-dried MAP samples

As shown in Figure 5.1, after 7 days of room temperature storage, the average number of detectable MAP cells in samples A and C (10% (w/v) trehalose and 10% (w/v) trehalose and 5% (w/v) sorbitol) was found to have only dropped by $< 0.5 \log_{10}$ producing titres of 1.6×10^3 (A) and 1.2×10^3 (C). Meanwhile viability of samples B and D was found to have dropped by $\sim 2 \log_{10}$ yielding 5.2×10^1 (B) and 3.7×10^1 (D) detectable cells per ml^{-1} .

When samples were rehydrated and titred after 30 days of storage at room temperature a large decrease in the number of detectable MAP cells was seen compared to the first storage time point. Sample A produced 2.2×10^1 pfu ml^{-1}

however no MAP cells were detected in samples B, C and D. This steady drop in detectability was attributed to MAP cells entering a viable but non- actively growing state as a response to the dry environment surrounding them. This would render such cells undetectable by phage based diagnostic tools. Therefore none of these freeze-dried MAP preparations retained enough detectable MAP cells to be used as a positive control in the Actiphage rapid phage assay kits. Additionally, samples of FD MAP containing trehalose + sorbitol (C) or sucrose, dextran and sorbitol (D) produced very similar numbers of detectable cells compared to their non sorbitol containing counterparts (A and B) thus indicating that the addition of sorbitol does not improve viability or long term stability of FD MAP preparations. It was thus decided to discontinue the use of sorbitol in future freeze drying experiments.

Figure 5.1. Room temperature stability of freeze-dried MAP containing various excipients



Samples of a MAP culture, containing various excipients (see graph labels), was freeze-dried and stored for up to one month at room temperature. Detectability was determined after 0, 7 and 30 days of room temperature storage using the phage enumeration assay ($n=3$). Error bars represent standard deviation within each set of triplicate samples.

* Having a Log_{10} value of 0 is mathematically impossible and represents detectable MAP titres of $< 1 \times 10^1 \text{ pfu ml}^{-1}$. The scale of the graph was left unchanged to improve data representation.

5.2.6. Investigating effect of trehalose on phage D29 plaque formation

In order to gain a better understanding of how excipients were interfering with the phage enumeration assay was decided to perform an efficiency of plating (E.o.P) experiment. To do this *M. smegmatis* lawns were prepared (section 2.3.4) which contained different concentration of trehalose. A pre-defined number of phage particles were then spotted onto the surface of the lawn and the number of resulting plaques used to measure how many phage particles managed to productively infect their *M. smegmatis* host cells in each case.

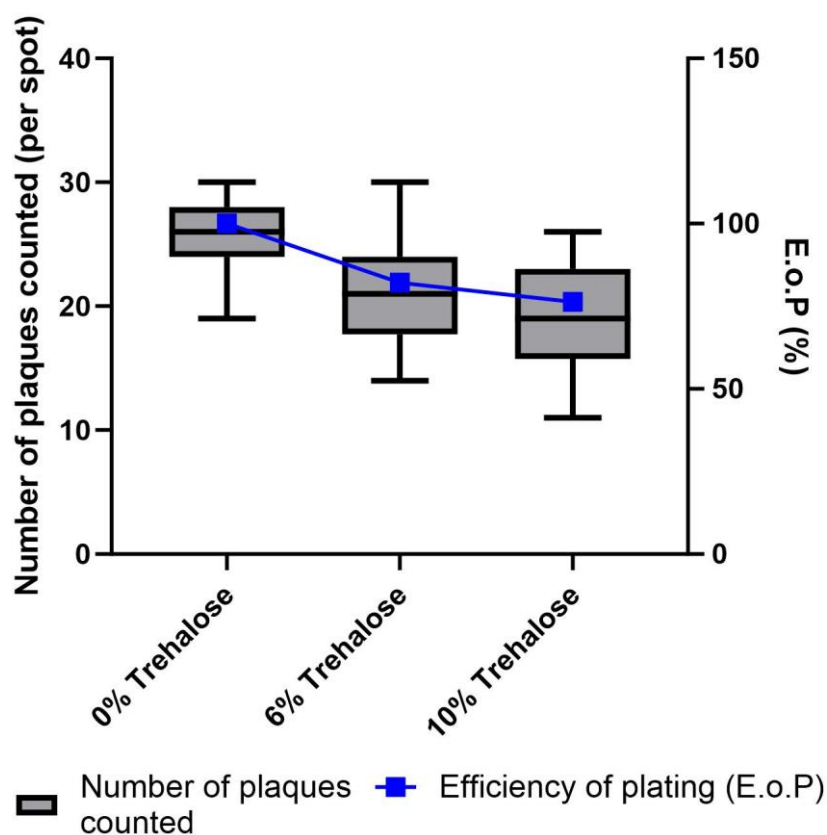
M. smegmatis lawn plates were produced as normal but 6%, 10% (w/v) or no trehalose was added to the mixture before the agar was poured in the petri dishes. Once completely solidified 50 individual drops (10 µl each) of bacteriophage D29 suspension with a titre of 1.4×10^3 pfu ml⁻¹ (as determined on a standard *M. smegmatis* lawn plate) were spotted on to each lawn plate and incubated at 37 °C for 24 h. The results are shown in figure 5.2. After incubation an average of 24.9 plaques formed on the plates containing no trehalose while the plates containing 6% and 10% (w/v) trehalose produced an average of 21.0 and 19.0 plaques, respectively. The standard deviation within each set of sample was calculated to be < 5 plaques (~ 20%). The efficiency of plating was calculated using the formula:

$$EP = \frac{\text{average \# of plaques counted}}{\text{average \# of plaques counted with no trehalose present}} \times 100$$

The E.o.P of phage D29 in the presence of 6 % (w/v) trehalose was found to be 84.5 % while and addition of 10 % (w/v) trehalose resulted in an efficiency of plating of 76.4 % indicating a clear inverse correlation between trehalose concentration and the number of plaques formed by the bacteriophage ($P < 0.01$). To confirm that increasing concentrations of trehalose did decrease the E.o.P, this experiment was repeated but now including an extra *M. smegmatis* lawn plate containing 15 % (w/v) trehalose (w/v). The phage D29 suspension used for this experiment had a titre of 2×10^3 pfu ml⁻¹ and the data is shown in Figure 5.3. When no trehalose was present the average

number of plaques recorded on each spot was 16.4 while each spot on the lawns made using 6%, 10% and 15% (w/v) trehalose produced an average of 14.9, 14.2 and 13.1 plaques respectively.

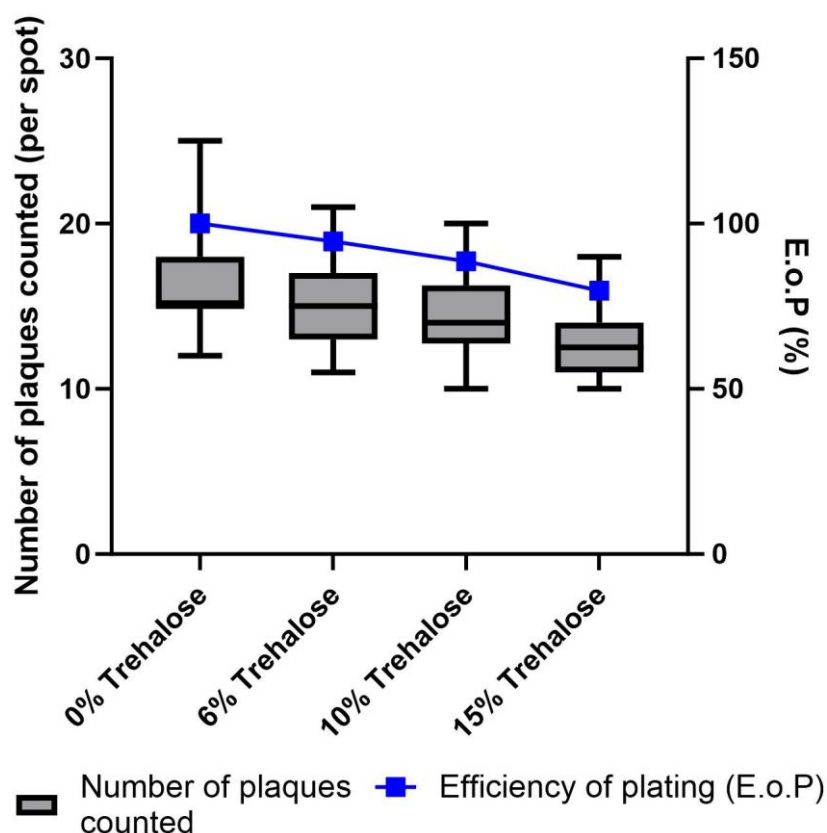
Figure 5.2. Effect of trehalose on the efficiency of plating of Bacteriophage D29



The efficiency of plating (E.o.P) of phage D29 in the presence of trehalose was determined by plating 10 μ l ($n=50$) spots of phage D29 suspension with a known titre on to *M. smegmatis* lawn plates containing 0, 6 and 10% (w/v) trehalose. The number of plaques per spot was counted and E.o.P was calculated.

This gave E.o.P values of 90.1% in the presence of 6% (w/v) trehalose, 86.5% in the presence of 10% (w/v) trehalose and 79.5% in the presence of 15% (w/v) trehalose in the lawn plate ($P < 0.01$), confirming the results of the previous experiment. Again it was observed that the plaques were generally smaller in size when grown on lawn plates containing trehalose compared to plaques grown without the addition of trehalose (data not shown). Since the E.o.P was decreasing when performing a standard phage titration experiment when the cells are known to be in an infectable state, this suggested that the trehalose was inhibiting the ability of the phage to infect the mycobacterial cells.

Figure 5.3. Repeat of experiment to determine effect of trehalose on the efficiency of plating of phage D29



The efficiency of plating (E.o.P) of phage D29 in the presence of trehalose was determined by plating 10 μ l spots ($n=50$) of phage D29 suspension with a known titre on to *M. smegmatis* lawn plates containing 0, 6, 10 and 15% (w/v) trehalose. The number of plaques per spot was counted and E.o.P was calculated. The standard deviation was calculated to be > 3 ($\sim 15\%$) for all groups of samples. Plaque numbers at 15% trehalose concentration were significantly ($p < 0.01$) smaller than with 0% trehalose.

5.2.7. The presence of eDNA in freeze-dried MAP preparations

Since mycobacteriophage D29 can only infect actively growing cells and will not cause productive infection in dead or stationary MAP cells. The ability to differentiate between viable and non-viable cells is one of the main advantages of the Actiphage assay compared to traditional PCR-based detection methods purely relying on the detection of MAP DNA present in the sample (Cangelosi and Meschke, 2014). The results in the previous section suggested that DNA detected by the qPCR at the end of the Actiphage assay using the freeze dried samples is not necessarily a result of productive phage D29 infection of MAP cells. One explanation for this would be that there was DNA present in the sample due to MAP cells lysing during the freeze-drying process or extracellular DNA (eDNA) present in the MAP culture used for producing the freeze-dried MAP preparation. Extracellular DNA is produced by a large number of bacteria, including mycobacteria, and is suspected to have important functions in biofilm formation, ranging from providing structural support to nutrient acquisition or even horizontal gene transfer (Rose *et al.*, 2015; Ibáñez de Aldecoa *et al.*, 2017). Rose *et al.* (2015) demonstrated that *Mycobacterium avium ssp. hominissuis* (MAH), a mycobacterial pathogen closely related to *Mycobacterium avium ssp. paratuberculosis*, produces and excretes large quantities of extracellular DNA into its surrounding. It was also found that the eDNA produced by MAH was identical to its genomic (g) DNA.

To determine whether the DNA detected in the previous experiments originated from live MAP cells or from eDNA carried over from the original MAP culture, it was decided to perform the Actiphage assay and qPCR either with or without the addition of bacteriophage D29. In theory, the samples not having phage added to them should not produce a positive qPCR signal. Four vials of freeze-dried MAP preparation containing 10% (w/v) trehalose were rehydrated in their original volume (0.5 ml) of MP. Duplicate Actiphage samples were prepared using 10 µl of rehydrated freeze-dried MAP culture combined with either 110 µl of phage D29 suspension (titre of $> 1 \times 10^8$ pfu ml⁻¹) or 110 µl of sterile MP. Positive and negative controls were prepared by

mixing 110 μ l Actiphage reagent with either 10 μ l sterile MP (negative control) or 10 μ l of a fresh MAP culture approx. 100 cells (positive control). As before the lysate from the Actiphage assay was purified using the Zymo clean and concentrator kit and detection of MAP DNA was carried out using the Ingenetix BactoReal kit according to the manufacturer's instructions using 5 μ l of purified template DNA per reaction.

After amplification, it was found that the two samples of freeze-dried MAP challenged with phage D29 produced Cq values of 13.5 and 13.6 while the samples that had MP added instead of the Actiphage reagent produced Cq values of 18.0 and 17.4. The controls behaved as expected with the negative MP control being detected after 24.3 cycles while the positive control was detected after 11.2 amplification cycles (see Fig. 5.4). Due to the problem that was seen on several occasions when commercial testing was being performed, with negative control samples producing Cq values before the recommended end point of the PCR kit, it had been decided to adopt a Δ Cq method of analysis (i.e threshold setting based on a negative control sample; (see Ruiz-Villalba *et al.* 2021), with positive samples being identified on the basis of a difference in the Cq value between the sample and the negative control. Samples that were detected at least two amplification cycles before the MP negative control (Δ Cq $\geq$ 2) were recorded as positive while samples Δ Cq =1.5-2 were recorded as weakly positive.

This result showed that free DNA not due to the result of phage lysis of MAP cells was present in the freeze-dried sample which is detected by the qPCR regardless of successful phage lysis of the cells. However, the samples challenged with phage D29 gave a positive result significantly ($\sim$ 4 cycles) earlier than the control samples containing no phage indicating that viable MAP cells are present in the freeze-dried samples and that freeze-dried MAP preparation containing trehalose can be detected by the Actiphage assay and qPCR.

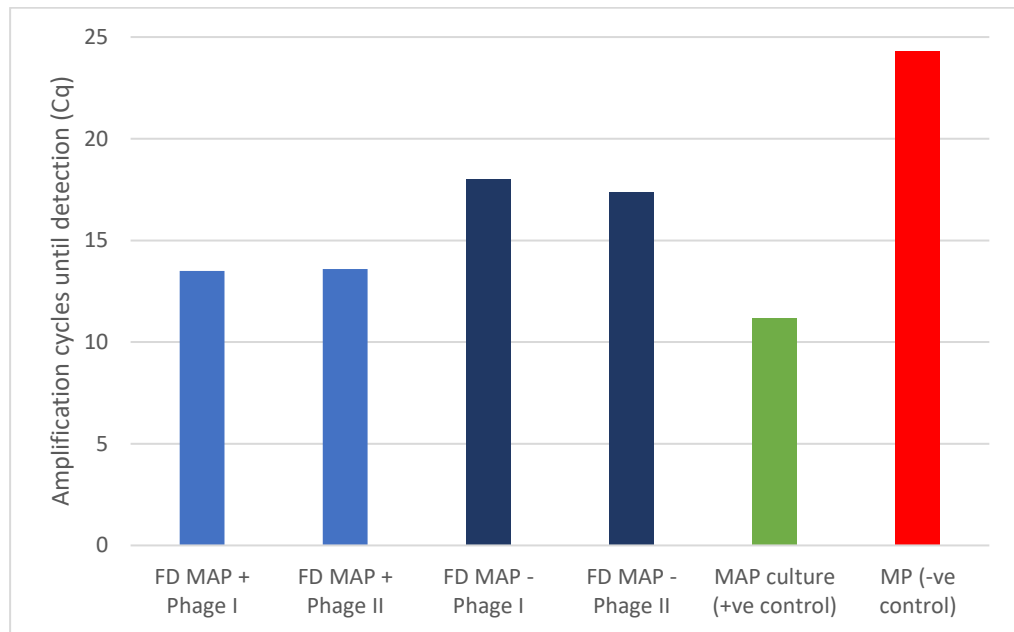
However, when analysing the experimental results it was noted that the controls used during the assay did not produce the expected values. For example, the 10 µl sterile MP -ve control produced a Cq value of 24.3 while it would normally be expected to produce a Cq value of 30-35. Meanwhile the positive control containing 100 MAP cells produced a Cq value of 11.2, significantly earlier than the 21 cycles expected and effectively overestimating the number of cells in the sample by $3 \log_{10}$. This and similar observations made in experiments performed by other members of the research group (data not shown) resulted in the discontinuation of the Ingenetix BactoReal kit in the Actiphage assay and the Biosellal Bio-T kit® for the detection of MAP (Biosellal, France) was used instead. This proved to be more consistent and reliable thus the exact mechanisms of failure of the Ingenetix BactoReal kit were not further investigated. However for reasons of consistency and comparability the remainder of MAP related experiments in this work was still carried out using the Ingenetix kit.

It was also noted that samples with no phage added were detected > 2 amplification cycles earlier than the MP negative control and would thus be interpreted as positive results indicating that the Actiphage assay worked flawlessly, which of course was not the case.

From the literature, two possible sources for this DNA contamination were identified. First, as mentioned above, this could be due to eDNA being produced by viable cells during growth in laboratory cultures. Second the extracellular DNA could result from MAP cells lysing and releasing their genetic material during freeze drying or storage. It is well known that freezing compromises the bacterial cell membrane and can lead to lysis (Didovyk *et al.*, 2017) and in previous experiments (see 5.2.5) it was found that freeze drying of MAP would incur titre losses indicating lysis of cells being an issue during freeze drying. Regardless of the origin, the high background levels of free DNA carried over from MAP culture made interpretation of results challenging and, so that the freeze-dried MAP preparation could be a useful positive

control in the Actiphage kits, work needs to be done to eliminate - or at least reduce -the amount of eDNA present in the final preparation.

Figure 5.4. Determining the presence of eDNA in freeze-dried MAP preparations



Free MAP DNA from the Actiphage assay was detected using qPCR. Duplicate samples (I & II) of FD MAP preparation underwent the Actiphage assay either with (+; light blue) or without (-; dark blue) phage added. Positive and negative controls consisted of 10 μ l sterile MP (-ve; red) or 10 μ l MAP culture containing 100 cells (+ve; green).

5.2.8. Washing of culture to remove eDNA

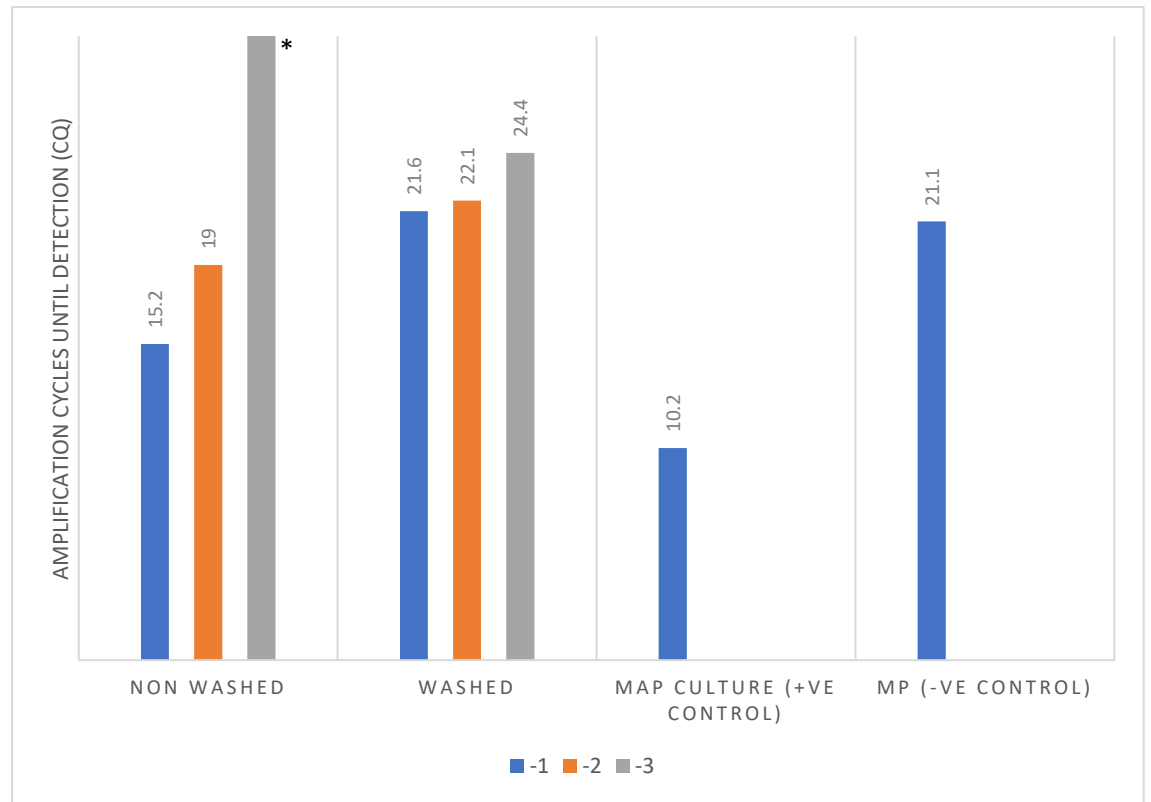
If the free DNA was present in the samples was eDNA produced due to the culture conditions used to grow the MAP cells prior to freeze drying, washing the MAP cells before freeze-drying could be a viable option for eliminating or at least reducing the amount of undesirable background signal seen in qPCR. To test this, five millilitres of high titre MAP culture ($\sim 1 \times 10^4$ pfu ml⁻¹) was serially diluted to 10^{-1} , 10^{-2} and 10^{-3} dilution levels before being fortified with 10 % (w/v) trehalose. A further 5 ml of the same MAP culture (undiluted) was washed as described in section 2.3.13. The washed MAP culture was then prepared for freeze drying the same way as the unwashed culture. Aliquots (0.5 ml) of both the washed and the unwashed cultures were then freeze-dried (section 2.3.10) and samples were subsequently rehydrated in double their original volume using MP. From each vial, a 10 µl sample of each freeze-dried MAP was tested using the Actiphage assay, with the standard positive and negative controls prepared according to the manufacturer's instructions (section 2.8). The results are shown in figure 5.5.

For this experiment, the standard assay negative control (MP alone) produced a Cq value of 21.1. This was again quite a high value for a negative control, but investigations carried out within the lab indicated that this was not due to contamination of the negative control sample, but may be due to PCR bridging (Pääbo *et al.*, 1990), and this again was addressed by using the ΔCq method of analysis.

The freeze-dried MAP cultures that had not been washed produced Cq values of 15.2 and 19.0 at the 10^{-1} and 10^{-2} dilution respectively while the 10^{-3} dilution did not produce a detectable qPCR signal after 45 amplification cycles. For the washed MAP cells, there was the expected increase in Cq values, but all of these were higher than the Cq value of the negative control (21.6, 22.1 and 24.4 at the 10^{-1} , 10^{-2} and 10^{-3} dilutions, respectively) and were therefore all scored as negative. These results indicate that washing of MAP culture by centrifuging and resuspending in fresh media can be used to reduce the concentration of eDNA in the culture, but the washing resulted in very high Cq

values that made these samples of limited use as positive controls due to problems being seen with high Cq values for the negative control samples.

Figure 5.5. Effect of washing on eDNA levels present in FD MAP cultures



MAP culture with a titre of $> 1 \times 10^4 \text{ pfu ml}^{-1}$ was either not washed or washed by pelleting at $4000 \times g$ and resuspending in fresh MP thrice before being 10-fold diluted (10^{-1} ■, 10^{-2} ■ and 10^{-3} ■). After dilution the samples were fortified using 10% (w/v) trehalose. All samples were then freeze-dried and rehydrated before being tested using the Actiphage assay.

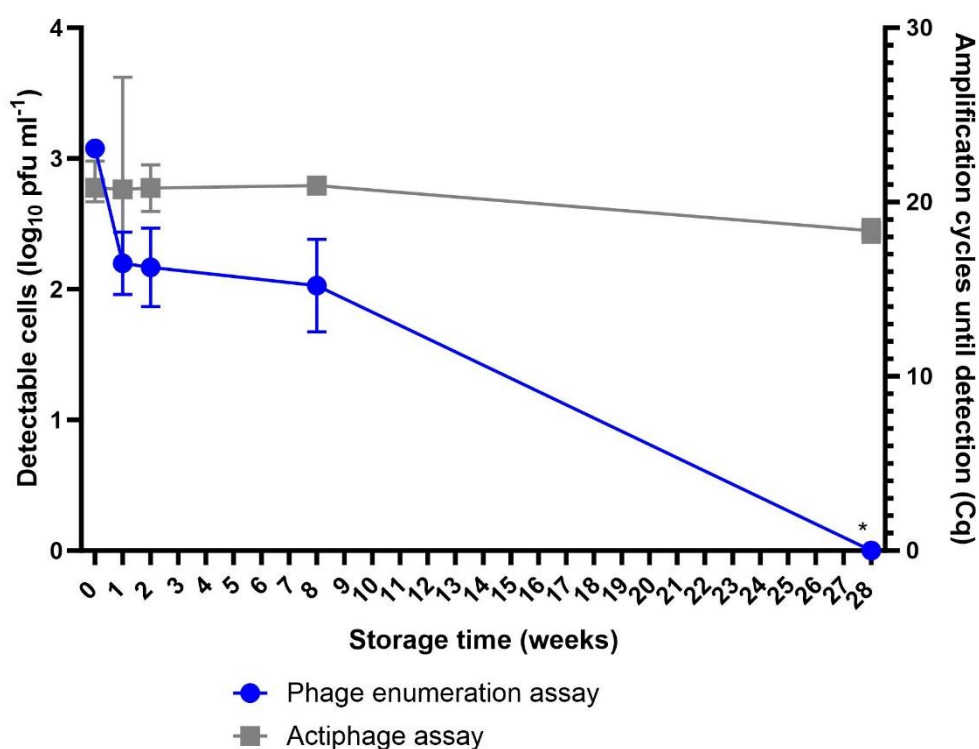
* Sample never crossed detection threshold after 45 cycles of amplification.

5.2.9. Stability of freeze-dried MAP preparations

To further investigate the e-DNA issue and the stability of the freeze-dried MAP samples during storage at room temperature over prolonged periods of time, a new batch of freeze-dried MAP preparations containing trehalose was prepared. For these experiments it was decided to compare the results gained using the Actiphage assay with the results gained using the two-day assay for the enumeration of MAP. This was done to allow live/dead differentiation as the two-day phage amplification assay would only be able to detect viable and actively growing MAP cells while the Actiphage assay could conceivably produce a false positive result due to detecting DNA of MAP cells that lysed during freeze-drying or storage rather than as a result of phage infection during the assay. The method to prepare the cells was also changed to include wash steps that were shown to reduce levels of eDNA prior to freeze drying the bacteria.

To this end, MAP culture with a titre of 4.6×10^4 pfu ml⁻¹ (titre of culture was determined by phage enumeration assay thus it expressed in pfu ml⁻¹) was washed by centrifuging at 4000 x g for 5 min at room temperature followed by resuspension of the pellet in its original volume of fresh MP, with this process being repeated three times. The washed MAP suspension was combined with 10 % (w/v) trehalose and 0.1 ml aliquots were freeze-dried (section 2.3.10) and then were stored in a dark place at room temperature. 0.1 ml aliquots were used as this was one of the intended volume options for use in the assay kits as it allowed one detection assay per vial of phage. To determine the number of detectable cells in these samples, six vials were rehydrated in 0.1 ml of MP. Three of the rehydrated MAP samples were tested using the Actiphage assay (section 2.8) using 10 µl samples from each vial. In parallel, the remaining three samples of rehydrated MAP preparation were mixed with 0.9 ml of MP and the number of detectable MAP cells determined using the phage enumeration method (section 2.6.2). To monitor the effect of storage on the detection events, this process was repeated at t= 1 week, 2 weeks, 8 weeks and 28 weeks. These results are shown in figure 5.6.

Figure 5.6. Comparing detectability of freeze-dried MAP culture stored at room temperature using the two-day phage amplification and Actiphage® Rapid assay



Freeze-dried MAP culture containing 10% (w/v) trehalose was stored at room temperature for up to 7 months. Detectability of MAP cells was then determined using the phage enumeration assay (●) and the Actiphage assay (■).

* Having a Log₁₀ value of 0 is mathematically impossible and represents detectable MAP titres of $< 1 \times 10^1$ pfu ml⁻¹. The scale of the graph was left unchanged to improve data representation.

5.2.9.1. Freeze-dried MAP tested without storage

Immediately after freeze-drying the enumeration assay was used to determine the number of detectable cells. Since we expect trehalose added as an excipient to cause sensitivity loss (see section 5.2.5 and 5.2.6) in the two-day assay when used at the 10^0 dilution all titre calculations were based on the number of plaques obtained using the 10^{-1} dilution. The number of detectable cells in the three vials tested was consistent (1.1 , 1.2 and 1.4×10^3 pfu ml $^{-1}$) but showed that a ten-fold reduction in the number of detectable MAP as a result of the freeze-drying process had occurred (original titre of MAP culture was 4.6×10^4 pfu ml $^{-1}$).

After performing the Actiphage assay for the detection of MAP and qPCR analysis (as described in section 2.8 and 2.9.2) the freeze-dried MAP samples produced strong positive results ($\Delta Cq \geq 2$; negative control $Cq = 30.45$) with Cq values of 20.08, 20.30 and 22.35 which were earlier than the assay positive control sample ($Cq = 25.82$) indicating that at least 100 cells had been detected.

5.2.9.2. Effect of storage and temperature on Freeze-dried MAP samples

The method of sampling described above was repeated after one week, two weeks, two months and seven months of storage at room temperature and the results gained are summarised in figure 5.6. After 1 week of storage a further 1 log $_{10}$ reduction in the number of detectable MAP cells were detected using the enumeration assay (1.63×10^2 pfu ml $^{-1}$; range 9×10^1 - 2.7×10^2 pfu ml $^{-1}$) compared to the average value detectable immediately after freeze-drying (1.2×10^3 pfu ml $^{-1}$; see Table 5.3)

More variability in the results was seen when the samples were tested after 1 week using the Actiphage assay. Two of the three samples produced strong positive results ($Cq = 16.37$, 18.67) but one sample was detected significantly later ($Cq = 27.17$), but still more than 2 Cq units earlier than the negative control ($Cq = 29.3$) and therefore was also scored as a positive result.

Table 5.3. Summary of storage trial results of freeze dried MAP cultures

Time of storage	Room temperature storage (~21 °C)				4 °C storage			
	average ⁽¹⁾ pfu.ml ⁻¹	Δtitre [log ₁₀] ⁽²⁾	Cq ⁽³⁾	ΔCq ⁽⁴⁾	average ⁽¹⁾ pfu.ml ⁻¹	Δtitre [log ₁₀] ⁽²⁾	Cq ⁽³⁾	ΔCq ⁽⁴⁾
1 wk	1.63 x 10 ²	n/a	20.08 20.30 22.35	10.37 10.15 8.1	n/a	n/a	n/a	n/a
2 wks	1.65 x 10 ²	+0.01	19.47 22.14	10.04 7.37	2.85 x 10 ²	+0.5	21.24 23.19	8.27 6.32
2 mths	1.25 x 10 ²	-0.12	20.56 21.35	9.48 8.69	2.45 x 10 ²	+0.18	21.42 22.67	8.62 7.37
7 mths	0	0	17.7 19.0	8.10 6.8	0 ⁽⁵⁾	0 ⁽⁵⁾	nd ⁽⁵⁾	nd ⁽⁵⁾
7 mths washed	nd ⁽⁶⁾	nd ⁽⁶⁾	22.2 24.4	3.6 1.4	nd ⁽⁶⁾	nd ⁽⁶⁾	n/a	n/a

⁽¹⁾ Average from duplicate samples

⁽²⁾ Difference in titre recorded from samples tested in week 1 (1.2 x 10³ pfu.ml⁻¹)

⁽³⁾ Results recorded for each of the individual samples tested

⁽⁴⁾ Difference in Cq value from negative control sample in the same experiment; values above 2 were scored as positive detection events

⁽⁵⁾ Sample vacuum lost during storage

⁽⁶⁾ Enumeration assay with wash step not performed as eDNA does not affect plaque count

At this point, due to the rapid loss in detectable MAP cells seen after one week of room temperature storage, it was decided to alter the experimental design and to store half of the freeze-dried MAP preparations at 4 °C to see if this would help stabilise the samples. For the samples stored at room temperature, there was very little change in the number of MAP cells detected using the enumeration assay for up to 2 months (Table 5.2 and Fig. 5.6). Interestingly, storage at 4 °C actually seemed to be beneficial with an increase of 0.5 log₁₀ recorded in titre (Table 5.2 and Fig. 5.7). This result was unexpected because normally a reduction in temperature induces a transition into stationary phase growth and renders the cells less detectable. The ability to recover higher numbers of detectable MAP cells by altering storage conditions indicate that the loss in detectable MAP cells encountered during storage is due to MAP cells entering a viable but non-detectable state and is at least partially reversible.

When the Actiphage assay was carried out. It was found that all samples produced strong positive results (see ΔC_q values in Table 5.3), however the results for individual vials were quite variable for samples stored at both temperature (individual results presented in Table 5.3). For qPCR detection, no particular pattern was seen in the results, again suggesting that issues with eDNA maybe making the results difficult to interpret.

The last sample point (2 months of storage) was performed in March 2020. It was initially planned to monitor the number of detectable MAP cells in the freeze-dried preparations after 3 and 6 months of storage. However due to the global pandemic caused by Covid19, and the resulting lock down measures, testing at these points could not be performed and the samples were tested in September of 2020. An additional problem was that during the laboratory closure, a complete power outage occurred that affected the refrigerators used for storing the samples of freeze-dried MAP preparations which were therefore exposed to temperatures above 4 °C for an undetermined period of time, as well as severe temperature fluctuation and unfortunately reduces the reliability of the results obtained for this set of

samples. When inspecting the vials of freeze-dried MAP preparation stored at 4 °C prior to sampling it was discovered that moisture had entered the vials and had partially re-hydrated the freeze-dried culture indicating a failure of the vacuum. This was confirmed when no characteristic hissing sound from air filling the vacuum was audible upon opening the vials. Nevertheless, the freeze-dried samples stored at 4 °C were re hydrated and titred using the phage enumeration assay for the detection of MAP but no cells could be detected at any dilution. In contrast the samples stored at room temperature appeared to be in perfect condition even after 7 months of storage and the vacuum was found to be intact upon opening of the vials. However, when the phage enumeration assay was performed on these samples no detectable MAP cells were detected in either.

To try and determine whether the variable results seen in earlier tests using the Actiphage assay were due to eDNA, a further four samples of freeze-dried MAP stored at room temperature for 7 months were re hydrated in their original volume and two samples underwent the Actiphage assay and qPCR for the detection of MAP without washing while the cells in the other two were washed three times by centrifuging at 13 000 x g for 5 min at room temperature before replacing the supernatant with an equal volume of MP.

For the unwashed samples, the results gained were similar to those seen at the other sampling points (Table 5.3) and these results did not correlate with the phage enumeration assay results which suggested that no detectable MAP cells remained (see Fig. 5.6). One possible explanation for this discrepancy is that the longer incubation period used when performing the Actiphage assay (3.5 h compared to 1 h) allowed the cells present in the vials to resuscitate into a phage-infectable state.

As seen before, the washed samples were detectable between 4-5 cycles after the unwashed samples which represents a ~100 fold reduction in detectable MAP cells based on the previously made observation that a 1:10 dilution of MAP culture results in detection delayed by ~2 cycles. This change again suggested that washing the cells helped remove free DNA in the samples

which was possibly released by MAP cells lysed during storage. Despite the increase in the Cq values, the ΔCq analysis still scored these results as positive ($\Delta Cq \geq 2$ and $\geq 1.5-2$, respectively) when compared to the negative control indicating that viable cells had been detected after phage lysis.

5.2.10. Pre incubation as a means to increase the number of detectable MAP cells

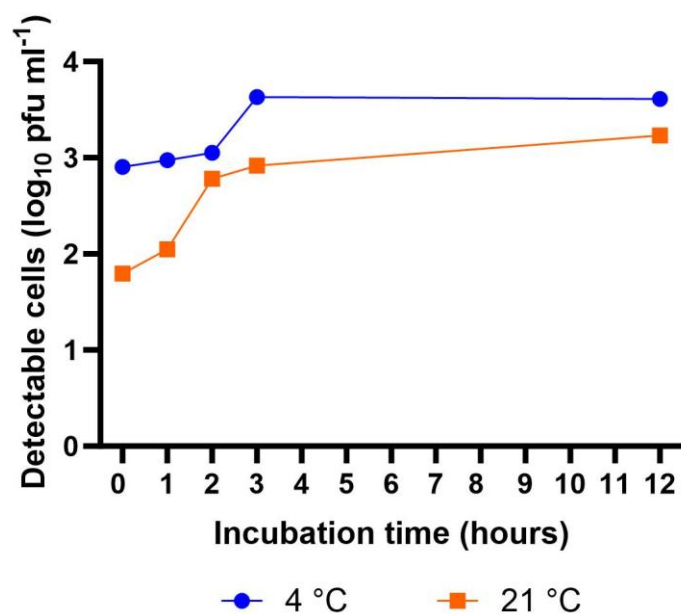
In the previous experiments it was noted that storage at 4 °C could reverse some of the titre loss seen when samples were stored at room temperature. This supported our hypothesis that the titre loss associated with freeze-drying and subsequent storage of MAP preparations was due to the MAP cells entering a viable but non-infectable state and could therefore not be detected by phage based detection methods. However, both fast and slow growing mycobacteria have the ability to quickly emerge from this stationary phase and resume active growth once conditions are favourable. Cultures of the fast growing mycobacterium *M. smegmatis*, starved for up to 250 days, have been shown to fully emerge from stationary phase and resume active growth within 5-10 hours of providing suitable growth conditions once again (Smeulders *et al.*, 1999). Meanwhile Nyka showed in a 1974 paper that *M. tuberculosis*, a slow growing mycobacterium, would resume active growth 9-14 days after providing nutrients when starved for up to 12 months. However, these results were based on the appearance of visible growth and it is reasonable to assume that productive phage infection and thus detection using phage based diagnostic tools could be achieved significantly earlier. These findings led to the hypothesis that the difference in the phage enumeration results and the Actiphage test results after 7 months of storage could be due to a difference in the incubation times, with the longer resuscitation period allowing cells to return to an active growth state that then supported D29 infection.

To investigate this it was decided to rehydrate 10 vials (n=2 for each set) of freeze-dried MAP preparation either stored at 4 °C or 21 °C (3 months old at

time of experiment). These were rehydrated in double their original volume (1 ml) of MP and pre incubate at 37 °C for 1 hour, 2 hours, 3 hours, and 12 hours before determining the number of detectable cells using the phage enumeration assay. The potential issue of MAP cells actively replicating and interfering with the results gained was deemed irrelevant since in the laboratory MAP is a particularly slow growing organism with a doubling time of 22-26 hours (Bannantine *et al.*, 2003).

The results of this experiment are shown in figure 5.7. The number of detectable MAP cells was initially (at time point 0) found to be significantly lower ($p < 0.05$) for vials stored at 21 °C compared to samples stored at 4 °C. However, after 12 hours of pre incubation at 37 °C there appeared to be no significant difference between the two samples ($p > 0.05$). Freeze-dried MAP cells stored at 4 °C required at least 2 hours of incubation before detectability increased significantly ($\sim 1 \log_{10}$ increase seen) whereas the samples stored at 21 °C started regaining detectability immediately. Hence, the number of MAP cells detectable in FD MAP samples stored at 4 °C reached $> 10^3$ pfu ml⁻¹ after 3 hours of pre incubation while the FD MAP preparation stored at 21 °C required overnight incubation to reach a similar number of detectable cells.

Figure 5.7. Effect of pre-incubating freeze-dried MAP cells on detection using the phage enumeration assay



Freeze-dried MAP cultures stored at 4 °C (●) or ~21 °C (■) were pre incubated at 37 °C for up to 12 hours and then the number of detectable MAP cells determined using the phage enumeration assay, n=2.

These results show that the number of detectable MAP cells can be increased greatly by incubating the rehydrated freeze-dried MAP sample at 37 °C for ~3 – 4 hours. It was also discovered that MAP cultures reached titres close to the levels in the original MAP culture used for freeze-drying (4.6×10^4 pfu ml⁻¹) when incubating the freeze-dried MAP preparations for up to 12 hours. These results confirmed that a large part of the titre loss associated with freeze-drying and storing mycobacteria is due to the mycobacteria entering a non-phage detectable growth state. From the results obtained it also appears that freeze-dried MAP preparations stored at 4 °C not only maintain higher numbers of detectable MAP cells but also higher numbers of MAP cells in overall terms since even after 12 hours of pre incubation the titre of MAP cells stored at 4 °C was $> 0.5 \log_{10}$ higher than the titre obtained from samples stored at room temperature. When comparing the increase in detectable MAP cells of the samples stored at 21 °C and 4 °C during incubation it was noted that the number of detectable MAP cells increased earlier and more gradually in the samples stored at room temperature with an increase in detectable cells from 1.2×10^2 to 6.3×10^2 pfu ml⁻¹ being recorded between one and two hours of incubation representing the fastest rise in detectable MAP cells. Meanwhile the increase in detectability in the samples stored at 4 °C was delayed by one hour but very rapid with an increase in detectable MAP cells from 1.1×10^3 to 1.7×10^4 pfu ml⁻¹ being recorded after 3 hours of incubation but no further increase was seen thereafter confirming that the MAP cells were not actively growing in the samples during the resuscitation period.

5.3. Freeze-drying of *Mycobacterium bovis* (BCG)

The Actiphage kits are used for both the detection of MAP in blood samples and *Mycobacterium bovis*. This section will deal with how the information gained when trying to develop a stable, freeze dried sample of MAP was used to try and make a freeze-dried sample of *M. bovis* BCG that could also be included in the commercial kits.

5.3.1. Introduction

The Actiphage® Rapid Blood assay has great potential for application in the control of bovine TB, however a live positive control consisting of detectable *M. bovis* cells is required as part of the kit to ensure the accuracy and reliability of the phage based detection assay. The requirements for a *M. bovis* positive control intended for use in the Actiphage® Rapid Blood assay kits are essentially the same as with MAP. The positive control needs to contain enough cells to allow reliable detection using the Actiphage assay using qPCR to detect released DNA from viable cells and maintain a stable number of detectable *M. bovis* cells for up to 6 months of storage at ambient temperature. In addition the bacterial cells included as a positive control also need to be in an infectable state for phage D29. As *M. bovis* is able to cause clinical TB in humans it must be handled under BS L3 containment conditions so it would be impractical to use this as a positive control sample. It was therefore decided to use the attenuated BCG vaccine strain of *M. bovis* which is sensitive to phage D29 and contains the insertion sequence IS6110 used for the identification of the MTBC complex but only requires BS L2 containment conditions (Advisory Committee on Dangerous Pathogens, HSE, 2023) .

5.3.2. Results

5.3.2.1. Initial experiments on freeze-drying *Mycobacterium bovis* (BCG) with and without trehalose

As 10 % (w/v) trehalose proved successful when freeze-drying MAP cultures, the first experiment performed was to try the same approach to freeze-dry *M. bovis* (BCG). To this end, trehalose was added to 3.5 ml of *M. bovis* (BCG) culture with a titre of 8.9×10^3 pfu ml⁻¹ (as determined using the phage enumeration assay; section 2.6.2) to a final concentration of 10 % (w/v). Samples of this (0.5 ml), and also samples of the BCG culture without excipients, were freeze-dried as before (section 2.3.10). Duplicate samples were rehydrated in double their original volume using MP and the number of detectable cells determined using the phage enumeration assay.

The results are shown in Table 5.4 and unexpectedly there seemed to be no beneficial effect of adding trehalose when freeze-drying the *M. bovis* BCG cells as both sets of samples lost $\sim 2 \log_{10}$ in detectability and the difference in detectability between both sets of samples is not statistically relevant ($p=0.29$).

Table 5.4. Summary of data for initial *M. bovis* BCG samples

Sample type	No. detectable cells ^{(1) (3)} pfu.ml ⁻¹	Δtitre [log ₁₀] ⁽²⁾	Cq ⁽³⁾	ΔCq ⁽⁴⁾
Initial BCG culture	8.9 x 10 ³	n/a	17.5	n/a
<i>M. bovis</i> BCG without excipients	1.4 x 10 ² 3.3 x 10 ²	-1.8 -1.43	17.3 18.1	15.7 14.9
<i>M. bovis</i> BCG with trehalose	7 x 10 ¹ 1.2 x 10 ²	-2.1 -1.9	18.8 18.2	14.2 14.8

⁽¹⁾ Number of cells detected per sample using the phage enumeration assay

⁽²⁾ Difference in titre recorded from initial *M. bovis* BCG culture

⁽³⁾ Results recorded for each of the individual samples tested

⁽⁴⁾ Difference in Cq value from negative control sample in the same experiment; values above 2 were scored as positive detection events

For the Actiphage assay, as before 10 µl samples were used but DNA was detected using the Biosellal BioT kit (MTC) (Biosellal, France; section 2.9.3) which targets the MTBC specific insertion sequence IS6110. As seen when using the other PCR kits, non-specific amplification was seen with the MP negative control giving a signal after 33 cycles (the nuclease-free water sample was detected after 40 cycles). For this experiment, a sample (10 µl) of the non-freeze-dried BCG culture was also tested and gave a detectable signal after 17 cycles and the negative control (10 µl of sterile MP that underwent the Actiphage assay) gave a positive result after 33 cycles. The expected Cq value for 89 cells (predicted cell number based on a culture with titre of 8.9×10^3 pfu ml⁻¹) is dependent on the number of copies of the IS element in the target cell. *M. bovis* BCG is known to have only 1 or 2 copies of this IS element per genome (Fomukong *et al.*, 1992), therefore it would be expected that the signal from this number of cells would be lower, especially as the Biosellal kit positive control (BCG DNA) was detected after 26 cycles of amplification. This result again suggested that there may be free DNA, either produced as eDNA or due to release of DNA by cell lysis during the freeze drying process, in the samples that was decreasing the Cq value. The results obtained indicated that the addition of trehalose seemed to not have a significant effect on the retention of detectability of *M. bovis* BCG culture during freeze-drying when compared to cultures freeze-dried without excipients. However, the addition of trehalose may still offer benefits in regard to improving shelf stability of freeze-dried BCG preparation stored at room temperature and therefore it was decided to use trehalose in further experiments.

5.3.2.2. Freeze-drying *Mycobacterium bovis* (BCG) using trehalose as an excipient

Given the difficulties cultivating these slow growing mycobacteria, to produce a freeze dried sample that could be included in a commercial kit that contained a standardised number of detectable bacteria, it was decided that the easiest approach was to grow a culture to high cell density, and then

dilute this to give a standard number of detectable mycobacteria per vial. To this end, a fresh culture of *M. bovis* BCG was grown in MP until the titre reached 2.25×10^5 pfu ml⁻¹ as determined using the phage enumeration assay. Trehalose was then added to a final concentration of 10 % (w/v) and samples were freeze dried in 0.5 ml aliquots (section 2.3.10). Samples were rehydrated in their original volume using MP and then tested using the phage enumeration assay and the Actiphage assay. The samples analysed using the phage enumeration assay were brought up to a final volume of 1 ml before undergoing the assay. This was accounted for when calculating the total pfu ml⁻¹.

Using the enumeration assay it was found that the number of detectable cells in two samples tested had dropped by $\sim 0.5 \log_{10}$ approximately (7.5 and 6.9×10^4 pfu ml⁻¹). This means that the reduction in number of detectable cells was not consistent between experiments, and therefore it is difficult to predict the number of viable cells remaining after this process.

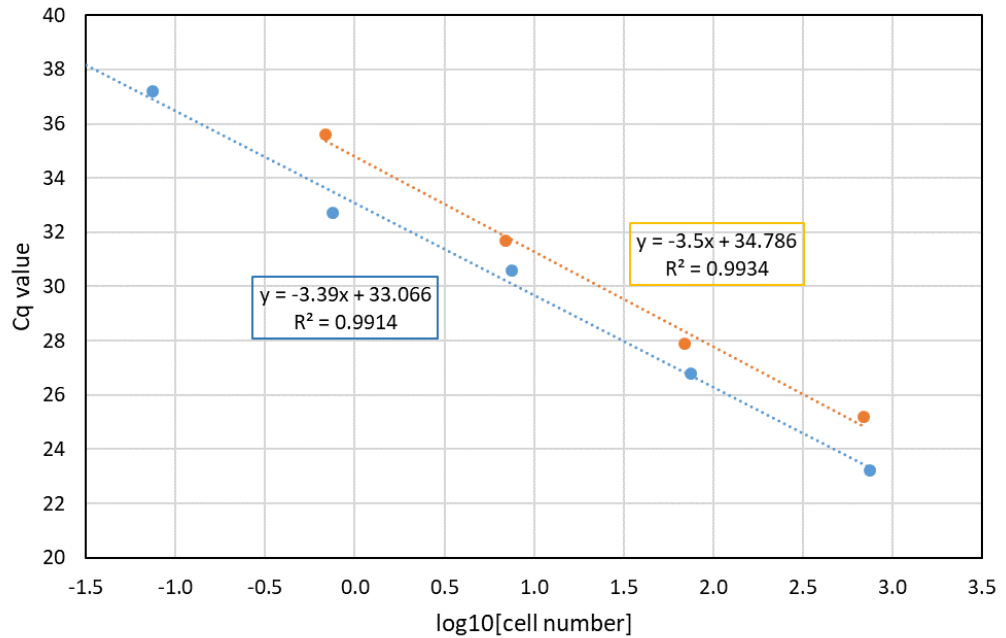
To identify how many cells in a sample would give a suitable signal as a positive control when using the Actiphage assay (the aim was to give a strong positive result but not with a Cq value less than 15 so that the signal was in the range of the other positive control samples in the qPCR kits), two rehydrated samples were diluted in 10-fold steps (10^{-1} to 10^{-4}) before the Actiphage assay was performed. 1 ml of diluted BCG culture with a titre of $\sim 2 \times 10^3$ pfu ml⁻¹ was used as a positive control while sterile MP served as a negative control.

The non-freeze-dried BCG culture with a titre of 2×10^3 pfu ml⁻¹ used as a positive control produced a Cq value of 30.7 while the MP negative control sample never crossed the detection threshold (total number of cycles = 40). The Cq values of the freeze-dried BCG cultures diluted to approximately the same cell number (10^{-2} dilution; Cq = 30.6 and 31.7) and the non-freeze-dried BCG culture (Cq = 30.7) were compared and it appeared that there was no significant reduction in the number of detectable *M. bovis* cells present in the two samples, which was not in agreement with the phage enumeration result.

Again, this may be due to the longer incubation period required for the Actiphage assay that allows cells to resuscitate and therefore be infected by the phage.

The results for the diluted samples are shown in Figure 5.8. In each case the PCR efficiency values were in the expected range (target range = 90-110%; sample 1 = 97.2%; sample 2 = 93.1%) indicating that no PCR inhibition was occurring. For sample 2, no signal was detected when the sample was diluted to 10^{-4} , however this was to be expected since, based on the phage enumeration results, this sample was predicted to contain only 0.069 detectable cells. The fact that the efficiency curves produced linear responses for all dilutions detected ($R^2 = <0.99$) again supports the idea that the phage enumeration assay is underestimating the number of cells in the samples that can be detected when using the Actiphage assay, otherwise it would be expected to see a non linear response at the lowest dilutions. However it is also possible that mycobacterial DNA from the culture or extracellular DNA (eDNA) was simply carried over and amplified in the PCR and this was just diluted out in a linear manner. Thus work was still required to determine the levels of eDNA contamination present in the freeze dried samples.

Figure 5.8. PCR Efficiency plots for the diluted *M. bovis* BCG samples



The *M. bovis* BCG samples were diluted in 10-fold steps before the Actiphage assay was performed and the DNA was detected using the Biosellal BioT kit (MTC; Biosellal, France). To determine PCR efficiency, the Cq values for each sample was plotted against the $\log_{10}$ cell number based on the results of the phage enumeration assay (sample 1 (●); sample 2 (●)) and the formulas for the trendlines generated for each sample set are shown in colour coded boxes. PCR efficiency was calculated according to the formula $(10^{(-1/\text{slope})} - 1) * 100$.

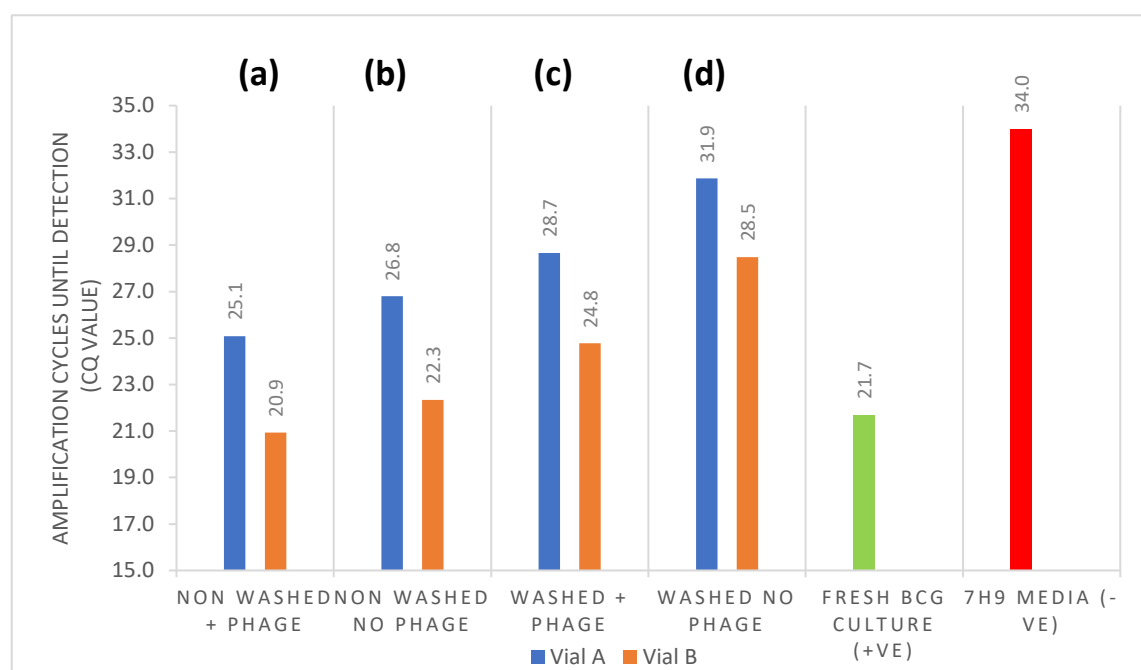
5.3.2.3. Evidence for the presence of eDNA in freeze-dried *M. Bovis* (BCG) samples

In previous experiments performed using MAP it was found that washing the cells helped reduce levels of eDNA present in samples (5.2.10). Based on these observations an *M. bovis* BCG culture was prepared with a titre of $\sim 1 \times 10^3$ pfu ml⁻¹, supplemented with trehalose (10 % (w/v) final) and 0.5 ml aliquots were freeze-dried. Two vials (A and B) were rehydrated in the original volume of MP. Half (250 µl) of each of the rehydrated samples were then transferred to a 1.5 ml microfuge tube. Cells were pelleted (13 000 x g for 5 min, room temperature) and then washed twice using 250 µl of MP before the cells were finally resuspended in 250 µl of MP. Actiphage assays were then performed using 10 µl samples of the washed and unwashed cells. Additionally, 10 µl samples of washed and non-washed cells were mixed with 110 µl of MP to create a “no phage” sample. This was done to assess the amount of background DNA present in the FD culture before and after the washing steps. After the Actiphage assays were complete *M. bovis* DNA was detected using the Biosellal BioT kit and the results are shown in figure 5.9.

For this set of experiments, the MP negative control gave a Cq value of 34, and all samples that contained *M. bovis* BCG cells produced Cq values lower than this and therefore were scored as positive test results ($\Delta Cq \geq 2$). For both samples, washing the cells resulted in an average increase in the Cq value of 3.75, which equates to ~ 13 -fold reduction in the level of DNA detected (see Fig. 5.9 panels (a) and (c)). When phage were omitted from the Actiphage assay process, again there was a reduction in the level of DNA detected in each case (compare panels (a) and (b) or (c) and (d) in Fig. 5.9). For the washed cells, the average increase in Cq value was 3.45, equating to a ~ 10 -fold reduction in DNA levels detected. For the washed cells the average increase in Cq value when phage was omitted was 1.55, which equates to only a 3-fold reduction in the levels of DNA detected. These results indicate that washing of the freeze dried cells in fresh media prior to use in the Actiphage assay appears to be a viable strategy of reducing the eDNA signal. However,

even when residual free DNA is present, the decrease in the C_q values seen between the test results gained with and without phage present can be used to show that the phage are active since more bacterial DNA is being released into the sample.

Figure 5.9. Effect of washing on the detectability of BCG culture using the Actiphage® Rapid phage detection assay



Duplicate samples of FD *M. bovis* BCG cells (Vial A ■ and B ■) were rehydrated and half of each sample underwent 3 rounds of washing to produce the washed samples (c and d). In addition samples of washed and non-washed cells underwent the process of the Actiphage assay but with no phage being added to the sample (b and d). After the Actiphage assay, *M. bovis* BCG DNA was detected using the Biosellal BioT kit. The last two panels show the results of the standard positive (fresh culture of *M. bovis* BCG) and negative controls (MP) prepared for the Actiphage assay.

5.4. Discussion

5.4.1. Freeze-drying of MAP and *M. bovis* (BCG)

The ultimate aim of this work was to develop a freeze-dried MAP culture that could be used as the positive control in the Actiphage assay kits for the detection of MAP developed by PBD Biotech LTD. The requirements for this positive control were A) to produce a standardised qPCR result that shows that the kit components are active and B) be stable for 6 months when stored at room temperature. Different excipient combinations including trehalose, sucrose, sorbitol and dextran were explored early on in the study, and ultimately trehalose was chosen as an excipient since it proved to be moderately successful at retaining higher numbers of infectable MAP cells after freeze drying and trehalose was already being used for freeze-drying and preserving the phage supplied in the Actiphage kit, so simplifying the manufacturing process. During these experiments a limitation of the use of trehalose was discovered in that an increase in trehalose concentration would generally result in a decrease the efficiency of plating when phage D29 suspension was spotted onto *M. smegmatis* lawns. It was not possible to determine whether these observations were caused by the trehalose impeding phage infection of host cells or were a result of another mechanism not currently understood, but does indicate that perhaps ultimately trehalose will not be the best excipient to use when preparing the positive control samples for the Actiphage kits.

Regardless of the excipient used, subsequent room temperature storage resulted in a large reduction in the number of detectable MAP cells based on the results of the phage enumeration method. However these samples still produce strong positive qPCR results after up to 7 months of room temperature storage. This result also led to the discovery that the samples contained significant levels of eDNA or free DNA. Washing the samples by centrifuging and re suspending cells in fresh media could negate this problem and produced samples that gave a large change in Cq value when phage were

added to the assay which could be used to indicate that the phage component of the Actiphage kit was active and therefore met the requirements of a positive control sample.

The exact *modus operandi* of eDNA production and utilization is still debated (Rose *et al.*, 2015) with some authors suggesting eDNA is the result of lysed cells (Qin *et al.*, 2007; Das and Manefield, 2012) while others suggested that eDNA is actively produced by live bacteria and exported into their environment. Indeed Barnes *et al.*, (2012) reported that *Enterococcus faecalis* would actively produce and export eDNA during biofilm formation without mass cell lysis. The role that eDNA plays in biofilm formation and cell aggregation suggests that eDNA binds to cell surfaces. This has been confirmed by Kavanaugh *et al.* (2019) who reported the presence of lipoprotein anchoring sites on the surface of *S. aureus* allowing eDNA binding. Based on these findings it seems very likely that eDNA anchored to the surfaces of mycobacterial cells will not be removed by pelleting and washing of cells and is instead carried over into the final freeze-dried positive control. Hence a possible approach for eDNA removal could be chemical degradation of DNA by using chemical agents such as sodium hypochlorite or DNase enzymes. However the former is a strong oxidizing agent and disinfectant likely to harm the MAP or BCG cells present while the latter is potentially expensive and untested.

5.4.2. Pre-incubation of freeze-dried culture as a means of increasing detectability

It has been known for a while that cold shock proteins (CSPs) play an important role in the adaptation of many bacterial species including *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* to environmental stresses. CSPs have been shown to facilitate the transition from active growth to stationary phase when bacterial cells are starved or experience a sudden drop in temperature and back to active growth when conditions improve (Bae

et al., 2002; Derzelle *et al.*, 2003). CSPs are mainly involved in the regulation of genes controlling metabolism and nutritional uptake but some also prevent misfolding of mRNA by bind to the mRNA molecule and acting as chaperones (Yamanaka and Inouye, 2001; D`Auria *et al.*, 2010). Most of this research has been carried out using *E. coli* but the mycobacterial organism *M. smegmatis* has also been shown to produce cold shock proteins facilitating its growth at 10 °C and transition into stationary phase when the temperature was reduced further (Shires and Steyn, 2001). In these experiments its was noticed that freeze-dried MAP preparations stored at 4 °C would consistently produce higher detectable MAP titres and would transition from stationary phase to active growth faster than freeze-dried MAP stored at room temperature. Hence it could be hypothesized that storing MAP at 4 °C induces a cold shock response and production of CSPs in a way similar to that previously reported in *M. smegmatis* which allows the MAP cells to survive in greater numbers and emerge from stationary phase faster. The samples of freeze-dried MAP that were stored at ambient temperatures (~21 °C) did not experience a significant temperature stress and thus would not have induced the cold shock response. The idea that the lack of CSPs present limited the transition to active growth once optimal growth conditions were restored is supported by the observation that even when optimal conditions were restored the MAP cells not exposed to 4 °C would resume active growth at a considerably slower rate compared to the cells that experienced refrigeration at 4 °C.

One limitation for exploiting this observation is that it was also noted that the freeze-dried MAP cells stored at 21 °C started recovering immediately, although at a slower rate, while the cells stored at 4 °C did not start to emerge from stationary phase until ~2 hours of incubation at 37 °C, but then regained more of its pre freeze-dried titre (over 90 %) within another one 1 hour of incubation. In contrast, the freeze-dried cultures stored at 21 °C only regained up to 80% of their pre-freeze-dried detectable titre even after 12 hours of incubation. This raised the question why a cold shock response is not initiated during the freeze-drying process where MAP cells were cooled and kept

frozen for multiple hours. However, cold shock adaptation seems to require at the very least 4 hours in *E. coli* (Sharma and Westman, 2004) and up to 24 hours in *M. smegmatis* (Shires and Steyn, 2001). The freeze-drying process allows no time for the cold shock adaptation and the expression of CSP genes before all biological activity including protein synthesis and enzyme activity ceases (Gottesman, 2018). From a practical perspective it was shown that the apparent loss in detectability when performing the two-day phage amplification assay on freeze-dried MAP and *M. bovis* BCG cultures stored at room temperature is almost entirely reversible and close to full viability of the culture can be regained simply by adding fresh media and incubating at 37 °C for ~3 hours which can easily be done in any BS L2 microbiology laboratory.

5.5. Conclusion

In this work we have shown that freeze-drying is an effective method for the preservation of MAP intended as a positive control in the Actiphage® Rapid Blood Test kits for the detection of MTC and MAP over multiple months without requiring refrigeration. The main focus must now be to optimize the freeze-dried MAP formulation developed in this project for large scale production and distribution as part of a commercial detection assay. More work needs to be carried out on developing methods to produce MAP culture reliably and consistently with the required number of detectable cells on a large scale. Our experiments on freeze-drying BCG have produced promising results however the shelf life at room temperature of this freeze-dried BCG preparation remains undetermined. Additionally, the large amount of background signal observed during the qPCR stage of the assay caused by eDNA present in the freeze-dried preparation remains a problem. Future work must therefore be done on reducing the eDNA contamination in the freeze-dried positive control as to make a false positive more distinguishable from a true positive result. Washing of MAP and BCG culture by centrifuging and resuspending of cells in fresh media thrice has been shown to reduce eDNA contamination significantly in our experiments. Additional cycles of washing and re suspending of culture in fresh media has the potential of reducing the amount of eDNA present further. An alternative approach would be to destroy free DNA present in the culture before freeze-drying by chemical means such as oxidizing agents or nuclease enzymes. Potential future work may also include the development of a combined freeze-dried positive control containing MAP and BCG to be supplied in one vial. This would make the Actiphage® Rapid Blood Test kits more user friendly as well as cheaper and easier to manufacture and distribute. Furthermore as the Actiphage® Rapid Blood Test kit is intended to be used and sold in countries with potentially extreme climates and/or lack lustre infrastructure it will be important to gain data on the behaviour of freeze-dried MAP preparations stored at sub optimal conditions like extreme temperatures (35+ °C) or high humidity.

Chapter 6

Evaluation of ACK lysis buffer for the isolation of MAP from blood samples for use phage based detection assays

6.1. Introduction

Mycobacterium avium ssp. paratuberculosis (MAP) is the causative agent of Johne's disease in a range of farmed ruminants including cattle, sheep, goats and deer (Bauerfeind *et al.*, 1996). In farmed, captive and free-living deer clinical signs of disease are diarrhoea, loss of weight and body condition (Palmer *et al.*, 2019) and 2 clinical syndromes have been described in red deer: sporadic disease with low morbidity and high mortality, and severe outbreaks in young (8–15 mo. old) deer resulting in both high morbidity and high mortality (Mackintosh *et al.*, 2004). In infected animals, MAP is increasingly shed in faeces as the disease progresses, and contaminated faeces then act as a source of transmission within herds by the faecal-oral route (Raizman *et al.*, 2005), but can also be transmitted through ingestion of colostrum from infected dams or *in utero* via the placenta and this is reported to be very high in both symptomatic and asymptomatic red deer (van Kooten *et al.*, 2006; Thompson *et al.*, 2007).

Although there are no good estimates of the economic impact of Johne's disease for commercial deer farming, it is known that the disease does pose a significant cost to other farmed species susceptible to Johne's disease (de Lisle *et al.*, 2003; Whittington *et al.*, 2019). For control of the disease in farmed deer it is important to have accurate tests that can detect infection at an early stage of infection (Schiller *et al.*, 2010; Whittington *et al.*, 2019). There is also the potential of non-farmed deer serving as a reservoir for MAP in the wild with the risk of transmitting the pathogen to herds of domesticated animals such as cattle or sheep. However, neither the prevalence of MAP in wild deer populations nor the interactions of these wild deer with domesticated animals have been thoroughly studied so there is currently no clear answer as to what extent wild deer serve as a reservoir for MAP. There also appear to be considerable geographical differences in the prevalence of MAP in wild deer. For example, Kopečna *et al.* (2008) found that MAP could be detected in between 1 and 5% of sampled wild deer in the Czech Republic while Robino *et al.* (2008) found that up to 32% of red deer and 22% of roe deer in

northwestern Italy were MAP positive. Carta *et al.* (2012) however found no evidence that deer served as a wild reservoir for MAP on the Iberian Peninsula. Meanwhile MAP Culture-based diagnostic approaches that can be used for other pathogenic organisms are not appropriate for mycobacterial pathogens due to their very slow *in vitro* growth rates. Hence culture-based tests require from 4 weeks (automated liquid culture) to 20 weeks (culture on solid media; Whittington *et al.*, 1998). The main diagnostic tool currently employed for the rapid detection of paratuberculosis in veterinary samples is an antibody ELISA which detects the presence of MAP-specific antibodies in either blood or milk produced by the animal in response to infection. In cattle populations, the MAP ELISA is often used as a front line test due to its availability and fast sample throughput, but its shortcomings include low sensitivity and accuracy, and it is often unable to detect the early stages of the infection (Schiller *et al.*, 2010; Whittington *et al.*, 2019). However, IGg ELISA tests that have been optimised for cattle samples and do not retain their performance characteristics when used to test blood samples from other species (Reyes-García *et al.*, 2008; Prieto *et al.*, 2014). An IgG1 ELISA for detection of paratuberculosis in deer (Paralisa™) has been developed in New Zealand and has been shown to be of value for screening deer herds to identify high shedding animals (O'Brien *et al.*, 2013), although this was not found to have a good predictive value in earlier stages of infection.

When performing the Actiphage® assay in cattle (Swift *et al.*, 2013; 2020) blood mononucleocytes are the target sample since these are the main targets of infection of mycobacterial pathogens in blood (Silver *et al.*, 1998; Rathnaja *et al.*, 2017). When developing the phage-based assay, it was found that one of the critical steps is the removal of red blood cells (RBCs) from the sample as these can inhibit phage infection (Swift *et al.*, 2013). Traditionally density gradient separation, such as the Ficoll buoyancy gradient method, is widely used for the separation of monocytes and other white blood cells (Menck *et al.*, 2014). This method has also successfully been employed for the isolation of PBMCs and the detection of *M. tuberculosis* in clinical samples (Su

et al., 2019). However this method is time consuming, requiring several centrifugation steps, and is sensitive to variation in both sample age and also temperature. Recently, it was shown that differential sedimentation methods developed for the isolation of human white blood cells are compatible with the Actiphage assay (Verma *et al.*, 2020) and that recovery of infected white blood cells from milk by centrifugation is also a suitable method to prepare samples prior to the phage assay (Gerrard *et al.*, 2018). Therefore the method used to isolate the white cells does not seem to be critical for the successful detection of mycobacteria using the phage assay.

6.1.1. Aims and Objectives

The first aim was to investigate whether a differential lysis approach to remove the RBCs from a blood sample using Ammonium Chloride-Potassium (ACK) lysis buffer was compatible with the Actiphage assay. The ACK lysis method lyses erythrocytes as a result of osmotic stress caused by the uptake of ammonium chloride before cell debris is removed by washing in a buffer that is osmotically balanced for the white blood cells and has been shown to be effective for obtaining purified white blood cells from whole blood samples (Chernyshev *et al.*, 2007; Brown *et al.*, 2016). Initially experiments were performed using bovine samples to determine the effectiveness of the new method by comparing results with the standard commercial test method that uses Ficoll isolation of PMBCs. Then the ACK lysis buffer method was evaluated for the isolation of blood mononucleocytes from deer blood to see if it could be used to detect MAP in the blood of naturally infected, farmed deer (results published in Kubala *et al.*, 2021)

6.2. Results

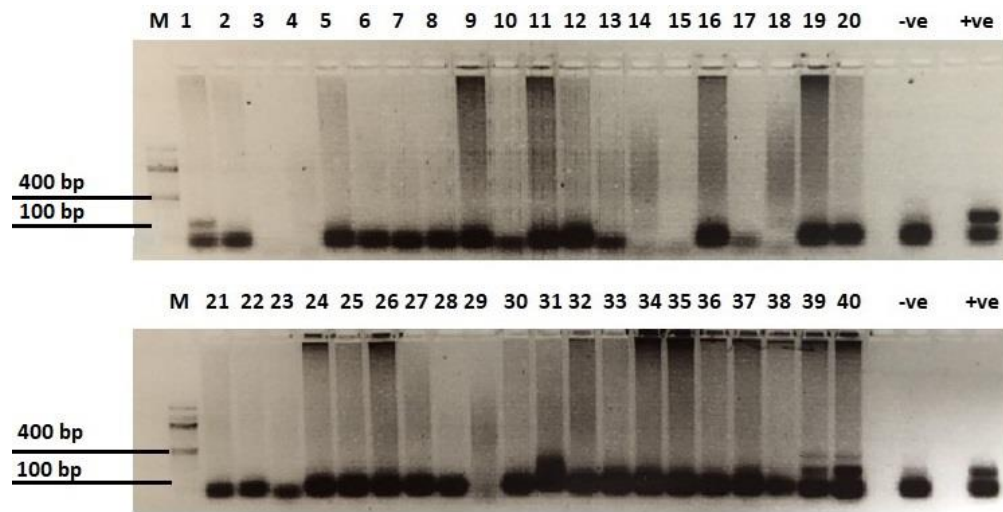
6.2.1. Phage based detection of MAP in deer blood using end point PCR

MAP infections in farmed and wild deer have become a key area of interest and concern in the last two decades due to the direct economic impact on the deer farming industry as a result of reduction in meat and fertility associated with Johne's disease as well as potential health risks to humans consuming meat contaminated with MAP (Whittington *et al.*, 2019). There is also a risk of wild deer serving as a reservoir for MAP with the potential of spreading it to cattle herds (Kopečna *et al.*, 2008; Robino *et al.*, 2008; Carta *et al.*, 2012). MAP control strategies rely heavily upon herd screening which requires a fast, reliable and ideally inexpensive diagnostic tool. It was therefore decided to attempt detecting MAP in deer blood using the Actiphage rapid detection assay.

Forty deer blood samples were obtained from a deer farm in Lincolnshire. Two millilitres of deer blood from each sample were treated using a standard Ficoll buoyancy gradient method for the separation of peripheral blood mononucleocytes (section 2.7.1). The PBMCs were subsequently re-suspended in 1 ml of MP and stored overnight at 22 °C in order to osmotically lyse the PBMCs and release any MAP cells potentially harboured within. The lysed PBMCs and any potential MAP cells were pelleted at 13000 x g for 1 minute before discarding the supernatant and re-suspending the pellet in 110 µl of phage D29 suspension with a titre of $> 1 \times 10^9$ pfu ml⁻¹ followed by the performance of the Actiphage assay for the detection of mycobacteria as per the manufacturer's instructions (section 2.8). Briefly: Isolated PBMCs resuspended in phage D29 were incubated at 37 °C for 3.5 hours to allow the phage to lyse any mycobacterial target cells present and release their nucleic acids. After incubation the reaction mixture containing raw DNA from lysed cells was filtered to remove any cell debris. The DNA obtained from the Actiphage assay was subsequently cleaned and concentrated using the Zymo clean and concentrator kit (Zymo, USA; see 2.8.1) and eluted in 14 µl of warm

(~50 °C) nuclease free water. 5 µl of cleaned and concentrated DNA was used as template for the IS900 PCR reaction (section 2.9.1). The expected size of the IS900 PCR product is 394 bp (Foddai *et al.*, 2020). It was found that two samples produced a positive result (#39 and #40), 37 samples produced a negative result, and one sample (#31) was inconclusive due to no clear separation of the bands on the agarose gel (see figure 6.1). This result showed that the method developed for use in cattle could also be used to detect MAP in deer samples.

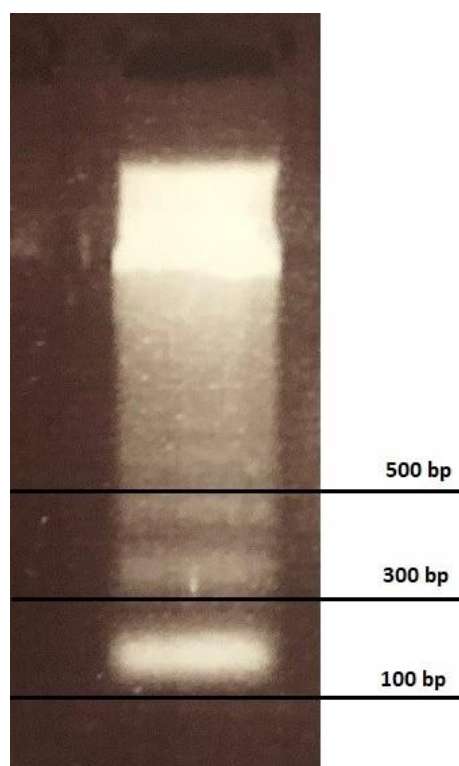
Figure 6.1. Detecting MAP in 40 deer blood samples



Gel image showing results of 40 deer samples tested for MAP using the IS900 PCR. PCR products from the Actiphage assay were analysed on a 1.5 % (w/v) TAE agarose gel containing ethidium bromide (125 μ M). The DNA was separated at 75V and the gel was subsequently visualised using UV light (section 2.9.1). Samples in lane 39 and 40 were recorded as positive, sample in lane 31 was inconclusive, all other samples were found to be negative. Lanes labelled "M" represent 100 bp molecular weight marker.

It was noted that most samples produced considerable amounts of background smearing, especially near the well used for loading the sample onto the agarose gel. This indicates that the contaminating DNA consists of strands much larger than the 100 bp marker used as a frame of reference and it was therefore concluded that it is most likely eukaryotic DNA carried over from the original deer blood sample. To test this hypothesis it was decided to run non-amplified template obtained from a deer blood sample that was determined to be MAP negative in the previous experiment. Thus, an agarose gel was loaded with 20 µl cleaned and concentrated, non amplified DNA obtained from an actiphage one day phage assay that produced a negative result for MAP. The agarose gel was subsequently run as described in section 2.9.1. When analysed (see figure 6.2), it was found that vast amounts of background DNA were present in the MAP negative deer sample causing significant smearing also seen previously (see figure 6.1) and supporting our hypothesis that the smearing is due to eucaryotic DNA present in the DNA obtained from the Actiphage assay. Meanwhile RNase free water -ve control behaved as expected and did not produce a band.

Figure 6.2. Gel image of non-amplified deer DNA.



Gel image showing visualized DNA of non-amplified deer sample run on a 1.5 % (w/v) TAE agarose gel containing ethidium bromide (125 μ M). The DNA was separated at 75V and the gel was subsequently visualised using UV light (section 2.9.1). Lines indicate band sizes relative to 100 bp molecular weight marker.

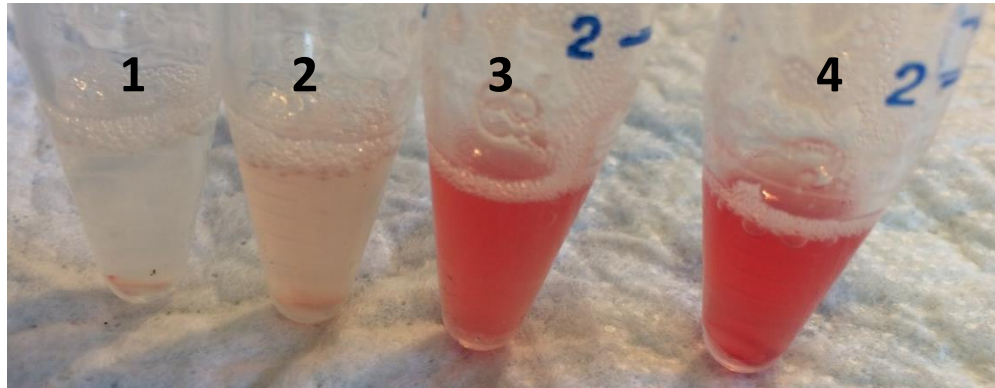
6.2.2. Use of ACK buffer for the Isolation of peripheral blood mononucleocytes

Since it has been shown that the method used to isolate the white blood cells does not seem to be critical (see section 6.1), it was decided to investigate whether a differential lysis approach to remove the RBCs from a blood sample using Ammonium Chloride-Potassium (ACK) lysis buffer was compatible with the Actiphage assay. Initially experiments were performed using bovine samples so the effectiveness of the new method could be determined by comparing results with a current commercial test method that uses Ficoll isolation of PMBCs. Then the ACK lysis buffer method was evaluated for the isolation of blood mononucleocytes from deer blood and evaluated to see if it could be used to detect MAP in the blood of naturally infected, farmed deer.

6.2.2.1. Initial experiments on cattle blood

At the time of this study the Actiphage test was being performed by PBD Biotech Ltd as a commercial blood test for detecting MAP in bovine blood samples. To determine whether the ACK lysis method could be used as an alternative method to purify white blood cells prior to performing the Actiphage assay, superfluous blood from bovine samples sent to PBD Biotech Ltd were used. On the day of commercial testing, white blood cells were also prepared from blood of 43 cattle of unknown infection status using the ACK lysis method described by Brown *et al.* (2016). Visible inspection showed that the ACK lysis method also produced purified white blood cells with low visible erythrocyte contamination (see Fig. 6.3) which is key indicator for samples to be successfully tested using the Actiphage kit. In fact the samples obtained using ACK appeared to have less erythrocyte contamination than the samples obtained using the Ficoll method.

Figure 6.3. Visual comparison of PBMCs obtained using ACK lysis buffer or Ficoll gradient



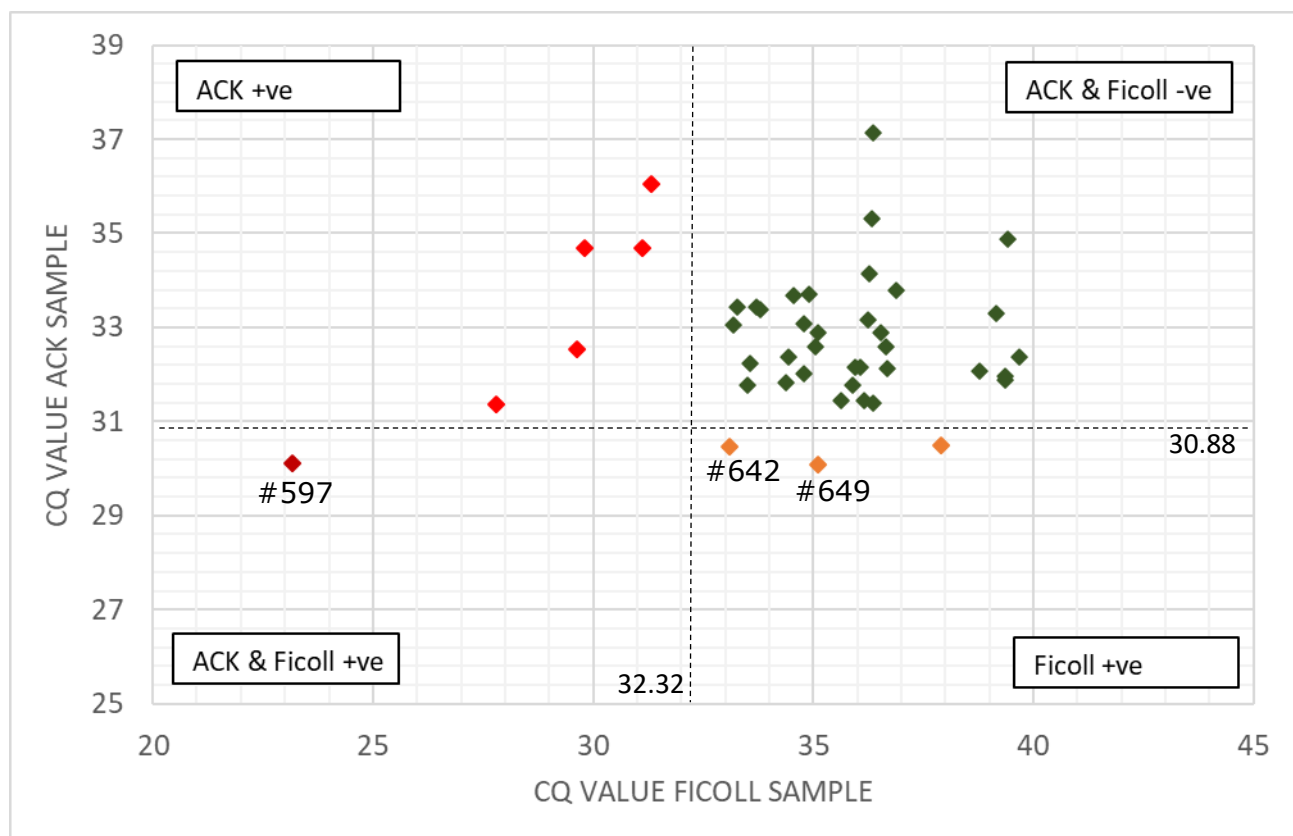
White blood cells obtained from cattle blood using the ACK lysis buffer method (samples 1 and 2) and the Ficoll buoyancy gradient method (samples 3 and 4).

After the PBMCs were isolated using the different methods, the rest of the Actiphage assay was performed on these samples. The Purified DNA resulting from the assay was tested for the presence of MAP DNA using a commercial qPCR kit (Bactoreal, Ingenetix). The signal from the internal positive control (IPC) indicated that no PCR inhibition was detected when the DNA samples prepared using either Ficoll or ACK method were tested, indicating that changing the extraction method did not result in a lower quality DNA sample (data not shown). The IS900 target DNA positive control sample provided in the kit that contained approximately 10,000 copies of the MAP-specific target sequence gave a Cq value of 20.54 (expected range 19-22). A positive control sample containing approximately 100 freshly grown MAP K10 cells in MP was prepared, and in this experiment this sample produced a Cq value of 21.13. Based on the Cq value of the positive control the expected range for the MAP positive control would be 21-22 as the MAP K10 strain contains 17 copies of the IS900 element (i.e. 100 cells contain 1700 copies of the IS900 target sequence). A negative control sample was also prepared consisting of just MP and the Actiphage reagent. This reagent still contains high levels of phage DNA and residual DNA from the *Mycobacterium smegmatis* strain used to propagate the D29 phage, and it is known that this may result in PCR bridging events. In this case the negative control samples give Cq values in the range of 30-32 and therefore these values were used to establish a base line for identifying positive test results (see Fig. 6.4). As in the previous chapter, analysis was done using a ΔCq method where samples detected at least two amplification cycles before the MP negative control ($\Delta Cq \geq 2$) were recorded as positive while samples $\Delta Cq = 1.5-2$ were recorded as weakly positive.

A comparison of the qPCR results of the 43 bovine samples prepared using the two different methods is shown in Figure 6.4. Using the standard Ficoll isolation method, MAP DNA was detected in 4 of the clinical bovine blood samples, two with a clear positive result ($\Delta Cq \geq 2$; #597, #649) and two gave a weak positive result ($\Delta Cq = 1.5-2$) indicating that very low levels of MAP (approximately 5 cells) had been detected. In comparison, using the ACK lysis

method, 6 positive results and no weak positive results were obtained. Comparing the pattern of results, the sample with the highest ΔCq value using the ACK group matched one of the two positive results for the Ficoll samples (Fig. 6.4; #597). Animal #642 gave a weak positive result with the Ficoll method and had a ΔCq of 1.2 for the ACK method, indicating that it was just below the threshold for a weak positive score. The two other positive results from the Ficoll set gave negative test results using ACK. It was not expected to get an exact correlation between positive test results when low levels of MAP are being detected in samples, since some stochastic variation in number of cells per sample is to be expected (see Gerrard *et al.*, 2018).

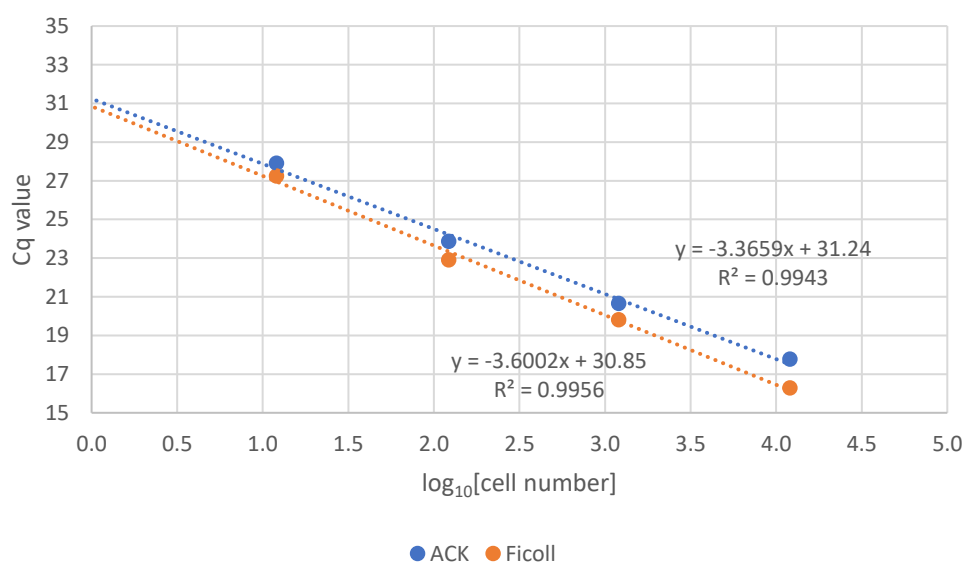
Figure 6.4. Comparison of Actiphage qPCR test results using ACK lysis buffer or Ficoll gradient for isolation of PBMCs.



The Cq values for the paired blood samples are compared using a scatter plot. Vertical and horizontal dashed lines are set at the cut off values for the Ficoll and ACK samples, respectively. Samples falling within each quadrant are colour coded and cut off values are given next to each line. The position of individual samples (#) referred to in the text are indicated.

For PCR efficiency determination, ten-fold dilutions of a MAP positive control culture with a starting titre of 1.2×10^4 cfu ml⁻¹ were used to inoculate white blood cells extracted using the two different methods with cell numbers from approximately 10 to 10^4 cells. For the Ficoll method the PCR efficiency value was 89.6% ($R^2 = 0.996$), with an intercept Ct value of 30.85, whereas for the ACK method the PCR efficiency value as 98.2% ($R^2 = 0.994$), with an intercept Ct value of 31.24 indicating that sensitivity achieved using the two sample preparation methods was very similar (Fig. 6.5). Overall from these results it was concluded that the ACK method was compatible with the Actiphage reagents as there was no evidence of inhibition and positive test results were achieved.

Figure 6.5. Comparison of PCR Efficiency when using samples prepared using either the ACK or Ficoll method



Comparison of Cq values for detection of PBMCs obtained using ACK buffer (blue) and Ficoll buoyancy gradient (orange) and spiked with 10^1 , 10^2 , 10^3 and 10^4 MAP cells. Trendlines connecting data points have a regression R^2 of 0.9943 (ACK) and 0.9956 (Ficoll) indicating good model fit. Intercept values were determined to be 31.24 (ACK) and 30.85 (Ficoll).

6.2.2.2. Determining lysis time of deer blood when treated with ACK buffer

When ACK lysis buffer is first added to blood it produces a cloud of turbidity that slowly dissipates over the course of 3-5 minutes as the red blood cells are being lysed. The end point of the reaction is reached when only a clear red solution with no visible sign of turbidity is left (see figure 6.6). As deer blood is known to exhibit unusual properties, for example the high prevalence of red blood cell sickling (Esin *et al.*, 2017), and the ACK lysis buffer method had only been used for human and cattle blood it was deemed important to determine the lysis time required for deer blood. This was investigated by measuring the turbidity of deer blood treated using ACK buffer using a spectrophotometer (650nm). To establish the base absorption of fully lysed deer blood, 1 ml of deer blood was added to 20 ml of ACK lysis buffer and allowed to react for > 10 min ensuring complete lysis before a sample was tested in the spectrophotometer. The resulting absorption (A_{650nm}) was found to be 0.039.

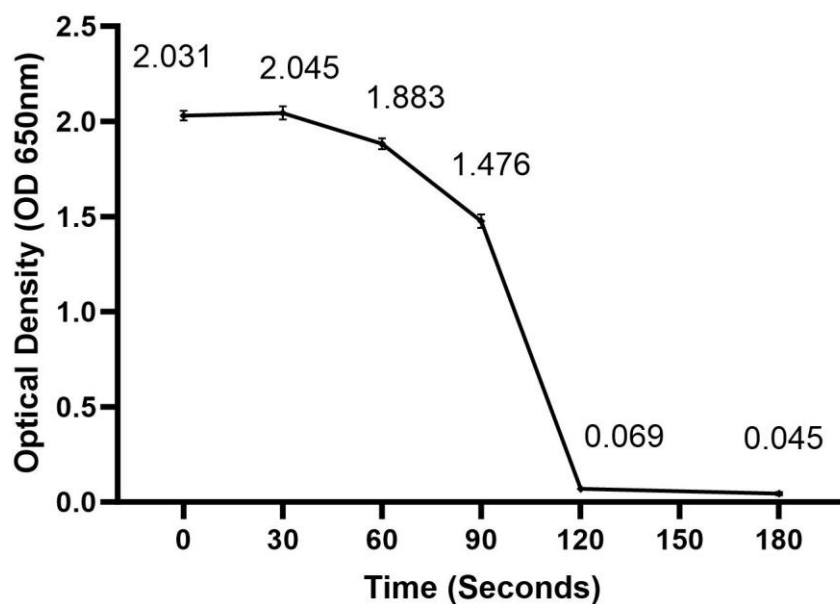
Figure 6.6. Deer blood in ACK lysis buffer before and after blood lysis



Deer blood in ACK lysis buffer, immediately after addition (left) and after ~4 minutes of agitated incubation (right). The turbidity encountered when adding blood to ACK buffer dissipates over the course of the reaction and a clear solution indicates complete lysis of all erythrocytes.

The experiment was repeated (in duplicate) but the absorbance was recorded at $t = 0, 30, 60, 90, 120$ and 180 sec after adding the ACK lysis buffer. As the undiluted sample was too turbid to allow a reading by the spectrophotometer it was diluted 1:10 in sterile water before measuring absorbance. After 120 seconds of agitated incubation at room temperature the decrease in optical density seen over time plateaued and by 180 seconds no further lysis seem to be occurring (see Fig. 6.7). These results indicate that the majority of the red blood cells present in the deer blood are lysed by the ACK lysis buffer after 2 minutes of agitated incubation.

Figure 6.7. Effect of lysis time of deer blood in presence of ACK buffer



The absorbance (A_{650nm}) of deer blood mixed with ACK lysis buffer was monitored over the course of 3 min with measurements being taken at $t = 0$, 30, 60, 90, 120 and 180 seconds after addition of ACK buffer ($n=2$).

6.2.3.2. Comparing the ACK lysis buffer and Ficoll buoyancy gradient method for isolating white blood cells from deer

Ficoll can be used to isolate white blood cells from many species of animal, including cervids, and ACK has been shown to be able to remove contaminating erythrocytes from cervid buffy coat preparations (Dugovich *et al.*, 2019). However deer blood is known to exhibit unusual properties due to sickling which can be associated with increased fragility of erythrocytes (Esin *et al.*, 2017). It was therefore deemed important to determine whether the ACK lysis method was an appropriate method for isolation of white blood cells from whole deer blood. To this end surplus material from 27 deer blood samples provided for commercial Actiphage testing were obtained and white blood cells were recovered from 2 ml samples using either the recommended Ficoll method or the ACK lysis method. Using Ficoll, the buffy coat layer was found to be faint or located below the frit in the Leucosep tubes, making extraction of the buffy coat layer more difficult (data not shown). In addition, the same pattern of erythrocyte contamination observed in the bovine blood samples (see Fig. 6.3) persisted even after washing with PBS, and many of the samples had a visible orange to red colour, indicating erythrocyte contamination. In contrast using the ACK lysis method, the purified blood mononucleocytes were off white to cream in colour indicating very little erythrocyte contamination was present.

After purification, the mononucleocytes were resuspended in MP to induce cell lysis and release any intracellular mycobacteria into the media. Then the Actiphage assay was carried out according to manufacturer's instructions. Due to a change in the reagents being used for the commercial Actiphage testing, on this occasion any MAP DNA present in both sets of samples was detected using the Bio-T kit® *Mycobacterium avium paratuberculosis* (Biosellal, France). Again no PCR inhibition was detected indicating that both methods of mononucleocyte isolation were compatible with the Actiphage assay reagents. Again the positive and negative control samples gave the expected results, with the MAP culture positive controls (approx. 10^2 MAP cells) giving

Cq values of and 22.2 for 20.8. In this instance none of the clinical samples produced a positive Actiphage result, however the Cq values for the paired samples were very similar with the average difference between the Cq values of the two sets being below 2% (Table 6.1) and were therefore not statistically different to each other ($p = 0.16$) indicating that the method of mononuclear isolation did not affect the quality of the DNA extracted from the Actiphage assay.

Table 6.1. Comparing qPCR results of deer blood samples treated using ACK lysis buffer and Ficoll

Sample	Blood Preparation method				Ratio <u>ACK:Ficoll</u> <u>Cq^c</u>
	ACK Cq	ΔCq^b	Ficoll Cq	ΔCq^b	
Act. +ve ^a	20.83	n/a	22.24	n/a	0.94
Act. -ve ^a	30.29	n/a	32.36	n/a	0.94
1	33.45	-3.2	40.72	-8.4	0.82
2	36.09	-5.8	35.49	-3.1	1.02
3	33.32	-3.0	32.51	-0.1	1.02
5	33.94	-3.7	33.00	-0.6	1.03
6	32.38	-2.1	32.33	0.0	1.00
7	34.40	-4.1	38.02	-5.7	0.90
8	33.57	-3.3	34.80	-2.4	0.96
9	28.38	1.9	33.76	-1.4	0.84
10	32.40	-2.1	34.00	-1.6	0.95
11	33.03	-2.7	34.35	-2.0	0.96
12	33.61	-3.3	33.86	-1.5	0.99
13	32.86	-2.6	31.86	0.5	1.03
14	33.63	-3.3	33.80	-1.4	0.99
15	32.79	-2.5	30.48	1.9	1.08
16	31.85	-1.6	33.44	-1.1	0.95
17	33.10	-2.8	35.57	-3.2	0.93
19	31.94	-1.7	33.84	-1.5	0.94
20	31.32	-1.0	31.96	0.4	0.98
21	43.93	-13.6	31.40	1.0	1.40
22	36.38	-6.1	35.09	-2.7	1.04
23	31.95	-1.7	36.73	-4.4	0.87
24	31.40	-1.1	35.03	-2.7	0.90
25	33.29	-3.0	34.40	-2.0	0.97
26	33.10	-2.8	33.20	-0.8	1.00
27	32.20	-1.9	30.82	1.5	1.04
29	31.95	-1.7	30.62	1.7	1.04

^a Actiphage positive control = Media Plus inoculated with approximately 10^2 MAP K10 cells; Actiphage negative control = Media Plus and Actiphage reagents alone.

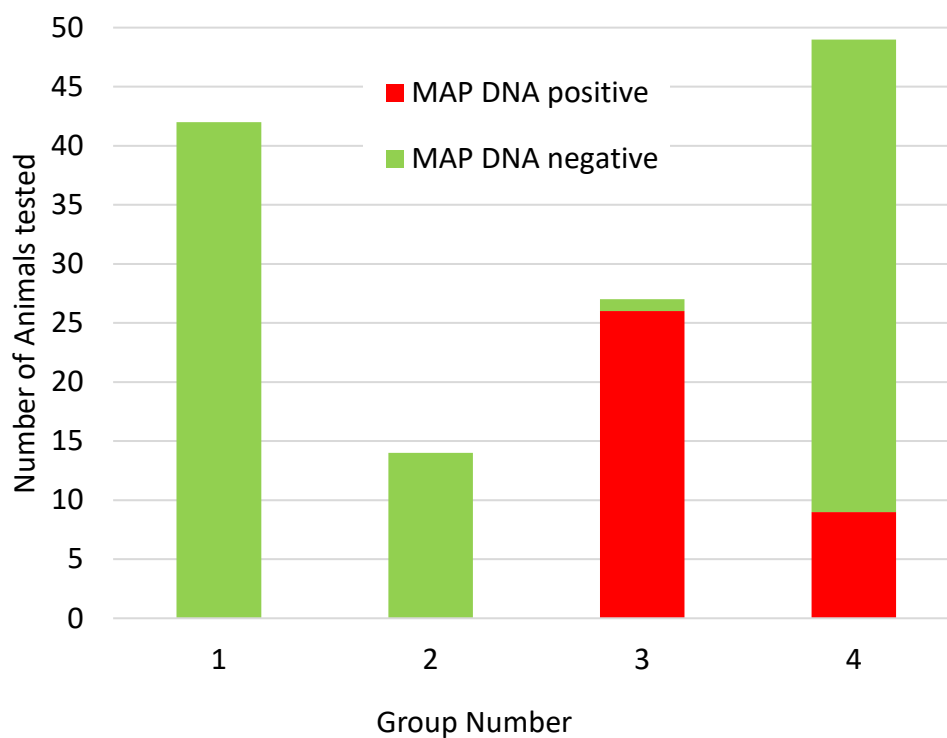
^b ΔCq ; difference in Cq sample compared to the Actiphage negative control. n/a = not applicable. Positive test result = $\Delta Cq \geq 2$; A weak positive test result = ΔCq 1.5-2 [weak positive results shaded in grey]

^c Ratio of Cq values = $Cq[ACK]/Cq[Ficoll]$ for each paired sample. Average value = 0.99. The one sample where this ratio was significantly larger highlighted in bold.

6.2.3. Detection of MAP in deer blood treated using the ACK lysis buffer method

After establishing that the ACK method could be used for the isolation and purification of mononucleocytes from whole deer blood, the method was used to test blood samples from a set of 132 female red deer that were obtained immediately prior to the autumn rutting season. These were treated using the ACK lysis buffer method and the purified peripheral blood mononucleocytes subsequently underwent the Actiphage assay again using the Bisoellal MAP kit (Biosellal, France) to detect MAP DNA. Again, the positive and negative control samples gave the expected results and no qPCR inhibition was detected. In this set of animals MAP was detected in the blood samples of 35 animals (27 %). Approximately 2/3 of the positive samples (21/31) produced strong positive results ($\Delta Cq > 5$), and the remaining 10 samples were scored as weak positive ($\Delta Cq 1.5-2$). Interestingly, when the animals were assigned to their production groups, it was found that all but nine of the animals that gave a positive test results belonged to one production group, and the rest were all found to belong to a second production group (see Fig. 6.8).

Figure 6.8. Distribution of positive actiphage MAP test results among commercial deer herd production groups



Blood samples were taken from 132 female deer prior to the rutting season. The samples were supplied to the lab blinded to production group. After the test results were available, the samples were unblinded and sorted by production group. Green bars indicate negative MAP test results; red bars indicate positive MAP test results.

6.2.4. Comparing the Actiphage assay + qPCR to MAP ELISA assay

The main alternative to culture for the detection of MAP in veterinary samples is the MAP antibody ELISA. As samples were being provided as superfluous material from part of a commercial testing program, it was not possible to obtain ELISA test results for the same set of animals. Therefore samples were obtained from a further 38 deer blood samples were treated using the ACK lysis buffer method as described. On this occasion all 38 deer blood samples gave a negative Actiphage test result, with the negative and positive test samples behaving as expected. The owner of the farm sent blood samples of the same 38 animals to Axiom Veterinary Laboratories (Devon, UK) who performed a MAP- specific antibody ELISA routinely used for the detection of MAP in veterinary samples. All samples were also found to be negative by ELISA confirming the results obtained using the Actiphage assay and ACK lysis buffer preparation method.

6.3. Discussion

6.3.1. The use of ACK buffer for the isolation of PBMCs from deer blood

In previous studies describing the use of the Actiphage assay to detect MAP in clinical blood samples, Ficoll gradients were used to recover blood mononucleocytes, however this method has its disadvantages in that it is laborious and requires costly reagents. The results in this chapter demonstrated that the ACK differentiation lysis method, developed for the rapid recovery of stem cells, is compatible with the Actiphage assay. Unlike INF- γ assays, the physiological state of the mononucleocytes is not important for phage-based detection assays. Rather it is the physiological state of the MAP cells that is critical, and the mononucleocytes are lysed during the first steps of the assay to release any intracellular MAP into a growth medium to keep them in a metabolically active state required for phage infection (Swift *et al.*, 2014). Indeed the additional osmotic stress that the mononucleocytes

experience during the ACK procedure may make them more susceptible to lysis which would also facilitate phage-based detection.

When preparing samples from deer blood, it was noted that there was less detectable erythrocyte contamination when using the ACK method.

Contamination of the Ficoll purified buffy coats may have been due to the fact that the buffy coat layer was formed very close to the surface of the erythrocyte layer, some of which may have been picked up while pipetting off the delicate buffy coat layer or may be due to the higher fragility of the erythrocytes causing premature lysis (Esin *et al.*, 2017). Hence for cervid blood samples, the ACK method had another advantage over standard Ficoll gradients.

6.3.2. Detection of MAP infection and JD in deer using the Actiphage® Rapid Blood Test kit

In this study, the Actiphage® Rapid Blood Test kit was used to screen animals from a commercial herd of farmed deer. The farm had sporadic problems with JD after some animals had been purchased, but no cases of clinical disease had been detected recently. Initially a set of female animals were tested, with blood samples provided simply in the order the animals entered the testing pen. In this set of results there was an unusual pattern, with a cluster of positive results occurring in samples collected at the end of the process. This pattern of results was explained when the test results were compared with information provided about the production groups, when it became evident that there was one main group (group 3) which had the highest level of MAP infection and a lower level of infection detected in group 4, whereas groups 1 and 2 did not give any positive test results. Since these groups are geographically separated on the farm, this information is now being integrated into the farm management plan to ensure that infected animals are not moved to the uninfected groups, and animals known to be infected are being prioritised for culling when numbers need to be reduced.

The variation in the levels of DNA detected in the same animal (Table 6.1) is to be expected as there will be stochastic variation in the number of MAP cells detected when only very low number of infected macrophage are present in the blood and only small (2 ml) blood samples are being tested (see Gerrard *et al.*, 2018). Our previous studies of MAP in bovine blood have detected only 5-50 MAP cells per sample using phage-based assays (Swift *et al.*, 2013, 2016) and therefore only a very small percentage of the PBMCs in a 1 ml sample (approximately 10^6) are likely to be infected. If larger samples could be tested, this would reduce the stochastic variation, but currently the viscosity of the sample which contains large amounts of bovine DNA derived from the lysed macrophages prevents larger volumes of blood processed using the Actiphage kit.

After this thesis work was completed, and when the farm testing program allowed, it was also possible to carry out some small scale test reproducibility evaluation work, and it was found that when repeat tests were performed within 1 month that the results gained were consistent (see Kubala *et al.*, 2021). When a set of animals that had previously given a negative Actiphage test result were tested again using both ELISA and Actiphage, both tests gave negative results. Since a commercial qPCR kit was used to detect the MAP DNA, and the specificity of the detection event comes from the qPCR step, this provides confidence that these are true positive results. However it was also clear that this non-optimised qPCR assay resulted in high background noise levels. The Bisoellal MAP kit (Biosellal, France) is optimised for the detection of MAP DNA in faecal samples and PCR bridging can lead to positive Cq values when the sample DNA contains high concentrations of off targets and primer concentrations have not been optimised (Lui *et al.*, 2002; Ruiz-Villalba *et al.*, 2017; Park *et al.*, 2020). If the PCR reagents are optimised, this may allow lower levels of cells to be detected with confidence.

6.4. Conclusion

The inherent specificity of bacteriophage D29 towards its Mycobacterial host cells, coupled with the high specificity and sensitivity of PCR-based detection methods has led to the development of the new, more sensitive Actiphage® Rapid Blood assay. The replacement of Ficoll gradients further simplifies the method and the chemicals required for the production of ACK lysis buffer (Ammonium chloride, Potassium bicarbonate and EDTA) are widely available and inexpensive. This proof of principle study demonstrated that Actiphage is a new tool that can directly detect the presence of MAP in cervid blood samples and clearly has potential for the control of Johne's disease. Further studies are required to fully validate the Actiphage method for detecting MAP infections in deer, including comparison with other standard diagnostic test methods such as the tuberculin skin test and MAP ELISAs. However, these results provided the first evidence that Actiphage is specific and in combination with the ACK lysis method can be used to detect MAP infection in farmed deer. Future work may also be carried out for optimizing the ACK lysis buffer and Actiphage® Rapid Blood assay for use in non-bovine and non-cervid animal species frequently suffering from MAP infections. MAP is a clinically and economically relevant pathogen in sheep (Singh *et al.*, 2014) and other domesticated ruminant animals such as camels (Salem *et al.*, 2019) and elk (Forde *et al.*, 2015). Johne's disease is of particular concern to the sheep industry with a 2020 review by Windsor and Whittington referring to ovine JD as "the most serious of concern to the (Australian) sheep industry". Control of MAP in sheep farming is especially difficult due to the large numbers of animals that require testing with Australia alone being home to over 60 million sheep (UNdata, 2020). The ACK lysis buffer and Actiphage® Rapid Blood assay could potentially play a significant role in the control of ovine JD by providing a sensitive and specific detection assay with results being available within a day.

Chapter 7

General discussion and future work

This project was instigated because of the need for a low cost, rapid, sensitive, and specific detection assay for MAP and *M. bovis* that could be used as part of the global effort to control these mycobacterial diseases. Successful control in farmed animals relies on herd surveillance and constant screening of animal populations for infection. This involves mass testing of individual animals, which in turn necessitates an inexpensive, fast, high throughput assay with high sensitivity and specificity. The Actiphage® Rapid Blood assay was developed in this laboratory and has shown great potential for the control of MAP and Johne's disease in farmed animals (Kubala *et al.*, 2021) as well as surveillance of wild animal population (Swift *et al.*, 2020) by offering sensitive and specific detection of mycobacterial cells in clinical blood samples in under 24 h. At the start of this project, PBD Biotech Ltd had just been established as a new spin out company of the University of Nottingham, and this provided the funding for this project. Hence, a key aim was to understand what needed to be done to move from a research methodology and create reagents that were appropriate for inclusion in a commercial test kit and also to refine the test method to make it more compatible with testing large numbers of samples.

7.1. Impact of the Covid19 pandemic on this project

The Covid19 pandemic and the lock down measures implemented by the UK government had a significant impact on this work. This is especially true for the work carried out on freeze-drying MAP and *M. bovis* which involved long term stability trials to determine culture detectability after storage at room- and refrigeration temperature. Detectability testing after 3 and 6 months of storage could not be carried out due to lock down and university closure. Additionally, the very nature of slow growing mycobacteria compounded the negative impact of the Covid19 pandemic on this work as MAP and *M. bovis* cultures had to be freshly set up after re-opening of the laboratory facilities and require 12-16 weeks before achieving titres sufficient for freeze-drying ($> 1 \times 10^5$ cfu ml⁻¹). In addition, as a fully industry sponsored student, I was placed on furlough by the company and therefore was unable to return to the

lab even when this partially reopened, further limiting my ability to complete some of the long term experiments.

A planned cooperation with the University of Jyväskylä in Finland (grant awarded by the Microbiology Society) to visualize freeze-dried phage and mycobacteria using a Scanning Helium Ion Microscope (SHIM) to better understand the nature of the sample produced had to be cancelled due to the pandemic. Using a SHIM would have allowed us to gain three dimensional images of freeze-dried phage particles and mycobacteria *in situ* thus possibly providing interesting insights into phage/bacteria interaction with each other and their surrounding matrix containing excipients. We were also interested in using the SHIM to explore phage D29 and TM4 interaction with host cells at low temperatures. Furthermore, a project with Professor Stephen Harding to investigate the effect of trehalose on phage efficiency of plating using molecular hydrodynamics and ultracentrifugation had to be postponed indefinitely.

The SHIM allows improved magnification to Scanning Electron Microscopes (SEM) or Scanning Gallium Ion Microscopes (SGIM) while also providing detailed 3-Dimensional images of phage and their surrounding matrix. SHIM technology has been used by Leppanen *et al.*, (2017) to produce images of *E. coli* bacteriophage T4 infecting host cells with a level of detail never seen before. It was planned to use SHIM imaging to gain detailed images of freeze-dried and frozen phage containing excipients and no excipients. This would provide visual data on phage-matrix/excipient interaction and potential physiological damage, i.e. burst phage capsids, damaged tail apparatus or tail fibres, inflicted on the phage particles during freezing and freeze-drying. High resolution images of phage frozen in plaques could also serve as an additional visual confirmation of the observation that phage would survive freezing in significantly higher numbers when embedded in agar compared to liquid suspension which is discussed in Chapter 3. In order to explain the observation that an increase in trehalose concentration would result in a reduction in the efficiency of plating of phage D29 on lawn plates it was

hypothesized that trehalose causes phage head aggregation, a phenomenon where phage bind to other phage thus forming macro molecular complexes. A study by Szermer-Olearnik *et al.* (2017) found that aggregation appears to be dependent on the ionic environment whereas a low ionic concentration (10 mM) would trigger spontaneous aggregation of 20-100 phage virions. This aggregation appears to be fully reversible by raising the ion concentration around the phage back to normal (150 mM) levels (Szermer-Olearnik *et al.*, 2017). Phage aggregates consisting of up to 100 virions reduce the number of free phage available to carry out infection of host cells while simultaneously reducing phage mobility in the agar due to increased molecular size and weight. To test this hypothesis it was planned to measure and compare the molecular weight of phage particles in the presence of different concentrations of trehalose by using molecular hydrodynamics and measuring molecular sedimentation rate during ultra-centrifugation. A trend towards faster sedimentation, and therefore larger molecules, in the presence of trehalose would lend credibility to the hypothesis of phage aggregating as trehalose concentration increases.

Completing these novel types of investigation would have added extra insights into the results that were gained during the thesis using more traditional microbiological methods.

7.2. Production of high titre phage lysate using SCLPs

Commercialisation and mass deployment of the phage based detection assays developed by PBD Biotech LTD. requires the large scale production of high titre phage lysate to be used in the assay kits. The phage lysate used in the phage assay needs to have a titre of $> 1 \times 10^8$ pfu ml⁻¹ however, in order to accommodate for the loss in phage titre expected during drying and subsequent storage it was decided that phage lysate with a titre of $> 1 \times 10^{10}$ should be produced. For many phage, this is achieved by preparing a large liquid lysate by infection of exponentially growing cells with a low number of

phage, allowing multiple rounds of replication and producing a large volume of high titre phage (see Bonilla et al., 2016). However this method is known not to work well for phage infecting many members of the phylum Actinomycetota, including the *Mycobacterium* genus, probably due to their very different cell wall architecture. This in itself was not a challenge on a small scale since it was known that picking plaques and eluting phage in a small volume of buffer would reliably yield phage lysate with the required titre. However picking of individual plaques, which was the main method employed for producing high titre phage lysate, was very time consuming and laborious (Fortier and Moineau, 2009). It was therefore decided to adapt a method by Bonilla *et al.* (2016) to produce SCLPs which would then be mechanically cut into small pieces and eluted in media. This method was found to be very effective with reliably yielding large volumes (> 10 ml per plate) of phage D29 suspension with titres of 10^{10-11} pfu ml⁻¹ while dramatically reducing the time and labour required for phage lysate production. Additionally, this method can easily be scaled up in size to accommodate the demand for larger quantities of high titre phage lysate. Disinfection of the phage lysate by centrifugation and filter sterilization is required to ensure no host cells are present in the final product. In the first stages of this project a method was successfully developed to be able to mass produce high titre phage suspensions that could be used in the Actiphage® Rapid Blood assay without the use of a bioreactor or the optimization work required to use such a system efficiently which were beyond the resources available to the company at the time. One unforeseen issue was that the lysates produced still contained high levels of *M. smegmatis* DNA and free D29 phage DNA, and this proved to be stable during the preservation processes. High levels of this target DNA were believed to be the reason for highly variable background amplification seen in the negative control samples prepared for the Actiphage assay. This has been attributed to PCR bridging (Liu *et al.*, 2002) which was originally described after the analysis of ancient DNA. It was found that damage to DNA promotes PCR bridging, and of course ancient DNA is inevitably in a damaged state when it is recovered from

preserved artefacts (Pääbo *et al.*, 1990). Recently Calcuttawala *et al.* (2022) have reported that infection of *M. smegmatis* by phage D29 induces apoptotic-like DNA fragmentation of the host cell genome, and this process may have resulted in the residual DNA present in the samples being highly fragmented and therefore even more likely to result in PCR bridging. This problem was overcome by the company by using a ΔCq approach to identifying positive samples. However, inevitably, this will lead to a loss of sensitivity of the Actiphage assay since samples with only low levels of target bacterial DNA will be lost against this background noise. Despite this, the reagents prepared using this method were shown to be of value when screening herds for Johne's disease (Chapter 6 and Kubala *et al.*, 2021). However, in the future, if a method could be developed, such as nuclease treatment, to remove the extraneous *M. smegmatis* and phage DNA from the Actiphage reagent without adding significantly to the complexity and cost of reagent production, it would be predicted that this would improve the assay performance.

7.3. Preservation of bacteriophage

The next challenge set by the company was to devise a method to stabilise the phage so that the high titre required was retained when the Actiphage[®] reagent was included in the commercial Rapid Blood assay kits. When the assay was developed, the phage were just prepared as a liquid phage lysate but this is not ideal due to the requirement of constant storage at refrigeration temperatures (4 °C) and the risk of contaminants such as fungi growing. The requirement of continuous refrigeration also poses further challenges for the application, distribution and overall commercialization of the phage based detection assays. Freeze-drying was found to be the most effective method for the preservation of phage D29 however, as previously reported in the literature (Leung *et al.*, 2018; Lavenburg *et al.*, 2020), a

significant reduction in the number of viable phage was encountered during freeze-drying if the samples were stored at room temperature storage.

Ultimately, trehalose was chosen as the excipient to be used in the freeze-dried D29 preparation. Merabishvili *et al.* (2013) also found that trehalose produced the best results for freeze-drying the *Staphylococcus* phage ISP (Myoviridae) when compared to other common excipients such as sucrose, mannitol, and glycine. Freeze-drying of another Mycobacteriophage, TM4 (Timme and Brennan, 1984) was attempted during this project and trehalose was also found to also protect this phage during and after freeze-drying and high titres ($> 1 \times 10^8$ pfu ml⁻¹) of phage could be recovered after freeze-drying and up to 6 months of room temperature storage (data not shown). TM4 is of interest to the company because its ability to infect mycobacteria that are not actively growing (Piuri and Hatfull, 2006; Swift *et al.*, 2016). Thus a combined phage D29 and TM4 preparation would be predicted to again increase assay sensitivity as it is predicted that D29 would not infect bacteria in stationary phase. The experiments completed in Chapter 5 support this conclusion, as clearly some of the samples contained cells that could not be detectable using D29, but they could be converted back into a detectable state when incubated in fresh media. Interestingly intracellular bacteria in clinical samples can be detected using D29 (Swift *et al.*, 2013; 2016; 2020) but it is possible that bacteria at other sites in the host may not be actively growing. It is known that MAP will enter a stationary phase *in vivo* when exposed to unfavourable conditions (Bacon *et al.*, 2014; Low *et al.*, 2020) and the ability to detect such stationary phase cells when testing other sample types could prove vital for successfully controlling JD. However, extensive verification and optimization will be required before phage TM4 can be included in the Actiphage® Rapid Blood assay kits and this is not currently seen as a priority for the company, but may prove to be an area of development in the future.

7.4. Freeze-drying of MAP

Developing a positive control for the Actiphage® Rapid Blood assay kits requires viable mycobacterial cells to demonstrate that the Actiphage® reagent is still active, thus proving that negative results are not due to kit failure and to allow confidence in the results obtained. Since phage D29 cannot infect and lyse mycobacterial cells that are dead or in stationary phase, these positive control samples also needed to be in an actively growing state. This requirement proved to be a major hurdle since MAP cells, in our experiments, tended to enter a stationary and non-detectable state during or quickly after freeze-drying. This issue was never fully resolved however it was discovered that rehydration of the freeze-dried cells in fresh MP followed by an incubation period of 3-5 hours at 37 °C would fully reverse the loss in detectability encountered during freeze-drying and storage with the number of MAP cells detectable using the one day phage assay for the enumeration of MAP reaching levels comparable to those obtained in non-freeze-dried MAP culture. Unfortunately, unlike the phage D29 samples, long term stability of the freeze dried cells stored at room temperature was not achieved. As mentioned above, a requirement for chilled storage is not ideal as it increases kit costs and limits distribution to areas with reliable cold store facilities. However the finding that the cells could tolerate short periods at room temperature as the detrimental effects seemed to be reversed when the cells were subsequently chilled means that these positive control samples would not have to be shipped under refrigeration conditions, which does simplify distribution issues for the company. Since the other carbohydrate-based excipients showed an equal ability to stabilise the MAP cells, it would be interesting in the future to see if any of these could be used to produce a product with better temperature stability.

The presence of large amounts of extracellular DNA (eDNA) in MAP and BCG cultures prior to freeze-drying has posed additional challenges as it was discovered that freeze-dried MAP and BCG positive control would produce a strong false positive result even when no phage was added during the assay.

The high copy number of free IS900 (MAP) and IS6110 (*M. bovis*) present in the freeze-dried preparation would not allow accurate differentiation between true and false positive controls. Repeated washing of culture prior to freeze-drying as well as washing of freeze-dried culture after rehydration has been shown to reduce the eDNA contamination significantly resulting in a detection of false positives ~3 amplification cycles after true positive results. However, washing of culture was only able to reduce the background signal caused by eDNA but not able to remove all eDNA present and samples of freeze-dried cells that underwent the Actiphage® Rapid phage assay without the addition of phage would still be detectable before the negative control. The most likely explanation for these observations is that washing will only remove eDNA freely suspended in the media used for growing the MAP or BCG culture. The role that eDNA plays in biofilm formation and cell aggregation suggests that eDNA binds to cell surfaces. This has been confirmed by Kavanaugh *et al.* (2019) who reported the presence of lipoprotein anchoring sites on the surface of *S. aureus* allowing eDNA binding. Based on these findings it seems very likely that eDNA anchored to the surfaces of mycobacterial cells will not be removed by pelleting and washing of cells and is instead carried over into the final freeze-dried positive control. Removal of eDNA bound to cell surfaces is likely going to be a particularly challenging endeavour as the mechanisms of eDNA production and binding in mycobacteria are not fully understood. A possible approach for eDNA removal could be chemical degradation of DNA by using chemical agents such as sodium hypochlorite or DNase enzymes. However the former is a strong oxidizing agent and disinfectant likely to harm the MAP or BCG cells present while the latter is potentially expensive and untested. However, overall the freeze-dried, washed MAP cells were shown to perform adequately as a first positive control for use with the Actiphage® Rapid Blood assay kits.

7.5. The use of ACK lysis buffer for the isolation of PBMCs intended to be used in the Actiphage® Rapid Blood assay kits

MAP, like other mycobacterial pathogens such as *M. bovis* and *M. tuberculosis*, is an intracellular pathogen that survives and replicates within macrophages thus evading detection by the hosts innate immune system (Nguyen and Pieters, 2005; Lim *et al.*, 2019). This is detrimental to the hosts ability to fight MAP infection, but it provides us with an opportunity to concentrate the low levels of mycobacterial cells found in a blood sample by isolating the peripheral blood mononucleocytes. The isolation of PBMCs is commonly done using the Ficoll buoyancy gradient method (Iralu *et al.*, 1993; Su *et al.*, 2019) which requires multiple centrifugation steps and a specialized density gradient media. The Ficoll gradient media is expensive making this method time consuming and expensive. Additionally, the Ficoll buoyancy gradient method has been found to be susceptible to temperatures, resulting in erythrocyte contamination, and also needs optimising for blood from different types of animals (Bøyum, 1976). PBMCs obtained using it were usually found to contain erythrocyte contamination. All these reasons make the Ficoll buoyancy gradient method not suitable for mass testing of veterinary samples in the context of controlling MAP in a commercial farm setting. Ammonium Chloride Potassium (ACK) lysis buffer was explored as an alternative method for the isolation of PBMCs as it has been reported to lyse erythrocytes efficiently and specifically but not macrophages or other blood mononucleocytes allowing those to be isolated by centrifugation (Chernyshev *et al.*, 2007; Brown *et al.*, 2016). In this study ACK lysis was found to successfully recover PBMCs from bovine and cervid blood and generally produced PBMCs with visibly lower levels of erythrocyte contamination. Direct comparison of the two methods for PBMC isolation and subsequent use in the Actiphage® Rapid Blood assay found that blood samples treated with ACK lysis buffer generally produced Cq values comparable to those obtained using the standard Ficoll buoyancy gradient method, with no evidence of PCR inhibition indicating that ACK lysis buffer can be used as an alternative to

Ficoll gradient centrifugation without losing sensitivity of the Actiphage[®] Rapid Blood assay. This represents a major improvement for the commercial test as it significantly reduces the time required to perform the assay (Ficoll buoyancy gradient takes around 60 min with centrifugation accounting for around 45 min; ACK lysis method takes around 20-25 min with centrifugation accounting for only 10 min), thus reducing costs and allows much higher sample throughput. Additionally the reagents required to produce ACK (ammonium chloride, potassium bicarbonate and EDTA di sodium salt) are cheap and readily available and simple to prepare, again reducing costs. Adopting this method also increases the flexibility of the Actiphage[®] Rapid Blood assay as it can be readily applied to a range of species without a need for sample preparation optimisation. This is particularly important in the veterinary diagnostic sector because MAP is able to infect a wide range of animal species, including numerous farmed animals and also exists in wild animal reservoirs. For instance it has been suggested that both badgers (Arsenault *et al.*, 2014) and deer (Balseiro *et al.*, 2008; Palmer *et al.*, 2019) act as potential reservoirs for MAP in the wild and this poses a challenge for any disease control programs. Hence the ability to use this method to recover PBMCs from a wide range of animal species opens new opportunities when designing control programs. For instance, it has been shown that the Actiphage[®] Rapid Blood assay can be used to test for *M. bovis* infections in captive lions (Molenaar *et al.*, 2020) and can also be used in combination with the ACK lysis method to detect *M. tuberculosis* in the blood of Indian Elephants (A. Hunyh, University of Nottingham, pers. Comm.). Hence optimization and validation of the ACK lysis buffer method for other animal species would be a good subject of future research.

7.6. Conclusion

The Actiphage® Rapid phage assay has great potential for aiding the global efforts to control JD in farmed and wild animals due to its ability to detect MAP rapidly and specifically in a wide range of veterinary and clinical samples with a high degree of sensitivity. Freeze-drying the biological assay components allow easier application and distribution of the assay, especially in developing countries. Optimization of the Actiphage® Rapid blood assay procedure by replacing Ficoll gradient centrifugation with ACK lysis buffer considerably reduced the cost and improved sample throughput for the detection of MAP in blood samples. These innovations will hopefully allow wider use and success of these phage-based diagnostic tools in combatting mycobacterial disease in general and JD in particular.

Chapter 8

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Chapter 9

Appendices

**Appendix 1: Paper published: Development of a method to detect
Mycobacterium paratuberculosis in the blood of 2 farmed
deer using Actiphage® Rapid**



Development of a Method to Detect *Mycobacterium paratuberculosis* in the Blood of Farmed Deer Using Actiphage[®] Rapid

Anton Kubala^{1,2}, Tania M. Perehinec¹, Catherine Evans¹, Andrea Pirovano², Benjamin M. C. Swift³ and Catherine E. D. Rees^{1,2*}

¹ School of Biosciences, University of Nottingham, Loughborough, United Kingdom, ² PBD Biotech Ltd., Link House, Elm Farm Park, Thurston, United Kingdom, ³ Pathobiology and Population Sciences, The Royal Veterinary College, Hatfield, United Kingdom

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*Correspondence:

Catherine E. D. Rees
cath.rees@nottingham.ac.uk

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Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, which is an economically and clinically relevant pathogen for commercial deer production. The purpose of this study was to develop a method that could be used to rapidly detect MAP infection in deer using the Actiphage Rapid blood test. This test has previously been used to detect MAP in cattle blood following the purification of buffy coat using Ficoll gradients, however this method is quite laborious and costly. The purpose of this study was to develop a simpler method of blood preparation that was also compatible with deer blood and the Actiphage test. Initially differential lysis of RBCs using Ammonium Chloride-Potassium (ACK) blood lysis buffer was compared with the Ficoll gradient centrifugation method using cattle blood samples for compatibility with the Actiphage reagents, and it was found that the simpler ACK method did not have an impact on the Actiphage test reagents, producing an equivalent sensitivity for detection of low levels of MAP. When the two methods were compared using clinical blood samples from farmed deer, the ACK lysis method resulted in a cleaner sample. When a blinded test of 132 animals from 4 different production groups was carried out, the majority of the positive test results were found to be from animals in just one group, with a small number identified in a second group. The test results were found to be reproducible when a small set of positive animals were tested again 1 month after their initial testing. Finally a set of negative animals which had been previously screened using an ELISA test, all animals gave a negative Actiphage result. This study shows that this improved sample preparation method and Actiphage blood testing can be used to test blood samples from deer, and the full diagnostic potential of the method can now be evaluated.

Keywords: *Mycobacterium paratuberculosis*, Actiphage, deer, bacteriophage, qPCR

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow growing acid-fast bacterium that is the causative agent of Johne's disease in a range of farmed ruminants including cattle, sheep, goats and deer (1). Johne's disease is a chronic inflammation (granulomatous enteritis of the intestine primarily affecting the jejunum and ileum) resulting in inhibition of nutrient absorption and

leading to chronic wasting of the animal resulting in reduced meat yields, reduced fertility and premature death of the animals (2). Although good estimates of the economic impact of Johne's disease for commercial deer farming are not available, it is known that the disease does pose a significant cost to other farmed species susceptible to Johne's disease (3, 4).

In farmed, captive and free-living deer, diarrhoea, loss of weight and body condition are clinical signs of disease (5). Two clinical syndromes have been described in red deer: sporadic disease with low morbidity and high mortality in adult populations, and severe outbreaks in young deer (8–15 months old) resulting in both high morbidity and high mortality (2). In infected animals, MAP is increasingly shed in faeces as the disease progresses [see (6)]. Contaminated faeces then acts as a source of transmission within herds by the faecal-oral route (7). MAP can also be transmitted through ingestion of colostrum from infected dams or *in utero* via the placenta and this is reported to be very high in both symptomatic and asymptomatic red deer (8, 9). Therefore, for control of the disease in farmed deer, it is important to have accurate tests that can detect infection at an early stage of infection (4, 10).

Culture-based diagnostic approaches that can be used for other pathogenic organisms are not appropriate for mycobacterial pathogens due to their very slow *in vitro* growth rates. Culture-based tests require from 4 weeks (automated liquid culture) to 20 weeks [culture on solid media; (11)]. The main diagnostic tool currently employed for the rapid detection of Johne's disease is an antibody ELISA which detects the presence of MAP-specific antibodies in either blood or milk produced by the animal in response to infection. In cattle populations, the MAP ELISA is often used as a front line test due to its availability and fast sample throughput, but its shortcomings include low sensitivity and accuracy especially during early stages of the infection (4, 6, 10). As intracellular pathogens, MAP is known to infect and replicate inside macrophages and have the ability to evade immune surveillance and signalling pathways, allowing them to persist in the intracellular environment for long periods of time before shedding becomes evident. In cattle animal's immune response to MAP is not consistent over time and antibody production is slow to develop [see (6)]. However, it has been shown that IgG ELISA tests that have been optimised for cattle samples do not retain their performance characteristics when used to test blood samples from other species (12, 13). In contrast to cattle, farmed deer do seem to raise a detectable antibody response (14), and surveys have been performed to evaluate the performance of different ELISA tests (13) but there are still limited numbers of commercial validated tests available for deer. The IgG1 ELISA for detection of paratuberculosis in deer (Paralisa™) has been developed in New Zealand and has been shown to be of value for screening deer herds to identify high shedding animals (15), although this was also not found to have a good predictive value in earlier stages of infection. Therefore, new tests that can directly detect the bacterium in the early stages of infection could be used to help implement control or eradication programmes.

Phage-based assays, such as the Actiphage® assay (PBD Biotech Ltd., UK), have been shown to be able to detect MAP

in the blood of cattle (16, 17). The assay uses mycobacteriophage D29 as a lysing agent to efficiently release genomic DNA from low numbers of mycobacterial cells which can be detected by signature specific PCR assays (16, 17). In addition to using phage-based assays to detect MAP in blood as a sign of infection, we have previously shown that the appearance of detectable levels of mycobacteria in the circulating blood is a good marker of disease for infections caused by both *Mycobacterium bovis* and *Mycobacterium tuberculosis* (16–20). In all species of animal, mononucleocytes are the main targets of infection of mycobacterial pathogens in blood (21–23). When developing the phage-based assay, it was found that one of the critical steps was the removal of red blood cells (RBCs) from the sample as these can inhibit phage infection (16). Traditionally density gradient separation, such as the Ficoll buoyancy gradient method, has been widely used for the separation of monocytes and other white blood cells (24). However, this method is time consuming, requiring several centrifugation steps, and is sensitive to variation in both sample age and temperature. Recently, we have shown that differential sedimentation methods developed for the isolation of human white blood cells are compatible with the Actiphage assay (18). In addition we have shown that recovery of infected white blood cells from milk by centrifugation is also a suitable method to prepare samples prior to the phage assay (25).

In this study we wanted to investigate whether a differential lysis approach to remove the RBCs from a blood sample using Ammonium Chloride-Potassium (ACK) lysis buffer was compatible with the Actiphage assay. The Ammonium Chloride-Potassium (ACK) lysis method lyses erythrocytes as a result of osmotic stress caused by the uptake of ammonium chloride before cell debris is removed by washing in a buffer that is osmotically balanced for the white blood cells and has been shown to be effective for obtaining purified white blood cells from whole blood samples (26, 27). The performance of ACK lysis buffer method for the removal of RBCs was first compared to the traditional Ficoll method using cattle blood. The ACK method was then evaluated to see if it could be used to detect MAP in the blood of naturally infected, farmed deer.

MATERIALS AND METHODS

Bacterial Strains and Media

MAP (strain K10; ATCC BAA-968) was used as the positive control for Actiphage assays. MAP was propagated in Middlebrook 7H9 broth (Difco, UK) supplemented with oleic albumin dextrose catalase (OADC) at a ratio of 1:10 and Mycobactin-J (2 µg ml⁻¹; Synbiotic Corporation, France), grown with aeration at 37°C (28). The Actiphage (PBD Biotech Ltd., UK) phage reagent and Media Plus were prepared according to the manufacturer's instructions. Briefly Media Plus is prepared by supplementing the base media supplied in the kit with a sterile supplement at a 1 in 10 dilution rate. Media Plus is then used to reconstitute the freeze dried Actiphage reagent.

Blood Samples

All clinical blood samples were handled under BS level 2 containment. For method optimisation experiments superfluous material from commercial bovine blood samples sent to PBD Biotech Ltd. for Actiphage testing was used. The clinical cervid blood samples originated from farmed deer in the East Midlands area of the UK. The deer were kept on outdoor pastures and in four different production groups. Blood was collected for diagnostic purposes by veterinarians in Heparin Vacutainer tubes (Beckton Dickinson, UK) and stored and transported at ambient temperatures (15–20°C) to ensure that the mycobacteria remained in an active growth phase required for productive D29 infection (28). The blood separation procedures were carried out within 12 h of collection. Samples for MAP blood ELISA assays were sent to Axiom Veterinary Laboratories (Devon, UK).

Animal Groups for Testing

All the experiments in this study was performed using superfluous blood samples sent to PBD Biotech Ltd. for commercial testing and therefore were samples of convenience rather than taken from particular cohorts of animals of known infection status. Initial comparison of the Ficoll and ACK methods was carried out using superfluous blood from 43 cattle of unknown infection status, but with a known low herd prevalence of MAP infection.

All the deer samples were from females that were part of the commercial breeding stock on the farm which had experienced low levels of sporadic MAP infection in the past. Set 1 included 29 animals of a range of ages that were being screened before being sale. Blood samples from these animals were used to determine if the ACK method was compatible with deer blood, and to confirm that this method did not affect the performance of the Actiphage reagents. Set 2 included most of the mature breeding animals on the farm (ages 5–10 years) and were from different breeding groups and animals within these groups had shown signs on John's disease on previous occasions. These animals were used to determine whether the ACK/Actiphage method could detect MAP in the deer. Set 3 included only young animals that were being screened prior to the rut to identify which animals would be retained on the farm. This set was also used to determine whether MAP could be detected in the blood of these animals. A small number of animals from this group that gave a positive Actiphage results were included in a set of animals (set 4) that were screened using Actiphage 1 month later and allowed the reproducibility of the test results to be examined. Animals in set 5 were selected by the farm to be screened before sale using both MAP ELISA tests and Actiphage and included only animals that had previously been given a negative test result using Actiphage.

Blood Preparation Methods

For separation of peripheral blood mononucleocytes (PBMCs) using Ficoll gradients (Ficoll paque Plus; GE Healthcare), a 10 ml Leucosep™ gradient tube (Greiner, Austria) was filled with 3 ml Ficoll density medium. 2.5 ml of Phosphate Buffered Saline (PBS) was gently mixed with a 2 ml sample of whole heparinised blood sample. The sample was then layered onto the top of the leucosep filter membrane. The sample was centrifuged (300

× g, 20 min, 19°C, swing out rotor with no deceleration). The buffy coat layer located above the filter membrane, was collected and mixed with 6 ml PBS before centrifuging (200 × g, 10 min, 19°C, swing out rotor with deceleration set at maximum. The pellet containing the blood mononucleocytes was resuspended in 1 ml of Actiphage Media Plus to induce lysis of the white blood cells and release the intracellular mycobacteria into the media. The samples were then either processed immediately (samples inoculated with laboratory cultured MAP) or incubated at room temperature for up to 12 h (clinical samples) prior to performing the Actiphage assay.

For purification of blood leukocytes (both mononucleocytes and granulocytes) using ACK buffer, a modification of the method described by Brown et al. (27) was used. Briefly, a sample (2 ml) of whole blood was added to 40 ml of ACK buffer (an aqueous solution of 150 mM Ammonium chloride, 10 mM Potassium bicarbonate, 0.1 mM EDTA, filter sterilised using 0.45 µm pore size). On addition of ACK the blood samples became more opaque, but this disappeared after gently agitating the sample for 5 min. The lysis process was determined to be complete when the sample became a dark red, transparent solution. The blood leukocytes were recovered by centrifugation (300 × g, 5 min at 20°C) and the supernatant discarded. The pellet was then washed twice to remove traces of erythrocyte debris using 5 ml of PBS with the intact white blood cells being recovered after each wash by centrifugation (300 × g, 5 min at 20°C). Finally the pellet was resuspended in 1 ml of Actiphage Media Plus and was stored at room temperature for upto 2 days before testing as described above.

Actiphage Assay and qPCR

The Actiphage assay was carried out according to manufacturer's instruction. Briefly, after lysis of the white blood cells, any mycobacteria released into the Actiphage Media Plus were collected by centrifugation (13,000 × g, 3 min). The pellet was resuspended in 110 µl of rehydrated Actiphage reagent and then transferred into an Actiphage Rapid tube before incubating at 37°C for 3.5 h to allow phage absorption, infection and complete lysis of the mycobacterial cells. After the phage lysis step, the Actiphage Rapid tubes were centrifuged (13,000 × g, 3 min) and the lysate containing the released mycobacterial DNA was recovered from the collection tube. For the assay positive controls (Actiphage Media Plus inoculated with ~10² MAP K10 cells) and negative controls (Media Plus and Actiphage reagent) were prepared according to the manufacturer's instructions.

DNA recovered from the Actiphage assay was concentrated using a Zymo™ clean and concentrator kit (Zymo, USA) and eluted in a total volume of 14 µl using 55°C water pre-warmed to 55°C. Detection of MAP DNA was carried out using the BactoReal kit for the detection of MAP (Ingenetix, Austria) which targets the IS900 element. qPCR reactions were performed according to the manufacturer's instructions using 5 µl of purified DNA per reaction. The positive control DNA sample provided with this kit contains ~10,000 target copies per reaction and the PCR Mastermix includes an Internal Positive control (IPC) labelled with VIC-TAMRA for detection of PCR inhibition. A test sample was determined positive if its Cq-value

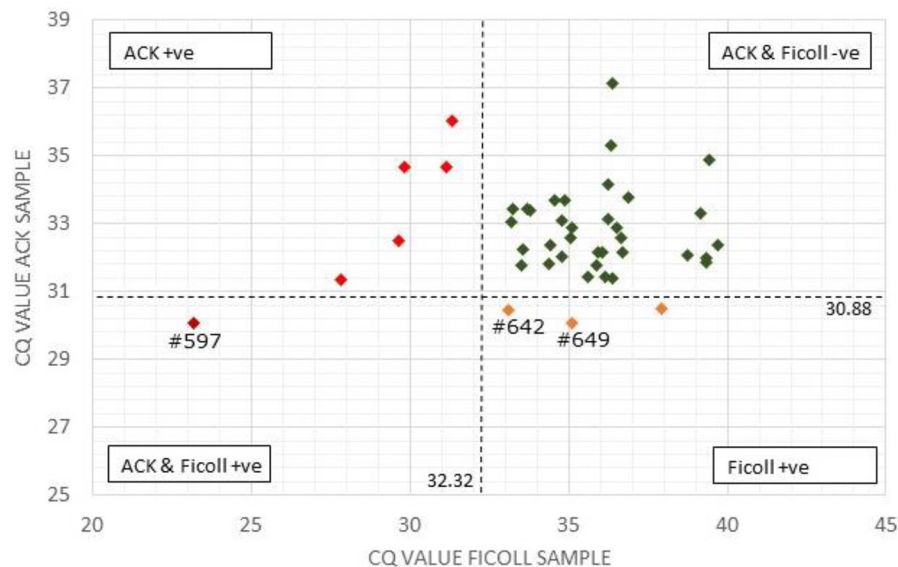


FIGURE 1 | Comparison of Actiphage pPCR test results using Ficoll and ACK methods to purify white blood cells. The Cq-values for the paired blood samples are compared using a scatter plot. Vertical and horizontal dashed lines are set at the cut off values for the Ficoll and ACK samples, respectively. Samples falling within each quadrant are colour coded and cut off values are given next to each line. The position of individual samples (#) referred to in the text are indicated.

was 2 cycles lower than that of the Actiphage negative control sample ($\Delta Cq \geq 2$). A sample producing a Cq-value between 1.5 and 2 units lower than the negative control was scored as weak positive ($\Delta Cq = 1.5-2$).

Statistical Analysis

Excel 2010 statistical add on package was used carry out a correlated *T*-Test when values for paired samples was compared. Significance was determined at $p < 0.05$. PCR efficiency was determined by plotting Ct-value against $\log_{10}$ (cell number) using Excel 2013, and determining the slope of the curve from the trend line and R^2 -values for each set of data points (R^2 over 90% = acceptable). PCR efficiency was then calculated using the equation $\text{Efficiency (\%)} = [10^{(-1/\text{slope})} - 1] \times 100$; acceptable range = 90–110%.

RESULTS

Determining Compatibility of ACK Method With the Actiphage Assay

The Actiphage test is currently being performed by PBD Biotech Ltd. as a commercial blood test for detecting MAP in bovine blood samples and the kit instructions describe the isolation of white blood cells using a Ficoll density gradient method. To determine whether the ACK lysis method could be used as an alternative method to purify white blood cells prior to performing the Actiphage assay, superfluous blood from bovine samples set to PBD Biotech Ltd. were used. On the day of commercial testing, white blood cells were also prepared from blood of 43 cattle of unknown infection status using the ACK lysis method described by Brown et al. (27). Visible inspection showed that

the ACK lysis method also produced purified white blood cells with low visible erythrocyte contamination which is key indicator for samples to be successfully tested using the Actiphage kit so that the results could be compared with the results reported for the commercial tests using the Ficoll method. Purified DNA was tested for the presence of MAP using a commercial qPCR kit (Bactoreal, Ingenetix) which includes an internal positive control (IPC) in the qPCR Mastermix to detect PCR inhibition. The signal from the IPC indicated that no PCR inhibition was detected when the DNA the samples prepared using either Ficoll or ACK were tested indicating that changing the extraction method did not result in a lower quality DNA sample (data not shown).

The IS900 target DNA positive control sample provided in the kit that contained $\sim 10,000$ copies of the MAP-specific target sequence gave a Cq-value of 20.54 (expected range 19–22). For the Actiphage assay, a positive control containing $\sim 10^2$ freshly grown MAP K10 cells is used, and in this experiment produced a Cq-value of 21.13. Based on the Cq-value of the positive control the expected range for the MAP positive control would be 21–22 as the K10 strain contains 17 copies of the IS900 element (i.e., 10^2 cells contain 1,700 copies of the IS900 target sequence). For the Actiphage test, a negative control sample is also prepared using the media supplied in the kit and the Actiphage reagent alone but still contain high levels of phage DNA and residual DNA from the *Mycobacterium smegmatis* strain used to propagate the Actiphage reagent which may result in PCR bridging events. In this case the negative control samples give Cq-values in the range of 30–32 and therefore these values are used to establish a base line for identifying positive test results (see Figure 1).

TABLE 1 | Comparison of qPCR result for deer blood samples prepared using Ficoll and ACK methods.

Sample	Blood preparation method				Ratio ACK:Ficoll Cq ^c
	ACK Cq	ΔCq ^b	Ficoll Cq	ΔCq ^b	
Act. +ve ^a	20.83	n/a	22.24	n/a	0.94
Act. -ve ^a	30.29	n/a	32.36	n/a	0.94
1	33.45	-3.2	40.72	-8.4	0.82
2	36.09	-5.8	35.49	-3.1	1.02
3	33.32	-3.0	32.51	-0.1	1.02
5	33.94	-3.7	33.00	-0.6	1.03
6	32.38	-2.1	32.33	0.0	1.00
7	34.40	-4.1	38.02	-5.7	0.90
8	33.57	-3.3	34.80	-2.4	0.96
9	28.38	1.9	33.76	-1.4	0.84
10	32.40	-2.1	34.00	-1.6	0.95
11	33.03	-2.7	34.35	-2.0	0.96
12	33.61	-3.3	33.86	-1.5	0.99
13	32.86	-2.6	31.86	0.5	1.03
14	33.63	-3.3	33.80	-1.4	0.99
15	32.79	-2.5	30.48	1.9	1.08
16	31.85	-1.6	33.44	-1.1	0.95
17	33.10	-2.8	35.57	-3.2	0.93
19	31.94	-1.7	33.84	-1.5	0.94
20	31.32	-1.0	31.96	0.4	0.98
21	43.93	-13.6	31.40	1.0	1.40
22	36.38	-6.1	35.09	-2.7	1.04
23	31.95	-1.7	36.73	-4.4	0.87
24	31.40	-1.1	35.03	-2.7	0.90
25	33.29	-3.0	34.40	-2.0	0.97
26	33.10	-2.8	33.20	-0.8	1.00
27	32.20	-1.9	30.82	1.5	1.04
29	31.95	-1.7	30.62	1.7	1.04

^aActiphage positive control = Media Plus inoculated with $\sim 10^2$ MAP K10 cells; Actiphage negative control = Media Plus and Actiphage reagents alone.

^bΔCq, difference in Cq sample compared to the Actiphage negative control; n/a, not applicable; Positive test result = ΔCq = ≥ 2 ; A weak positive test result = ΔCq 1.5-2 (weak positive results shaded in grey).

^cRatio of Cq-values = Cq[ACK]/Cq[Ficoll] for each paired sample. Average value = 0.99. The one sample where this ratio was significantly larger highlighted in bold.

A comparison of the qPCR analysis of the 43 bovine samples prepared using the two different methods is shown in **Figure 1**. Using the standard Ficoll isolation method, MAP DNA was detected in 4 of the clinical bovine blood samples, two with a clear positive result (ΔCq = ≥ 2 ; #597, #649) and two gave a weak positive result (ΔCq = 1.5–2) indicating that very low levels of Map (~ 5 cells) had been detected. In comparison, using the ACK lysis method, 6 positive results and no weak positive results were obtained. Comparing the pattern of results, the sample with the highest ΔCq-value using the ACK group matched one of the two positive results for the Ficoll samples (**Figure 1**; #597). Animal #642 gave a weak positive result with the Ficoll method and had a ΔCq of 1.2 for the ACK method, indicating that it was just below the threshold for a weak positive score. The two other positive results from the Ficoll set gave negative test results using ACK. When low levels of MAP are being detected in samples, some stochastic variation in number of cells per sample is to be expected [see (25)].

For PCR efficiency determination, 10-fold dilutions of a MAP positive control culture with a starting titre of 1.2×10^4 cfu ml⁻¹ were used to inoculate white blood cells extracted using the two different methods with cell numbers from ~ 10 – 10^4 cells. For the Ficoll method the PCR efficiency value was 89.6% ($R^2 = 0.996$), with an intercept Ct-value of 30.85, whereas for the ACK method the PCR efficiency value as 98.2% ($R^2 = 0.994$), with an intercept Ct-value of 31.24 indicating that sensitivity achieved using the two sample preparation methods was very similar (**Supplementary Figure 1**). Overall from these results it was concluded that the ACK method was compatible with the Actiphage reagents as there was no evidence of inhibition and positive test results were achieved.

Comparison of Blood Processing Methods for Clinical Cervid Blood Samples

Ficoll can be used to isolate white blood cells from many species of animal, including cervids, and ACK has been shown

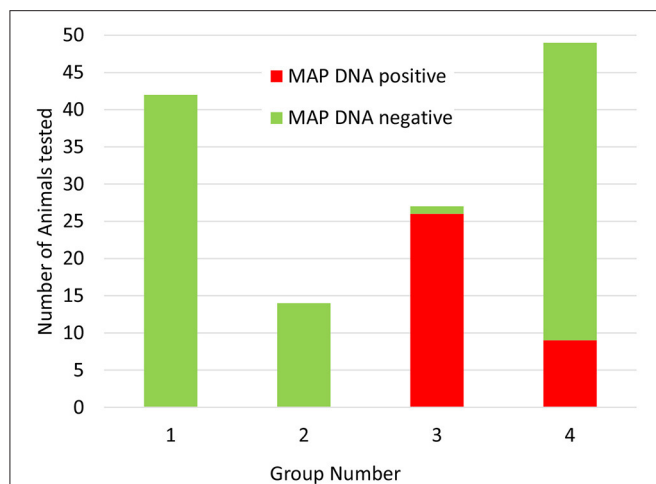


FIGURE 2 | Distribution of Actiphage MAP test results in commercial deer herd production groups. Blood samples were taken from 132 female deer prior to the rutting season. The samples were supplied to the lab blinded to production group. After the test results were available, the samples were unblinded and sorted by production group. Green bars indicate negative MAP test results; red bars indicate positive MAP test results.

to be able to remove contaminating erythrocytes from cervid buffy coat preparations (29). However, deer blood is known to exhibit unusual properties due to sickling which can be associated with increased fragility of erythrocytes (30). Therefore, to determine whether the ACK lysis method was an appropriate method for bulk isolation of white blood cells from whole blood, surplus material from 29 deer blood samples (set 1) provided for commercial Actiphage testing were obtained. White blood cells were recovered from 2 ml samples using either the recommended Ficoll method or the ACK lysis method. Although buffy coat was recovered from deer blood samples using Ficoll, the position of the buffy coat layer was inconsistent, and often formed a layer underneath the porous frit of the Leucosep tubes making extraction more difficult. In contrast a pellet of purified blood leukocytes was formed using the ACK method that on visible inspection was cream in colour indicating very little contamination with erythrocytes.

After the Actiphage assay had been performed, no PCR inhibition was detected by the IPC for either set of DNA samples, again confirming that the ACK method was compatible with the Actiphage assay reagents. Similarly the Actiphage assay positive control ($\sim 10^2$ MAP cells) and negative control (Actiphage reagents) samples gave Cq-values in the expected range (Table 1) but for this set of animals no clear positive test results were obtained, although tests were scored as weak positive for 4 animals (Table 1). The Cq-values for the paired samples was compared (Table 1) and were not found to be statistically different ($p = 0.16$), with an average ACK:Ficoll Cq of 0.99 with only 1 sample (#21) significantly deviating from this value. These results indicated that using the ACK method of white blood cell preparation did not affect the assay performance.

Detecting MAP in Deer Blood

Having established that the ACK method could be used to purify leukocytes from whole deer blood, the method was used to screen blood samples from a set of 132 female red deer immediately prior to the autumn rutting season (set 2). Again, positive and negative control samples gave the expected results and no qPCR inhibition was detected. In this set of animals MAP was detected in the blood samples of 35 animals (27%). Approximately 2/3 of the positive samples (21/31) produced strong positive results ($\Delta Cq > 5$), and the remaining 10 samples were scored as weak positive ($\Delta Cq 1.5-2$). Interestingly, when the animals were assigned to their production groups, it was found that all but nine of the animals that gave a positive test results belonged to one production group, and the rest were all found to belong to a second production group (see Figure 2 and Supplementary Table 1).

The following year, a set of 298 yearling animals from the same farm were tested using the ACK blood preparation method but as the farm only chose to test young animals on this occasion, this group did not include any of the animals tested the previous year (set 3). In this set of animals, 16% gave a positive test result (Supplementary Table 2). The following month a small set of 11 animals from this group that gave a positive test result were included in the blood samples set for screening (set 4; Table 2). At the second time of sampling all these animals again gave a positive test result, although 3 only gave a weak positive results ($\Delta Cq 1.5-2$).

A set of 38 animals were also chosen by the farm for commercial testing prior to sale that had given a negative test result the previous year and provided an opportunity to investigate the reproducibility of negative test results (set 5). On this occasion, blood samples were taken for both commercial MAP blood ELISA and Actiphage testing using the ACK method. In this case all blood samples from these animals produced a negative Actiphage test result and the ELISA test results were also all negative (data not shown).

DISCUSSION

We have previously shown that phage-based assays can be used to detect MAP in blood mononucleocytes of infected cattle (16) and more recently have described a new, more rapid and sensitive phage-based test that can be used to detect infection at any age [Actiphage; (17)]. In these previous studies Ficoll gradients were used to recover blood mononucleocytes, however this method has its disadvantages in that it is laborious and requires costly reagents. In this study we have demonstrated that the ACK differentiation lysis method, developed for the rapid recovery of stem cells, is compatible with the Actiphage assay. Unlike INF- γ assays, the physiological state of the white blood cells is not important for phage-based detection assays. Rather it is the physiological state of the MAP cells that is critical, and the leukocytes are lysed during the first steps of the assay to release any

TABLE 2 | Comparison of Actiphage-test results after 1 month.

Sample	Test date: 27/08/20			Test date: 22/09/20		
	Test result ^b	Cq-value	Δ Cq ^c	Test result ^b	Cq-value	Δ Cq ^c
Act. +ve ^a	+ve	25.72	n/a	+ve	18.68	n/a
Act. -ve ^a	-ve	30.43	n/a	-ve	32.44	n/a
1	+ve	21.44	9.0	+ve	29.75	2.7
2	+ve	22.34	8.1	+ve (w)	30.97	1.5
3	+ve	22.97	7.5	+ve	29.78	2.7
4	+ve	23.15	7.3	+ve (w)	30.59	1.9
5	+ve	24.98	5.5	+ve	29.25	3.2
6	+ve	26.02	4.4	+ve (w)	30.49	1.9
7	+ve	26.22	4.2	+ve	29.34	3.1
8	+ve	27.43	3.0	+ve	29.95	2.5
9	+ve	27.96	2.5	+ve	29.61	2.8
10	+ve	28.05	2.4	+ve	30.21	2.2
11	+ve	28.09	2.3	+ve	30.22	2.2

All animals tested were female, and were born in the same year (2019).

^aActiphage positive control = Media Plus inoculated with $\sim 10^2$ MAP K10 cells; Actiphage negative control = Media Plus and Actiphage reagents alone.

^bTest result; Positive = Δ Cq = ≥ 2 ; Positive weak (w) = Δ Cq 1.5-2.

^c Δ Cq; difference in Cq sample compared to the Actiphage negative control; n/a, not applicable.

intracellular MAP into a growth medium to keep them in a metabolically active state required for phage infection (28). Indeed the additional osmotic stress that the leukocytes experience during the ACK procedure may make them more susceptible to lysis which would also facilitate phage-based detection.

When preparing samples from deer blood, it was noted that there was less detectable erythrocyte contamination when using the ACK method. Contamination of the Ficoll purified buffy coats may have been due to the fact that the buffy coat layer was formed very close to the surface of the erythrocyte layer, some of which may have been picked up while pipetting off the delicate buffy coat layer or may be due to the higher fragility of the erythrocytes causing premature lysis (30). Hence for cervid blood samples, the ACK method had another advantage over standard Ficoll gradients.

In this study, the ACK method was used to screen animals from a commercial herd of farmed deer. The farm had had sporadic problems with JD after some animals had been purchased, but no cases of clinical disease had been detected recently. Initially a set of female animals were tested, with blood samples provided simply in the order the animals entered the testing pen. In this set of results there was an unusual pattern, with a cluster of positive results occurring in samples collected at the end of the process. This pattern of results was explained when the test results were compared with information provided about the production groups, when it became evident that there was one main group (group 3) which had the highest level of MAP infection and a lower level of infection detected in group 4, whereas groups 1 and 2 did not give any positive test results. Since these groups are geographically separated on the farm, this information is now being integrated into the farm management plan to ensure that infected animals are not moved

to the uninfected groups, and animals known to be infected are being prioritised for culling when numbers need to be reduced.

When the farm testing program allowed, it was also possible to carry out some small scale test reproducibility evaluation work, and it was found that when repeat tests were performed within 1 month that the results gained were consistent. The variation in the levels of DNA detected in the same animal (Table 2) is to be expected as there will be stochastic variation in the number of MAP cells detected when only very low number of infected macrophage are present in the blood and only small (2 ml) blood samples are being tested [see (25)]. Our previous studies of MAP in bovine blood have detected only 5–50 MAP cells per sample using phage-based assays (16, 31) and therefore only a very small percentage of the PBMCs in a sample ($\sim 10^6$) are likely to be infected. If larger samples could be tested, this would reduce the stochastic variation, but currently the viscosity of the sample which contains large amounts of bovine DNA derived from the lysed macrophages prevents larger volumes of blood processed using the Actiphage kit. Similarly when a set of animals that had previously given a negative Actiphage test result were tested again using both ELISA and Actiphage, both tests gave negative results. Since a commercial qPCR kit was used to detect the MAP DNA, and the specificity of the detection event comes from the qPCR step, this provides confidence that these are true positive results. However, it was also clear that this non-optimised qPCR assay resulted in high background noise levels. The Bactoreal kit is optimised for the detection of MAP DNA in faecal samples and PCR bridging can lead to positive Cq-values when the sample DNA contains high concentrations of off targets and primer concentrations have not been optimised (32–34). If the PCR reagents are optimised, this may allow lower levels of cells to be detected with confidence. Further studies are required to fully validate the Actiphage method for detecting MAP infections in deer, including comparison with

other standard diagnostic test methods, but these results provide first evidence that Actiphage is specific and in combination with the ACK lysis method can be used to detect MAP infection in farmed deer.

The inherent specificity of bacteriophage D29 towards its Mycobacterial host cells, coupled with the high specificity and sensitivity of PCR-based detection methods has led to the development of the new, more sensitive Actiphage assay. The replacement of Ficoll gradients further simplifies the method and the chemicals required for the production of ACK lysis buffer (Ammonium chloride, Potassium bicarbonate and EDTA) are widely available and inexpensive. This proof of principle study demonstrates that Actiphage is a new tool that can directly detect the presence of MAP in cervid blood samples and clearly has potential for the control of Johne's disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because Superfluous blood samples from commercial testing

program provided. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AK developed and evaluated the ACK method for use with deer blood, and contributed to writing the manuscript. AP and CE generated results for commercial samples using the Ficoll method. TP and BS contributed to the design, supervision of experiments, writing, and reviewing the manuscript. CR contributed to design of experiments, data analysis, and writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2021.665697/full#supplementary-material>

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Conflict of Interest: CR and BS are founder members and shareholders of PBD Biotech Ltd., and AP is an employee of the company. AP and CE are UoN students sponsored by the company, but they can declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. TP is an employee of UoN and has no commercial or financial relationship with the company.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix 2: Paper under review for publication: Freezing of plaques as a novel method for storing Bacteriophage

Introduction

Bacteriophage are the most abundant life form on earth and can be found in every environment from the poles to deserts, geysers and can be isolated from food products, soil, slurry, plants and animals. Although phage structure can be very variable, the majority of them belong to the order *Caudovirales* characterised by having a double-stranded DNA genome contained within in a protein capsid or head and possess a tail structure which is required for successful infection of bacterial cells (see Jończyk et al., 2011; Veerler and Cambillau, 2011). The challenge of antibiotic and disinfectant resistance in bacteria, has led to a significant increased interest over the last decade in isolation of new bacteriophage for use as antimicrobial agents (Gibson et al., 2019). Maintaining genetically stable and viable stocks of bacteriophage for prolonged periods of time has been challenging as it is known that a range of physical and chemical factors can inactivate a phage by causing damage to the structural head and tail elements (Maillard et al., 1995; Ackermann et al., 2004). Storing liquid suspensions of phage at 4 °C results in a continuous loss in viability (Clark and Klein, 1966; Ackermann et al., 2004). Storage properties of phage at 4 °C appear to be highly variable between phage type with virions of some phage maintaining viability for years as liquid suspension (Lobocka *et al.*, 2018) while Gonzalez-Menedez *et al.* (2018) found that different *Staphylococcus* phage (phiPLA88, phiPLA35, phiPLA-RODI and phiPLA-C1C) would lose between 1 and 3 log₁₀ in phage viability over a period of up to 24 months when phage virions were stored as liquid suspension at 4 °C. Furthermore, liquid stocks are prone to contamination by bacteria or fungi, potentially leading to loss of samples due to protease production by the contaminants (Ackermann et al. 2004). In order to maintain a high titre phage stock, continuous sub-culturing is necessary which is detrimental to the genetic stability of the phage as each sub-culture has the potential to select for mutations into the population.

The ability to freeze newly isolated phage to protect them from degradation would solve these problems as samples could be kept for many years, removing the need for sub-culture and the potential risk of titre loss and growth of contaminants. However, freezing of phage in suspension results in a high and sometimes almost complete - loss of phage viability and is therefore not an ideal solution (Clark and Geary, 1973). The main cause of damage to the bacteriophage during freezing is believed to be the crystallisation of water and the resulting

increase in volume damaging and destroying the phage capsid and or tail proteins and, thus, rendering the phage non-infective (Vandenheuvel et al., 2014). The complexity of the tail apparatus of bacteriophage has been suggested as the major factor determining the likelihood of bacteriophage survival during freezing (Warren and Hatch, 1969).

Bacteriophage intended for long term storage are commonly freeze-dried, but this process is also accompanied by substantial loss in phage titre due to exposing the phage particles to both, freezing and drying stress (Clark and Geary, 1973). Freeze-dried phage also tend to lose viability over time (Ackermann et al., 2004). Different attempts have been made in the past to add stabilizing agents to the phage suspension to protect the phage from damage. Mono- and polysaccharides, such as trehalose, mannitol and sucrose and glycerol and biodegradable polyester microspheres have all been evaluated as potential excipients to protect phage during the freeze-drying process (Puapermpoonsiri et al., 2009). It has been shown that chemicals such as trehalose can be used as a stabilizing agent giving structural support to the phage particle and protecting it from damage. It was found that phage particles in a trehalose solution can survive freeze drying in much higher numbers as the trehalose is giving structural support to the phage particles and “mimicking” the presence of water (Vandenheuvel et al., 2014)

This study tested the ability of agar to act as a stabilising agent by freezing bacteriophage plaques extracted from standard phage propagation plates. As plaques result from multiple rounds of host cell lysis, they contain high numbers of bacteriophage embedded in agar. Agar contains a mixture of two galactose-based polymers, agarpectin and agarose, which have a molecular backbone similar to known cryoprotectants such as trehalose or sucrose. We hypothesised that if agar could protect phage from structural damage during freezing, it would provide a simple and attractive method to preserve phage stocks with a relatively high titre.

Materials and Methods:

A list of bacteriophage, host strains and media used for culture used are given in Table 1. Bacteria were grown in liquid cultures, at 37 °C with aeration in the media specified. For the growth of *Mycobacterium smegmatis*, Middlebrook 7H9 broth was supplemented with OADC

(Abtek, UK) to a final concentration of 10 % (v/v). Plaques were produced by mixing 6 ml of the appropriate culture broth with 1 ml of bacterial culture (*M. smegmatis*; $\sim 10^8$ cfu ml⁻¹), 5 ml of 1.5 % (w/v) molten agar and between 200 and 400 phage particles per sample to allow clearly distinguishable plaques to form. This mixture was then poured into Petri dishes and incubated at 37 °C for 18-24 h. After incubation, agar containing plaques were excised using a biopsy 3 mm punch needle (Kai Medical, Japan) and transferred into microcentrifuge tubes.

Phage were eluted from individual plaques for titration by elution based on the method developed for recovery of phage M13 (Green and Sambrook, 2017). Briefly, individual excised plaques were immersed in 0.1 ml lambda phage buffer (6 mM Tris HCl, pH 7.2, 10 mM MgSO₄, 0.005 % gelatin) for 5 min (experiments were performed to show that after 5 min incubation, no further increase in phage titre occurred; data not shown). The resulting phage suspension was titred on bacterial lawn plates produced by adding ~ 1 ml of bacterial culture (approx. 1×10^9 cfu ml⁻¹) to 6 ml of broth and 5 ml of molten agar using the Miles and Misra technique.

“Artificial plaques” were produced by adding 5 μ l of high titre bacteriophage suspension to 5 μ l of either warm (~ 45 °C), liquid agar (either Nutrient Agar; Oxoid, UK or Middlebrook 7H10 agar; Sigma-Aldridge, USA) or warm, liquid 20 % (w/v) gelatine solution. After mixing with phage the sample was cooled to room temperature to allow the matrix to form a gel.

Excel 2010 statistical add on package was used carry out a correlated T-Test when values for paired samples were compared. A p-value < 0.05 was considered significant.

A complete list of bacteriophage, their bacterial hosts and culture media used in this work is given in table 1.

Table 1: List of bacteriophage, host strains and media used for culture

PHAGE NAME	PHAGE MORPHOLOGY	HOST STRAIN FOR PROPAGATING PHAGE	MEDIA USED FOR CULTURE	SOURCE REFERENCE FOR PHAGE
D29	Siphoviridae	<i>Mycobacterium smegmatis</i> mc ² 155	Middlebrook 7H9 broth** and 7H10 agar (Difco, USA)	(Rybniker et, al. 2006)
TM4	Siphoviridae	<i>Mycobacterium smegmatis</i>	Middlebrook 7H9 broth** and 7H10 agar (Difco, USA)	(Rybniker et, al. 2006)
PRD1	Tectiviridae	<i>E. coli</i> S17-1	Nutrient broth and agar (Oxoid, UK)	Bamford and Bamford, 1990)
BP	Siphoviridae	<i>Salmonella enterica</i> serovar Kedougou	BHI broth and agar (Oxoid, UK)	Bagheri, Hassan Moeiniyan PhD thesis UoN 2013
B1	Siphoviridae	<i>Listeria monocytogenes</i> ATCC 23074	Nutrient broth and agar (Oxoid, UK)	ATCC 23074-B1
FELIX	Myoviridae	<i>Salmonella enterica</i> , serovar Typhimurium DB7156	BHI broth and agar	Felix 01 Felix and Callow, 1943

** *Mycobacterial growth supplement OADC (Boval, USA) was added up to a final concentration of 10 % (v/v).*

Results

Freezing of mycobacteriophage D29 and TM4 plaques and suspension

To determine the effect of freezing on a liquid suspension of phage in lambda buffer, a sample of either phage D29 or TM4 phage suspension (approx. 10^9 pfu ml⁻¹) was stored at 4 °C overnight or was frozen at -20 °C and stored overnight. The frozen samples were thawed and both sample groups were brought to room temperature before being re-titred. After storage at 4 °C, as expected, there was no significant ($p > 0.1$) difference in the titre of either phage suspension. However, after freezing, the titre recovered from the frozen D29 suspension was 5.5×10^7 pfu ml⁻¹ indicating that freezing the phage in lambda buffer resulted in a $> 1 \log_{10}$ drop in phage titre ($p < 0.05$). A similar pattern of results was found when a liquid suspension of phage TM4 was frozen at -20 °C which resulted in a $2 \log_{10}$ drop in phage titre (2.6×10^6 pfu ml⁻¹).

To investigate whether agar could protect phage during freezing, plaques containing each type of phage were prepared by plating samples on a lawn of *M. smegmatis*. Individual plaques were then excised from the lawn for each phage and these were stored in microcentrifuge tubes without the addition of lambda buffer. The excised plaques containing phage were stored at either 4 °C or at -20 °C overnight. To provide a base line figure, phage were eluted from 20 individual phage D29 plaques by adding in 100 µl of lambda buffer and incubating for 5 min (see method section) immediately after excision, and this produced an average titre of 1.51×10^9 pfu ml⁻¹ (SD ± 0.38). To determine the effect of these storage conditions on phage, number of phage particles released from the plaques ($n=5$) stored under different conditions was then determined (Fig. 1). The average titre of phage D29 recovered from plaques stored at 4 °C was found to be 2.4×10^9 pfu ml⁻¹ (SD ± 0.28), and the titre of plaques stored at -20 °C was found to be 3.76×10^8 pfu ml⁻¹ (SD ± 0.3) indicating that only $1 \log_{10}$ reduction in titre had occurred compared to liquid phage suspension frozen at -20 °C where, on average, only 3.28×10^7 pfu ml⁻¹ could be detected (SD ± 0.24 ; $p < 0.01$). Similarly, when plaques containing phage TM4 were stored at 4°C, the average titre of phage recovered from the agar was 2.34×10^9 pfu ml⁻¹ (SD ± 0.11), and the titre of phage recovered from plaques frozen at -20 °C (2.42×10^9 pfu ml⁻¹) (SD ± 0.13) showed no significant decrease in phage titre ($p = 0.85$). This data is summarized in table 2 and visualized in figure 1+2. In summary, for both D29 and TM4, the drop in titre on freezing in agar was significantly less (p

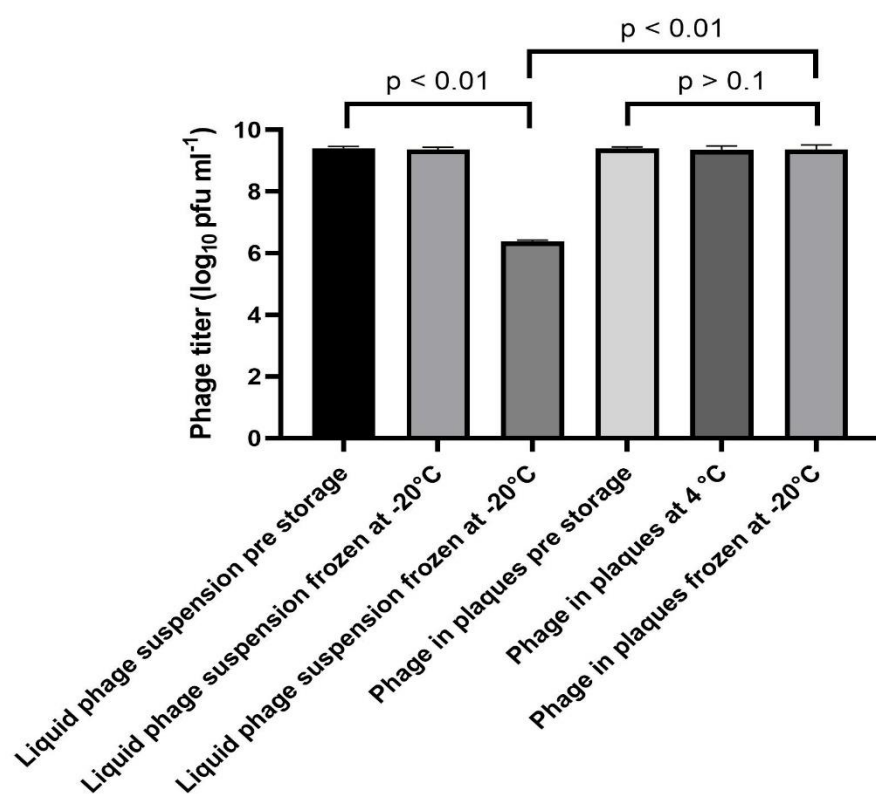
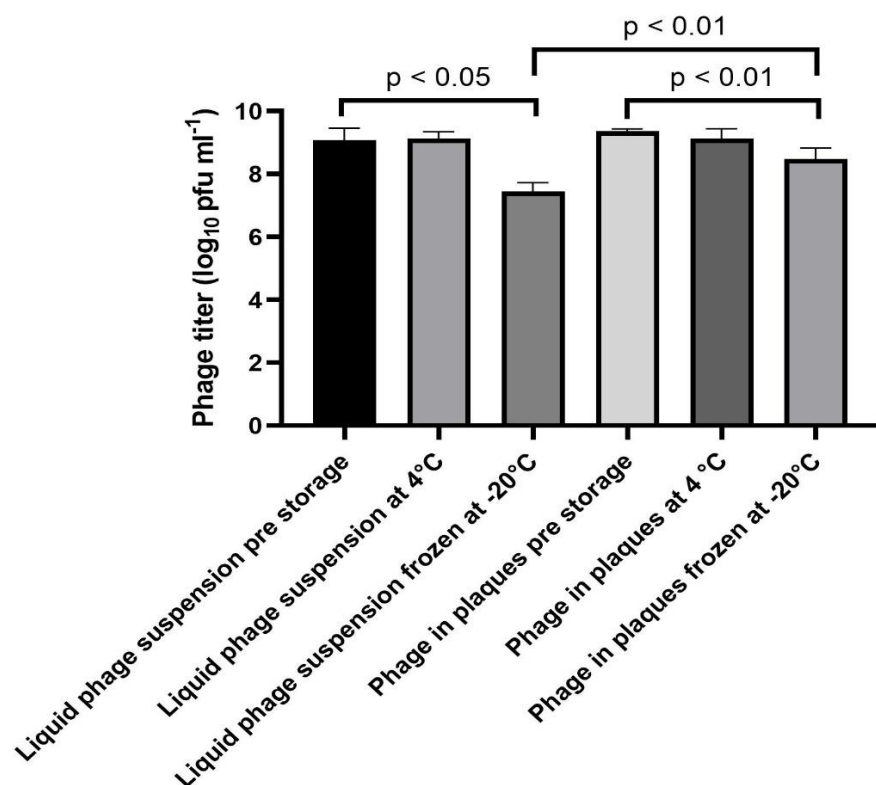
< 0.05) than that seen when liquid suspensions of the phage were frozen and suggested that plaque material was acting as a cryoprotectant.

Table 2: Comparing phage D29 survival in plaques or suspension at refrigeration and freezing conditions.

STORAGE CONDITIONS	TITRE PHAGE D29 (PFU ML ⁻¹)	SD± (LOG ₁₀)	TITRE PHAGE TM4 (PFU ML ⁻¹)	SD± (LOG ₁₀)
LIQUID PHAGE SUSPENSION PRE STORAGE	1.52 x 10 ⁹	0.38	2.54 x 10 ⁹	0.05
LIQUID PHAGE SUSPENSION STORED AT 4 °C	1.48 x 10 ⁹	0.19	2.34 x 10 ⁹	0.06
LIQUID PHAGE SUSPENSION STORED AT -20 °C	3.28 x 10 ⁷	0.24	2.44 x 10 ⁶	0.04
PHAGE PLAQUES PRE STORAGE	2.38 x 10 ⁹	0.06	2.54 x 10 ⁹	0.04
PHAGE PLAQUES STORED AT 4 °C	1.60 x 10 ⁹	0.28	2.34 x 10 ⁹	0.11
PHAGE PLAQUES STORED AT -20 °C	3.76 x 10 ⁸	0.30	2.42 x 10 ⁹	0.13

Table showing the average number of phage D29 and TM4 particles recovered from liquid samples and plaques pre storage, at 4 °C and at -20 °C as well as sample group standard deviation (log₁₀)

Figure 1 + 2: Comparing phage D29 and TM4 survival in suspension and plaques at refrigeration and freezing conditions.



Comparison of effect of different storage methods on phage D29 and TM4. Samples used were either liquid suspensions of phage D29 and TM4 or samples of the same phage embedded in agar (excised plaques). To determine the effect of storage conditions on phage viability, both types of sample were titred and then either stored at 4 °C for 24 h and then retitred or were frozen at -20 °C and then thawed and re-titred. For samples where phage were embedded in agar, prior to phage titration phage were eluted from the agar by soaking in λ buffer.

Establishing consistent phage concentration in excised plaques

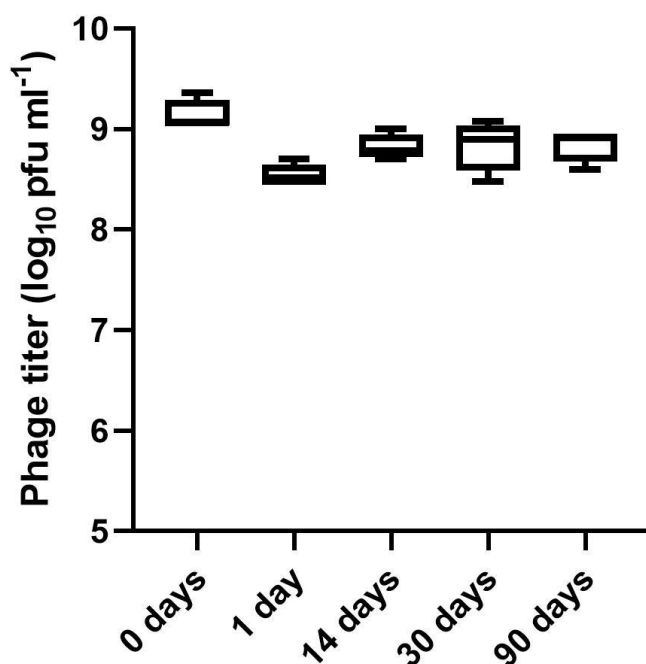
In this work plaques were harvested or “picked” using a 3 mm biopsy punch needle (Kai medical, Japan). Since phage replication and plaque formation are complicated processes where factors such as number and growth rate of the host bacteria, agar concentration, ion concentration and temperature can introduce a considerable amount of variability on the number of phage present in a plaque it was deemed necessary to confirm consistency in the number of phage that could be recovered from single 3 mm plaque extractions. To this end phage D29 plaques were grown on *M. smegmatis* lawn plates and two sets of plaques (n=20) were excised, eluted and titred as described in materials and methods. The average titre of the two sets was recorded as 1.84×10^9 and 2.24×10^9 pfu ml⁻¹ with intra-set standard deviations (log₁₀) of 0,39 and 0,38 respectively. These results indicate that there is a low degree of variability in the number of phage particles recoverable from individual 3 mm excised D29 plaques.

Viability over time of phage stored frozen in plaques

After establishing that the number of viable phage particles is maintained when freezing embedded in agar compared to when phage were frozen in liquid suspension, it was decided to investigate whether continual storage at -20 °C would result in any loss in phage viability. To do this, phage D29 plaques were excised and then transferred to individual microcentrifuge tubes. As a baseline the number of viable phage in plaques was determined by immediately immersing each plaque in 100 μ l of lambda buffer. Set of individual plaques (n=4) were stored at -20 °C for 1, 14, 30 or 90 days before samples were thawed and phage recovered from the plaques by elution. It was found that before freezing the average number

of phage recovered from plaques stored at 4 °C (n=4) was 1.43×10^9 pfu ml⁻¹ while after 1, 14, 30 and 90 days storage at -20 °C the average titre of the phage recovered from the plaques was determined to be 3.50×10^8 ; 6.75×10^8 ; 7.75×10^8 and 7.50×10^8 pfu ml⁻¹, respectively (Fig. 3). The standard deviation for all sets of samples was determined to be < 0.3 log₁₀ indicating that the results were very reproducible. Comparing the titre of phage samples stored at -20 °C for 1 day with those stored at -20 °C for up to 3 months, there was no significant drop in phage titre (p< 0,05) (Fig. 3) indicating low or no decay of phage titre during frozen storage.

Figure 3 Effect of long term frozen storage on stability of phage D29



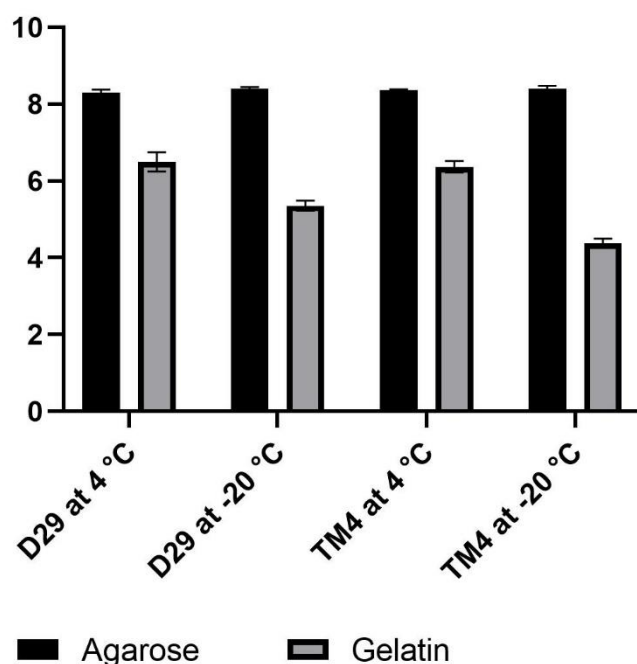
Sets of three phage D29 plaques were excised and phage were either immediately eluted from the agar or plaques were stored frozen at -20 °C for 1, 14, 30 and 90 days before phage were eluted from the agar. The average number of viable phage particles recoverable from each plaque sample was then determined.

Investigation of critical components of plaques for cryoprotection

The initial bacteriophage used in this study were those specific for mycobacteria. These bacteria are routinely grown in media supplemented with oleic acid and glycerol, which are also potential cryoprotectants. To test whether this was the case, “artificial plaques” were prepared by adding 5 µl of a purified D29 phage suspension to 5 µl of either molten Nutrient agar or molten Middlebrook 7H10 agar which was then allowed to set. Samples of each of these artificial plaques (n=3) were frozen and the number of viable phage that could be recovered from the agar after freezing was determined. The nutrient agar plaques produced phage suspensions with a titre of 8×10^8 pfu ml⁻¹ while the 7H10 agar yielded 2×10^8 pfu ml⁻¹, and therefore there was no significant difference in the number of phage recovered from the artificial plaques made using these two types of agar. Hence, the agar itself could confer the cryoprotective properties observed rather than the specific additives present in Middlebrook 7H10 agar.

To investigate whether the cryoprotection was the result of the formation of a gel, or due to the specific chemistry of the carbohydrate components of agar, artificial plaques (were created as described above using molten 1.6 % (w/v) agarose or a molten 20 % (w/v) gelatine solutions instead of agar. Agarose was chosen as it is a more purified form of one of the polysaccharides present in agar. Artificial plaques (n=3) were either stored at 4 °C or frozen at -20 °C and then the number of surviving phage determined after eluting them from the matrix as before. The average titre recovered from D29 plaques made with agarose was 2.00×10^8 pfu ml⁻¹ (4 °C) and 2.50×10^8 pfu ml⁻¹ (-20 °C) indicating that the phage were protected by this matrix. In contrast D29 plaques produced using gelatine only yielded titres of 3.35×10^6 pfu ml⁻¹ (4 °C) and 2.33×10^5 pfu ml⁻¹ (-20 °C) and in both cases the number of plaques recovered from gelatine was significantly lower than the sample prepared using agarose ($p < 0.01$; Fig. 3). This indicated that the phage did not survive well when mixed with molten gelatin held at the same temperature as the agar. A similar pattern was found using phage TM4 with average titres recovered from agarose plaques of 2.37×10^8 pfu ml⁻¹ (4 °C) and 2.50×10^8 pfu ml⁻¹ (-20 °C). The TM4 plaques created using gelatine were found to contain 2.43×10^6 pfu ml⁻¹ (4°C) and 2.47×10^4 pfu ml⁻¹ (-20 °C) ($p < 0.01$). Even though the number of phage surviving in the formation of the artificial plaques using gelatine was lower, it was still clear that this matrix did not have the same the cryoprotective properties as agarose.

Figure 4: Comparing the effect of gelatine and agarose on cryoprotection of phage.



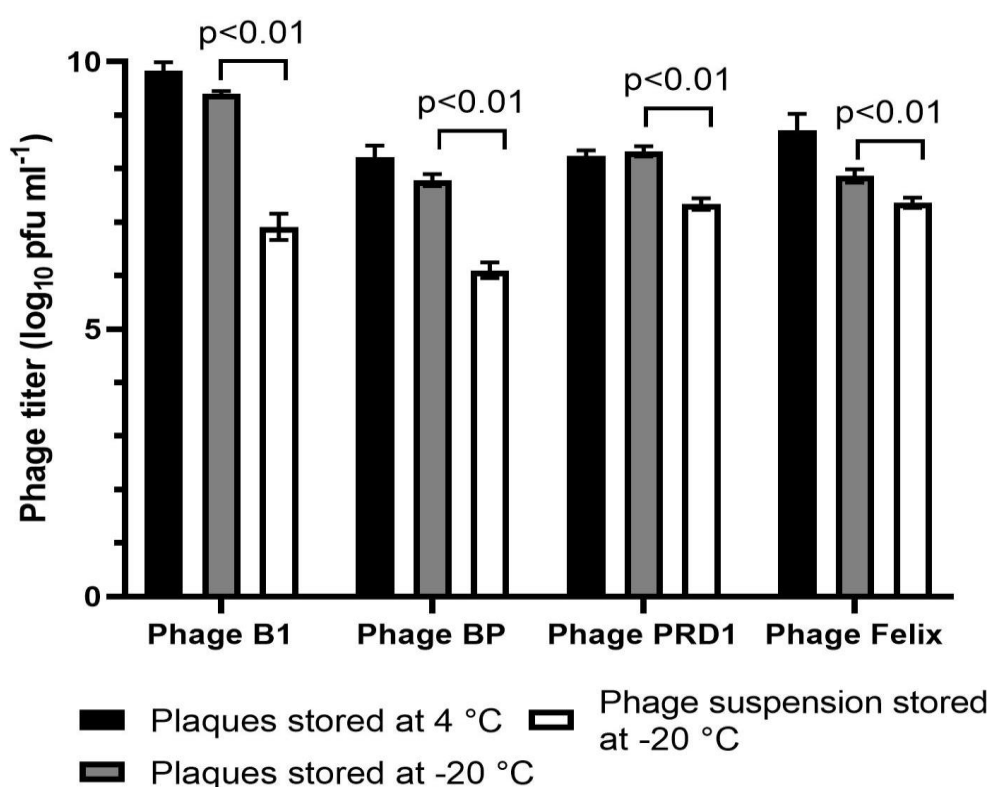
Phage suspensions of phage D29 and TM4 were solidified using a 1.6 % (w/v) agarose solution or a 20 % (w/v) gelatine solution. These “artificial” plaques were then either frozen at -20 °C or refrigerated at 4 °C for 24 h. To monitor the effect of the different conditions on phage survival, the phage within the “artificial” plaques were eluted and then titred.

Freezing of non-mycobacteriophage plaques and suspension

To determine whether this method of cryoprotection was specific to Mycobacteriophage or whether it could be applied to different phage types and morphologies, a range of other phage were used. Bacteriophage B1 and BP are both *Siphoviridae* that are specific for *Listeria monocytogenes* and *Salmonella* Kedougou, respectively. Felix 01 is a Myovirus specific for *S. Typhimurium*, and phage PRD1 is a Tectivirus that will infect cells that contain the IncP conjugation system. For each of these different phage, plaques were prepared using their host strain (Table 1), and agar containing phage excised as before (n=5). In addition, liquid suspensions of these phage with titres of approximately 10^8 pfu ml⁻¹ were frozen, and all samples were titred to monitor the levels of phage after the storage treatment (Fig. 5). In each case, the frozen liquid suspension of phage showed a marked reduction ($p < 0.01$) in titre (1-3 log₁₀ reduction recorded; see Fig. 5). In contrast, the titre of phage recovered from

the frozen plaques was always within 1 log₁₀ value of the sample of the same phage stored at 4 °C. Phage B1 plaques produced higher titre of phage per plaque compared to the other phage used in this study that consistently produced 10⁸-10⁹ phage per plaque. This was expected as this phage is characterised as having a short replication time (approx. 30 min) and a large burst size (approx. 100) and produces lysates with high phage titres (Bagheri, 2013). From the results it was concluded that this method of preservation was suitable for phage isolated from a range of different bacterial hosts and was also effective for phage from different morphologies groups.

Figure 5: Evaluation of plaque storage in different phage morphologies



Plaques of bacteriophage BP, B1, PRD1 and Felix were mechanically excised from bacterial lawn plates and either frozen at -20 °C or refrigerated at 4 °C. After overnight storage the phage within the “artificial” plaques were eluted and then titred. High titre phage suspensions of these phage were frozen at -20 °C for 24 h before being thawed and titred. Titres of different phage recovered from plaques stored at 4 °C or – 20 °C were respectively,

B1; 1.82×10^8 pfu ml⁻¹ and 6.32×10^7 pfu ml⁻¹, BP; 7.2×10^9 pfu ml⁻¹ 2.56×10^9 pfu ml⁻¹, PRD1; 1.78×10^8 pfu ml⁻¹ and 2.16×10^8 pfu ml⁻¹, Felix; 6.12×10^8 pfu ml⁻¹ and 7.6×10^7 pfu ml⁻¹.

Discussion

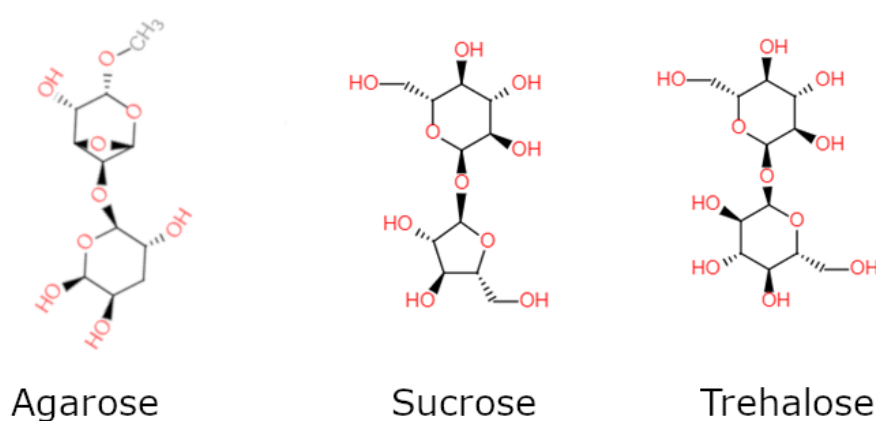
Embedding bacteriophage in resulted in great recovery of viable particles after freezing at -20 °C compared to phage in suspension. The main cause of damage to the bacteriophage during freezing is the crystallisation of the water molecules and the increase in volume damaging and destroying the phage capsid and or tail proteins - rendering the phage non-infective (Vandenheuvel et al., 2014). As previously mentioned, the complexity of the tail apparatus of bacteriophage has been suggested as one factor determining the survival of phage during freezing. Myoviridae such as phage Felix used here have complex tail fibres (Calendar, 2006) and are thus expected to suffer the most from freezing stress. However, in our experiments all phage tested, including the Myovirus (phage Felix), were able to survive freezing without resulting in a significant drop in phage viability when embedded in agar.

In studies of phage preservation, different approaches have been made to add stabilizing agents to the phage to reduce or neutralize the effect of the ice crystallization on the phage particles or to reduce crystal formation. Glycerol based stabilizing agents have been evaluated in the past (Clark, 1962; Ackermann et al., 2004), not only for freezing but also for freeze drying and for long term storage at 4 °C. As we have shown with the freezing of “artificial” plaques, and the use of nutrient agar for non-mycobacteriophage, the cryopreservation effect seen was not due to oleic acid or glycerol which are included in the 7H10 agar. In earlier studies disaccharide and polysaccharide sugars, such as sucrose, trehalose and mannitol, have been shown to protect phage particles from damage during freeze drying (Merabishvili et al., 2013; Vandenheuvel et al., 2014). This effect was attributed to these sugars giving the capsid and other structural proteins of the phage structural support and imitating the presence of water. Agar polysaccharide polymers are made up of galactose subunits and therefore its structure is not dissimilar to sucrose and trehalose molecules (Fig. 6). It is hypothesized that the molecules of the agar perform a similar role when a plaque is frozen. This is supported by observation that gelatine which

consists of polypeptides but no saccharides (Rawdkuen et al., 2013) did not confer any cryoprotective properties to the phage during freezing.

While this method works well with natural plaques extracted from phage isolation or titre plates, it has to be acknowledged that these samples will always also contain viable host bacteria which may survive freezing and can potentially then be transferred into any culture derived from the plaque when the phage are eluted from it after storage. However, the production of artificial plaques for the storage of bacteriophage isolates provides a simple method for the freezing of purified phage stocks so that the frozen samples are not contaminated with host bacteria. “Artificial” plaques can simply be produced by eluting phage from a primary plaque, filtering to remove any bacteria and then solidifying a purified phage suspension.

Figure 6: Schematic diagram comparing the molecular structure of Agarose, Sucrose and Trehalose



2- dimensional image of an agar polymer subunit and molecules of sucrose and trehalose.

Conclusion

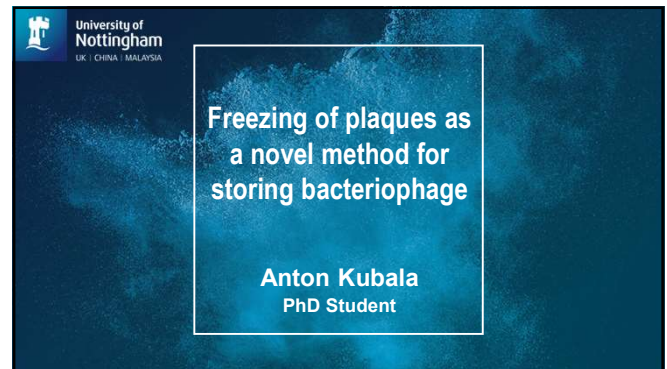
We can conclude that bacteriophage frozen in agar survived freezing in significantly higher numbers than bacteriophage frozen in liquid suspension. This makes freezing of plaques an easy cost-effective way of storing large numbers of phage isolates. It is not expected that

there will be any further drop in phage titre after the initial drop seen during freezing since all structural changes, including water crystallization have already occurred, and any enzymatic activity, such as protease or nuclease activity, will be inhibited in samples stored at -20°C (Sambu, 2015). The cryo-preservative properties of agar are likely derived from its molecular similarity to sucrose and trehalose which are known to act as stabilizing agents during freezing and drying of phage. This makes the method very attractive for programs that wish to store large number of new phage isolates before they are characterised for applications such as phage therapy. Since there is no need for continuous propagation to maintain stocks, the risk of introducing mutations to the laboratory phage isolate is greatly reduced. In addition, there is no risk of bacterial or fungal contaminants growing in and degrading the sample. The presence of viable host bacteria in the sample can be avoided by producing artificial plaques by using a pre-purified phage suspension. It must be noted that continuous freezing and thawing could prove detrimental to phage viability (Rivers, 1926) and should be avoided, and therefore it would be recommended that multiple samples are stored, so that individual tubes can be thawed out for initiating new growth of a phage.

**Appendix 3: Presentation slides 4th international scientific conference, 2018,
Nizhny Novgorod, Russia**



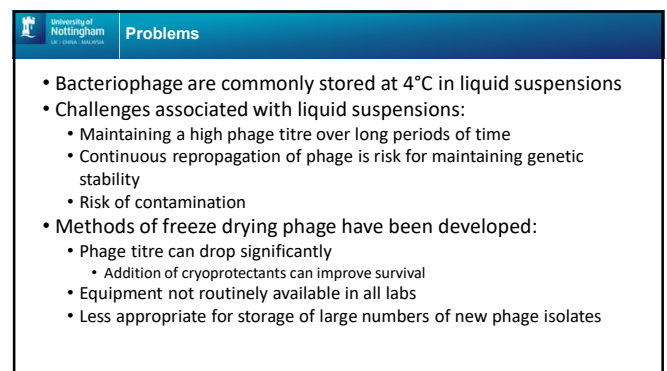
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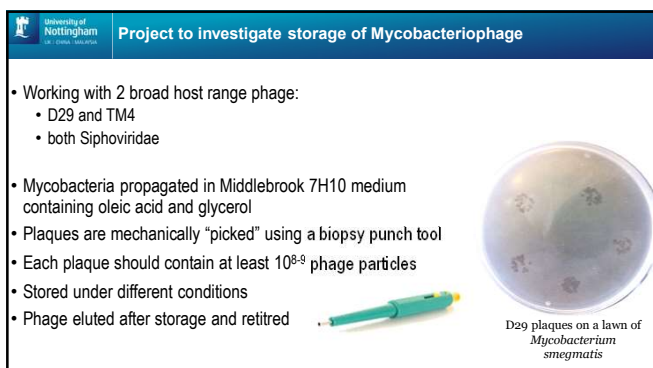
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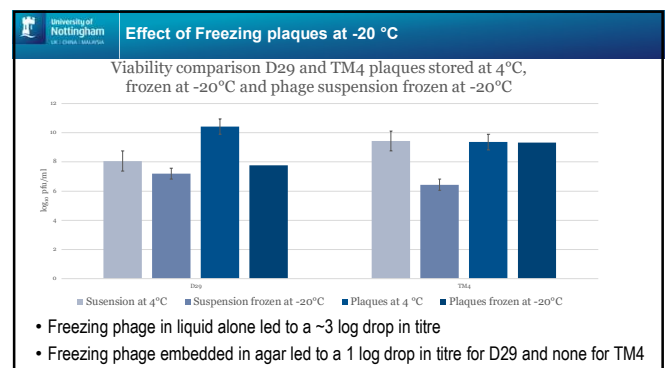
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Method applied to other phage and media

- Study extended to other Siphoviruses BP and ATCC 23075-B1

Phage	Phage morphology	Host strain
D29	Siphoviridae	<i>Mycobacterium smegmatis</i>
TM4	Siphoviridae	<i>Mycobacterium smegmatis</i>
BP	Siphoviridae	<i>Salmonella enterica</i> serovar Kedougou
ATCC 23074-B1	Siphoviridae	<i>Listeria monocytogenes</i> ATCC 23074

- Salmonella* and *Listeria* lawns formed using BHI agar

7

Tests using other Siphoviridae

Viability comparison of plaques stored at 4°C, frozen at -20°C and phage suspension frozen at -20°C

Phage	Plaques at 4°C	Plaques frozen at -20°C	Suspension frozen at -20°C
BP	~10.2	~10.1	~6.5
B1	~8.2	~7.8	~5.2

- Same pattern of protection against freezing seen when phage in agar
- Ruled out specific protective effect of 7H10 media components

8

Method applied to other phage

- Study extended to include Myovirus and Tectivirus to see if phage structure affects results

Phage	Phage morphology	Host strain
PRD1	Tectiviridae	<i>E. coli</i> S17-1
Felix	Myoviridae	<i>Salmonella typhimurium</i> DB7156

Siphovirus

Tectivirus

Myovirus

9

Tests using other Siphoviridae

Viability comparison of plaques stored at 4°C, frozen at -20°C and phage suspension frozen at -20°C

Phage	Plaques at 4°C	Plaques frozen at -20°C	Suspension frozen at -20°C
PRD1	~8.2	~8.1	~7.5
Felix	~8.8	~7.8	~7.5

- Same pattern of protection against freezing seen as with other bacteriophage tested before

10

Method applied to other phage

- Plaques for PRD1 (Tectivirus) and Felix (Myovirus) picked and frozen at -20 °C
 - High titres recovered from both phage types
- Method is also able to protect both Myoviruses and Tectiviruses against effects of freezing ($p < 0,05$)

11

Possible explanation for results seen

- Agar is very capable of protecting bacteriophage during freezing
- Trehalose is a commonly used cryopreservative
 - Disaccharide sugar with free -OH groups
 - Binds to proteins of phage and replaces water and stabilises macromolecules

Trehalose

Victor M. Baldoia a,b,*, Marta M.D.C. Vila, Structural and functional stabilization of protein ensembles: state-of-the-art, 2014

12

Structural similarities between trehalose and agar

- Agar is a **carbohydrate** polymer composed of galactose subunits
- Shares some structural similarities with trehalose

Agar

<https://pubchem.ncbi.nlm.nih.gov/compound/7437534#section=Top>

Trehalose

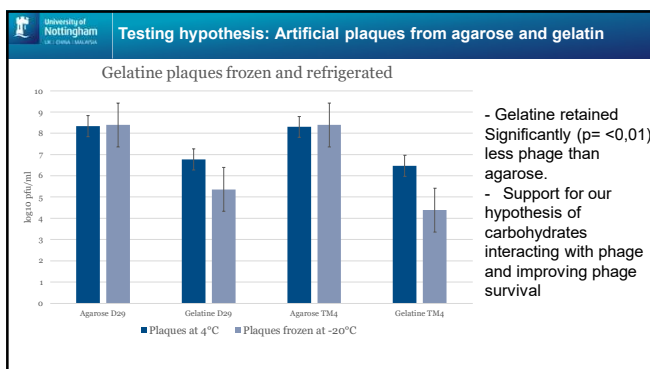
<https://pubchem.ncbi.nlm.nih.gov/compound/7427#section=Top>

13

Testing hypothesis: Artificial plaques from agarose and gelatin

- "Artificial plaques" produced by solidifying phage D29 and TM4 suspension with molten agarose or gelatine solution (20% w/v)
- Agarose is the purified carbohydrate polymer that provides agar with its water retaining abilities.
- Gelatine is a protein polymer
- Samples then either frozen at -20°C or refrigerated at 4°C for >24 h before phage were eluted and titred.
- If hypothesis is correct we should see a cryoprotective effect from agarose but not from gelatine.

14



15

Conclusion: Benefits of this method

- For all phage types tested, high numbers of phage preserved
 - Maximum of 1 log drop in titre recorded
 - Similar to losses seen when phage are freeze dried
 - BUT considerably lower than losses recorded when freezing liquid samples
- Frozen phage are stable over time
 - Removes the need to regrow stocks and therefore risk of genetic drift
 - No possible growth of contaminants
- Very simple method that can be performed in all laboratories
 - Low cost and very quick to perform
 - Compatible with storing large numbers of new phage isolates

16

Conclusion: Disadvantages

- Excising phage from lawn will mean sample contains bacterial cells
 - Artificial plaques removes this problem
- Repeated freezing and thawing could lead to loss of phage titre
 - Not useful for long term large scale storage of stocks

17

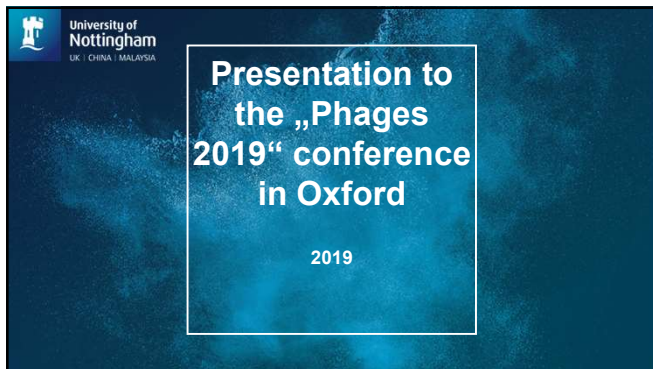
Conclusion

- Agar is a good cryoprotectant
- Method applicable to all phage types tested
- No special equipment, media or reagents required
- Method applicable to either plaques picked directly from agar OR for storing purified phage samples

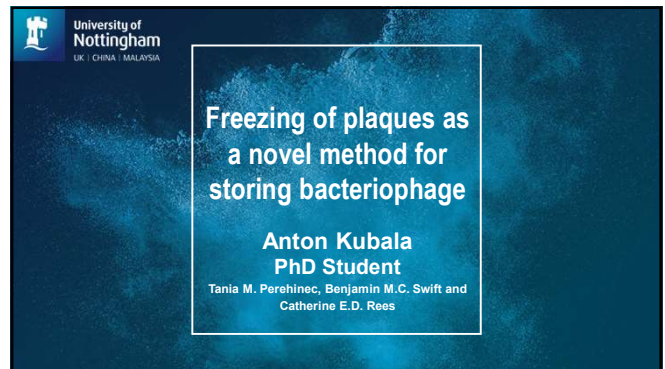
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**Appendix 4: Presentation slides Phages 2019 symposium, 2019,
Oxford, UK**



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2

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Problems

- Bacteriophage are commonly stored at 4°C in liquid suspensions
- Challenges associated with liquid suspensions:
 - Maintaining a high phage titre over long periods of time
 - Continuous repropagation of phage is risk for maintaining genetic stability
 - Risk of contamination
- Methods of freeze drying phage have been developed:
 - Phage titre can drop significantly
 - Addition of cryoprotectants can improve survival
 - Equipment not routinely available in all labs
 - Less appropriate for storage of large numbers of new phage isolates

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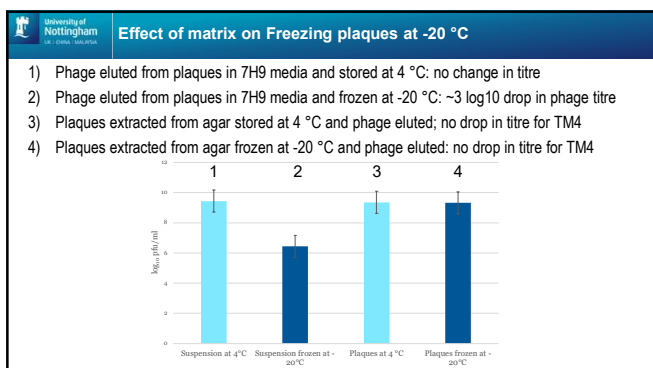
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Project to investigate storage of Mycobacteriophage

- Working with 2 broad host range mycobacteriophage:
 - D29 and TM4
 - both Siphoviridae
- Mycobacteria propagated in Middlebrook 7H9/10 medium
 - contains oleic acid and glycerol
- Plaques are mechanically "picked" using a biopsy punch tool
- Each plaque should contain at least 10^{8-9} phage particles
 - Phage eluted and stored under different conditions
 - Phage recovered after storage and retitred.

D29 plaques on a lawn of *Mycobacterium smegmatis*

4



5

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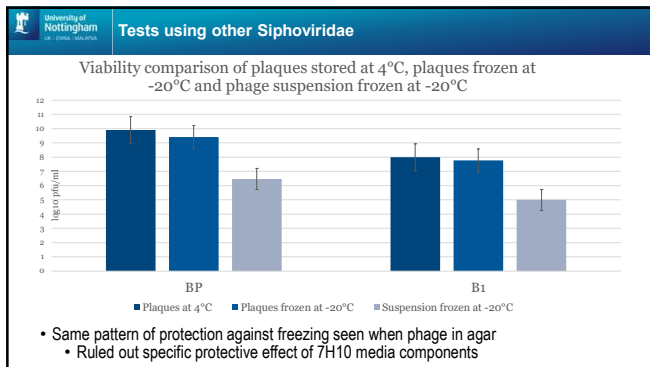
Method applied to other phage and media

- Study extended to other Siphoviruses BP (*Salmonella*) and ATCC 23075-B1 (*Listeria*)

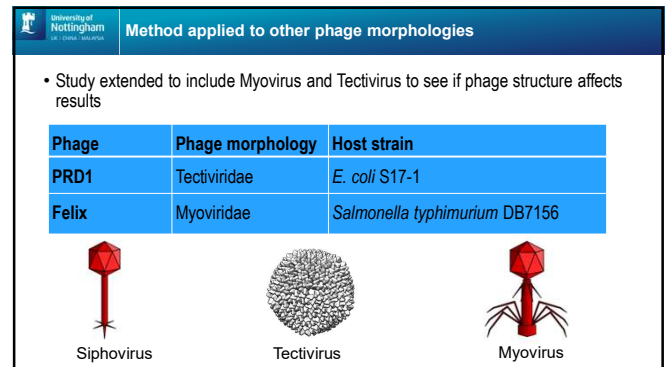
Phage	Phage morphology	Host strain
D29	Siphoviridae	<i>Mycobacterium smegmatis</i>
TM4	Siphoviridae	<i>Mycobacterium smegmatis</i>
BP	Siphoviridae	<i>Salmonella enterica</i> serovar Kedougou
ATCC 23074-B1	Siphoviridae	<i>Listeria monocytogenes</i> ATCC 23074

- *Salmonella* and *Listeria* lawns formed using BHI agar; no glycerol or oleic acid

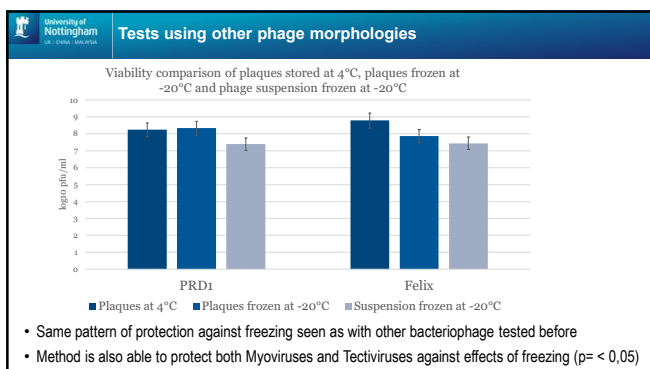
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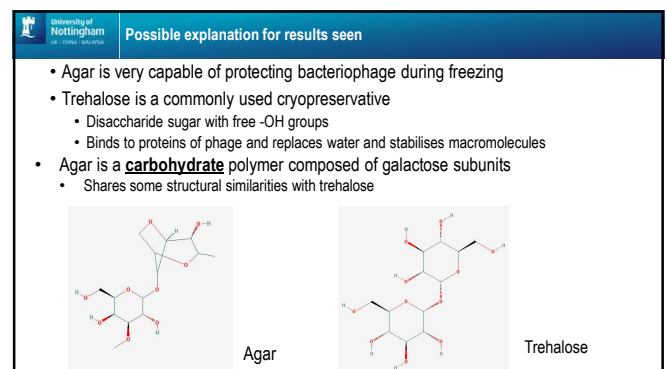
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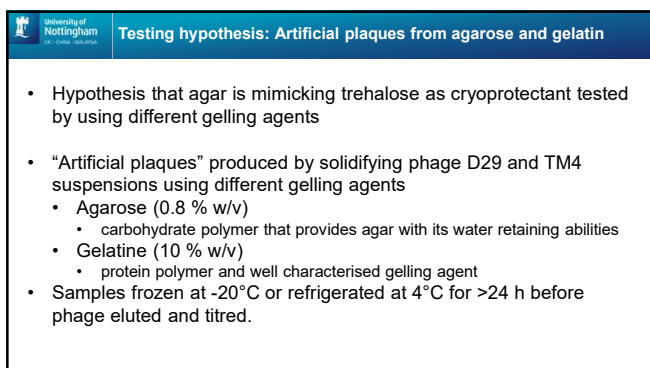
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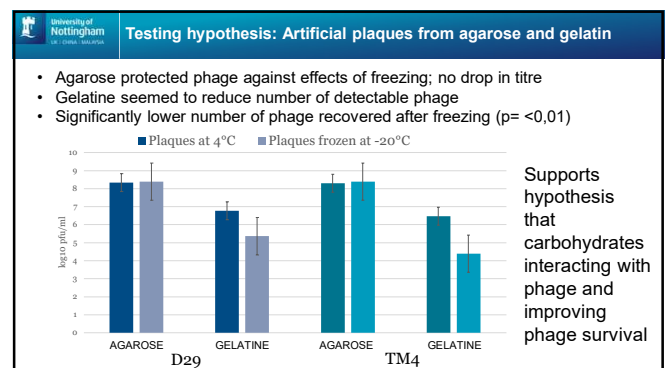
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10



11



12

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Conclusion: Benefits of freezing phage in agar as method of cryopreservation

- For all phage types tested, high numbers of phage preserved
 - Maximum of 1 log₁₀ drop in titre recorded
 - Similar to losses reported when phage are freeze dried
 - BUT considerably lower than losses recorded when freezing liquid samples
- Frozen phage are stable over time
 - Removes the need to regrow stocks and therefore risk of genetic drift
 - No possible growth of contaminants such as fungi during storage at 4 °C

13

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Conclusion: Disadvantages of freezing phage in agar as method of cryopreservation

- Excising phage from primary lawn will mean sample also contains bacterial cells
 - Preparing "Artificial" plaques removes this problem but is more laborious
- Repeated freezing and thawing could lead to loss of phage titre
 - Not as useful for long term large scale storage of stocks

However:

- Very simple method that can be performed in all laboratories
 - Low cost and very quick to perform
 - Compatible with initial storage of large numbers of new phage isolates prior to characterisation

14

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Acknowledgments

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15