Development and evaluation of new methods to optimise the Actiphage Rapid™ assay for detection of mycobacteria

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ABSTRACT

Pathogenic mycobacteria including *M. tuberculosis* complex and *M. avium* subspecies *paratuberculosis* are pathogens of global importance posing a risk to human health and contributing to large economic losses in the agricultural industry. Due to nature, severity and implications of mycobacterial infections, there is a need for rapid and sensitive diagnostics. The characteristic, slow-growing nature of pathogenic mycobacteria introduces a barrier to diagnosing infections quickly when using culture-based methods. Histology lacks the sensitivity to detect low cell numbers, nor does it allow for species specific identification. ELISA assays are most used for detection of mycobacteria due to their inexpensiveness and quick turnaround from sampling to results. Although ELISA tests are specific, they have low sensitivity, especially in early stages of infection. To overcome these issues with standard mycobacterial methods of detection, the Actiphage® assay was developed to provide a reasonably quick, sensitive identification method by using a broad host-range Mycobacteriophage to lyse mycobacterial cells recovered from white blood cells and using endpoint PCR to identify species-specific signature DNA sequences extracted from the mycobacteria.

This project was sponsored by PBD Biotech Ltd. to develop, evaluate and optimise Actiphage Rapid™-qPCR based approaches for detection of mycobacteria from clinical blood and milk samples. Whilst qPCR kits are currently commercially available for the detection of *M. avium* subspecies *paratuberculosis*, many of these kits have been optimised for detection from faecal, tissue or organ samples. Therefore, whether these kits were compatible with DNA samples prepared using

mycobacteriophage lysis and containing substances from blood known to inhibit PCR amplification remained to be explored. Evaluation of the use of commercial qPCR kits to detect mycobacterial DNA prepared from the Actiphage® assay was carried out. The results highlighted issues with using commercial kits including the need to implement a ΔCq analysis method due to unspecific binding events of the Actiphage Rapid™ assay negative control. Results also showed that trouble shooting was difficult with commercial qPCR kits, therefore, the company decided to develop and optimise an in-house qPCR assay to allow for more control over assay development. Whilst there was success in designing and using a novel qPCR kit for the detection of *M*. *avium* subspecies *paratuberculosis* and *M*. *tuberculosis* complex, results show that more optimisation is needed to efficiently incorporate an internal amplification control within the assay, something that is essential for a diagnostic kit. Nevertheless, experiments to detect *M. avium* subspecies *paratuberculosis* from blood of farmed goats by Actiphage Rapid™ assay and qPCR using a commercial qPCR kit demonstrated that Actiphage Rapid™-qPCR assay approaches offer a more sensitive detection of M. avium subspecies paratuberculosis than ELISA based assays.

A second area of research focused on elucidating further applications of the Actiphage Rapid™ assay. Areas of interest were using Actiphage Rapid™ assay as a physical lysin to extract mycobacterial DNA for sequencing experiments, investigating restriction fragment length polymorphisms found in *M*. *avium* complex and using Actiphage Rapid™ assay to aid in herd management. Exploiting single nucleotide polymorphisms within an insertion sequence of *M*. *avium* complex to develop a high-resolution melting analysis approach allowed for differentiation of

M. *avium* subspecies *paratuberculosis* subtype, something that can be beneficial as an epidemiological tool. Finally, through testing of bovine bulk milk and individual cattle with Actiphage Rapid™ assay, results were used to successfully isolate *M*. *tuberculosis* complex infected animals from a herd to control disease and work towards a disease-free status for the farm, validating that Actiphage Rapid™ assay is a useful diagnostic tool for control of mycobacterial disease.

IMPACT STATEMENT

The work in this thesis was disrupted due to the COVID-19 pandemic. Restrictions meant that the experimental period of the PhD were disrupted due to inability to access the laboratory during lockdown and restrictions on number of persons allowed in the laboratory at any one time. The work was additionally disrupted during COVID-19 due to the sponsor, PBD Biotech Ltd., requiring the research to change to accommodate their needs during this time. Research was also affected by changes in CEO of the company who sponsored the project, which led to several changes in direction of what was asked to be focused on.

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LIST OF ABBREVIATIONS

ACK	**Ammonium-Chloride-Potassium**
ADC	**Albumin Dextrose Catalase**
AGID	**Agar gel immune diffusion**
ATCC	**American Type Culture Collection**
AVHLA	**Animal Health and Veterinary Laboratories Agency**
BCG	**Bacillus Calmette-Guérin**
BLAST	**Basic Local Alignment Search Tool**
bp	**base pairs**
CA	**cellulose acetate**
cfu	**colony forming units**
Cq	**quantification cycle**
DEFRA	**Department for Environment, Food and Rural Affairs**
DMSO	**Dimethyl sulfoxide**
DNA	**deoxyribonucleic acid**
E	**Efficiency**
EDTA	**Ethylenediaminetetraacetic acid**
ELISA	**Enzyme-linked immunoassay**
FSA	**Food Standards Agency**
g	**grams**
gDNA	**genomic deoxyribonucleic acid**
h	**hours**
HRM	**High-resolution melting**
IAC	**Internal amplification control**
ID	**Identification**
IFU	**Instructions for use**
IL-12	**Interleukin 12**
IPC	**Exogenous internal positive control**
L	**litres**
LH	**Lithium heparin**
LOD	**limit of detection**
M	**molar**
MAA	**Mycobacterium avium subspecies avium**
MAC	**Mycobacterium avium complex**
MAH	**Mycobacterium avium subspecies hominissuis**
MAP	**Mycobacterium avium subspecies paratuberculosis**
MAS	**Mycobacterium avium subspecies silvaticum**
mg	**milligrams**
min	**minutes**
ml	**millilitres**

mm	**millimetre**
mM	**millimolar**
MP	**Media Plus**
MQ	**milli-Q**
MTBC	**Mycobacterium tuberculosis complex**
NCBI	**National Centre for Biotechnology Information**
NH	**Sodium Heparin**
nm	**nanometres**
NTC	**non template control**
NTM	**non-tuberculous mycobacteria**
OADC	**Oleic Albumin Dextrose Catalase**
pH	**potential hydrogen**
PBMC	**peripheral blood mononuclear cells**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**
pfu	**plaque forming units**
qPCR	**real-time polymerase chain reaction**
REA	**restriction enzyme analysis**
RFLP	**restriction fragment length polymorphism**
RNA	**Ribonucleic acid**
RO	**Reverse osmosis**
s	**seconds**
SD	**Standard deviation**
SICCT	**Single Intradermal Comparative Cervical Tuberculin test**
SNP	**Single nucleotide polymorphism**
TAE	**Tris-acetate-EDTA**
TB	**tuberculosis**
UK	**United Kingdom**
UoN	**University of Nottingham**
USA	**United States of America**
USP	**United States Pharmacopeia**
UV	**ultraviolet**
V	**volts**
WHO	**World Health Organization**
v/v	**volume/volume**
w/v	**weight/volume**
Δ	**delta**
°C	**Degrees Celsius**
µg	**micrograms**
µl	**microlitres**
µm	**micrometre**

µM	**micromolar**
%	**percent**
x g	**times gravity**

CHAPTER 1

INTRODUCTION

1.1 Background to *Mycobacterium*

Mycobacterium is a genus of fastidious, non-motile, rod-shaped bacteria containing multiple pathogenic and non-pathogenic species within the family of *Actinobacteria*. The *Mycobacterium* genus is a diverse group of organisms that is still growing as new members are identified. To illustrate this, the number of species rose from approximately 170 species to over 200 between 2017 and 2019 (Forbes, 2017, Armstrong and Parrish, 2021). A classification system to categorise mycobacteria into broad groups has been in place since around the 1950's and these are based on traditional microbiological methods such as phenotypic and culture traits and other observable characteristics. One criterion traditionally used, growth rate at optimal temperatures, is used to discriminate slow-growing organisms from fast-growing organisms. Typically, slow growing *Mycobacterium* species take more than seven days of incubation before visible colonies can be observed on solid medium, whereas fast growing *Mycobacterium* species take between two and five days for visible colonies to form (Kim *et al.*, 2013). A second grouping criteria is whether the organism is known to cause disease (pathogen vs non-pathogen), and the pathogens are further grouped according to their host range - including both humans and various animal species - and what type of infection is caused. More recently based on phylogenomic analyses, the *Mycobacterium* genus has been further split into five new genera; *Mycolicibacterium*, *Mycolicibacter, Mycolicibacillus, Mycobacteroides* and *Mycobacterium* (Gupta *et al.*, 2018). Although, this has been met with some resistance from research groups claiming this causes unnecessary confusion in health care settings with the renaming of important pathogens (Tortoli *et al.*, 2019,

Sparks *et al.*, 2023). For the purpose of this work, the original designation of *Mycobacterium* will be used henceforth to avoid confusion.

The non-pathogenic group of mycobacteria include *Mycobacterium smegmatis* (*M. smegmatis*), *Mycobacterium gilvum* (*M. gilvum*) and *Mycobacterium vanbaalenii* (*M. vanbaalenii*) all of which are not reported to cause disease in individuals including those individuals who are immunocompromised (Prasanna and Mehra, 2013). *M. smegmatis*, with a doubling time of 3 to 4 hours compared to up to 16 weeks for pathogenic mycobacteria, is commonly used in laboratories as a model organism for mycobacterial investigation due to the organisms fast-growing nature and the lack of risk of causing disease.

The pathogenic group of mycobacteria are sub-divided into three distinct categories based on the type of disease they cause: the *Mycobacterium tuberculosis* complex (MTBC) causes classical tuberculosis; *Mycobacterium leprae* (*M. leprae*) causes the chronic, infectious, peripheral nerve damaging disease, leprosy, and non-tuberculous mycobacteria (NTM) such as *Mycobacterium abscessus* (*M. abscessus*), *Mycobacterium marinum* (*M. marinum*) and *Mycobacterium avium* complex (MAC).

The general umbrella term of NTM encompasses over 150 species of disease-causing mycobacteria (Greif *et al.*, 2020). NTMs are ubiquitous in the environment and can be isolated in a diverse range of samples ranging from soil to drinking water to milk. NTMs that are most likely to inhabit soil or aquatic environments include *Mycobacterium avium* complex (MAC) and *M. abscessus*. Despite being typically less pathogenic than tuberculosis inducing *Mycobacterium* species, they are still capable of causing disease in humans and animals, with

immunocompromised individuals being more susceptible to infection. Disease can present as pulmonary infection, skin disease or lymphadenitis (Claeys and Robinson, 2018) with pulmonary infections being the most prevalent of the three seen in humans, accounting for between 65-90% of all NTM cases (Porvaznik *et al.*, 2017). Due to the large number of species categorised as NTMs, they are classified according to their growth rate as either slow-growing NTMs if they take more than seven days to grow under optimal conditions and rapid-growing NTMs if they can grow in optimal conditions in less than seven days.

1.1.1. Mycobacterial cell walls

Despite the mycobacterial genus having great pathogenic, genetic, and environmental diversity, they all share a common feature in possessing a complex and robust cell wall which plays an important role in the survival of *Mycobacterium* in hostile environments. All bacteria have a peptidoglycan complex situated on the outside plasma membrane to aid in the maintenance of cell shape integrity; however, mycobacteria are characteristically different to many other bacteria due to the presence of a further structure forming a complex with the peptidoglycan layer. The mycobacterial cell wall structure and composition is relatively unique although similar structures have been reported in members of the order *Corynebacteriales* (Marrakchi *et al.*, 2014). This complex structure features a mycolic acid layer to create a lipid rich, thick, hydrophobic cell wall. Mycolic acids are long chain fatty acids comprised of α-branched, β-hydroxy long chains. Within the mycobacterial cell wall, these mycolic acids are covalently bonded to the peptidoglycan-arabinogalactan complex (figure 1.1) creating a robust structure which assists in the resilience of mycobacteria to survive in harsh environments as

well as being more resistant to pH and temperature stresses. Mycolic acids are able to facilitate this by contributing to the low permeability and hydrophobicity of the mycobacterial cell wall to hydrophilic compounds such as antibiotics, many nutrients such as glucose and glycerol, and the host immune system (Jarlier and Nikaido, 1990). Mycolic acid also contributes to the acid fastness of *Mycobacterium* meaning the bacterium can resist carbol fuchsin stain decolourisation by acid alcohol solutions, allowing for identification by Ziehl-Neelsen staining.

Although all mycobacteria have mycolic acids in their cell walls, mycolic acid composition varies between species. These differences in mycolic acid composition contribute to differences in pathogenicity and resistance to host defences. Mycolic acids can exist in the non-oxygenated α-mycolic acid form or the oxygenated keto- and methoxymycolic acid forms (Di Capua *et al.*, 2022). Alpha-mycolic acids are the most common to be found in mycobacterial cell walls. Keto-mycolic acids contain a keto functional group, and these mycolic acids can be found in mycobacterial species such as *Mycobacterium avium* and *Mycobacterium intracellulare*. Methoxymycolic acids which can be found in species such as *M. smegmatis* contain methoxy groups in the mycolic acid. Dubnau *et al.* (2000) demonstrated the importance of oxygenated mycolic acids in the virulence of *M. tuberculosis* through creating a strain with inactivated *hma* gene, which contributes to the production of oxygenated mycolic acids, thus causing a loss of virulence to *M. tuberculosis*.

In addition to mycolic acids, mycobacteria have other features within their cell walls that contribute to their persistence and survival within the host. Lipoarabinomannan, a lipoglycan found in the cell wall of mycobacteria, contributes

to the inactivation of kinase activity and functioning of T-cells and phagocytic macrophages (Knutson *et al.*, 1998) whilst lipomannan induces the production of interleukin 12 (IL-12) and macrophage apoptosis (Ghazaei, 2018).

Figure 1.1 Structure of mycobacterial cell walls

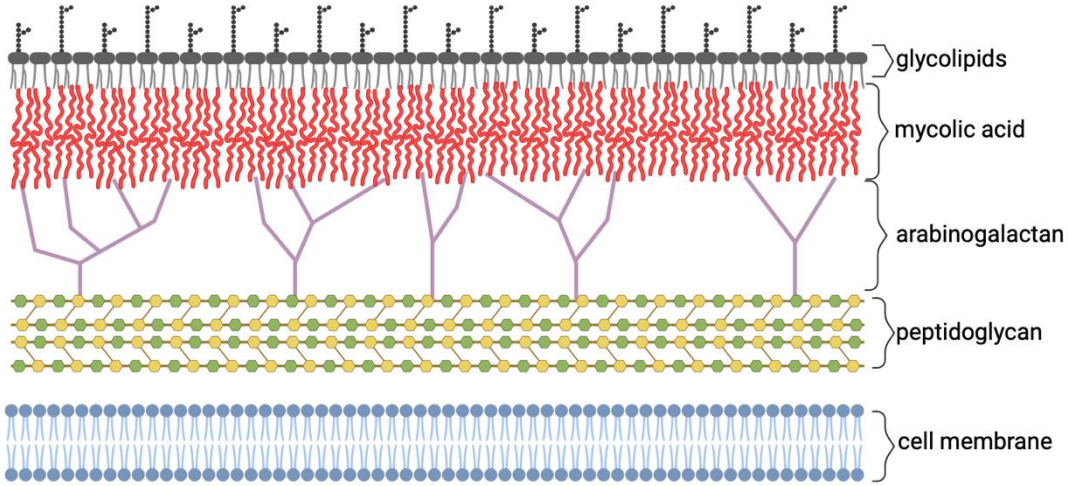


Image produced using biorender.com

A simplified schematic diagram of mycobacterial cell walls showing the complex structures of peptidoglycan, arabinogalactan, and mycolic acid complex. Cell membrane is on the inside of the structure. A layer of peptidoglycan made up of glycan chains connected by short peptides aids in maintenance of cell structure. Covalently bound to the peptidoglycan layer are protein arabinogalactan structures. Closer to the surface of the cell wall, mycolic acids in parallel chains are linked to the peptidoglycan-arabinogalactan complex. This myco-membrane of peptidoglycan-arabinogalactan-mycolic acid acts as the permeability barrier for the cell. On the outer surface of the cell wall are a layer of glycolipids consisting of glucan and proteins and very little lipid. The entire structure of the mycobacterial cell wall aids in the resilience of Mycobacteria to survive in hostile environments.

1.2. *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis complex (MTBC) is made up of obligate pathogens from the *Mycobacterium* genus that are known to cause tuberculosis infection in both humans and animals. Mycobacteria are grouped this complex due to their close genetic similarity, sharing greater than 99.9 % homology in 16S RNA sequences at a nucleotide level (Todd, 2023) and ability to cause similar disease in a range of hosts. Despite genetic and pathogenic similarities, members of MTBC have different epidemiology and host ranges. The complex comprises of seven characterised species; *Mycobacterium tuberculosis* (*M. tuberculosis*) which is the main cause of tuberculosis (TB) disease in humans, *Mycobacterium bovis* (*M. bovis*), known to cause tuberculosis infections in a wide range of animals, *M. bovis* BCG (Bacille Calmette-Guérin) which is used as the vaccine strain, both *Mycobacterium africanum* and *Mycobacterium canettii* which cause pulmonary tuberculosis most commonly in African countries, *Mycobacterium microti* which has been reported to infect dogs and cats and *Mycobacterium pinnipedii* that is known to cause disease in seals. Two of the more clinically significant members of MTBC are *M. tuberculosis* and *M. bovis*.

M. tuberculosis is the main cause of tuberculosis infections in humans responsible for over 90 % of cases of tuberculosis. Disease is primarily transmitted through droplets by coughing or sneezing however transmission through ingestion of contaminated foodstuff or direct contact of infectious material through breaks in the skin can also occur (Sartoris *et al.*, 2020, Coleman *et al.,* 2022). Tuberculosis is more prevalent in densely populated areas with poor hygiene with infants and immunocompromised people being at higher risk of infection due to the nature of

infection. *M. tuberculosis* is a disease of high global significance with it being estimated by the World Health Organization (WHO) that 10.6 million people were infected with *M. tuberculosis* and causing approximately 1.3 million deaths in 2022 alone (WHO, 2022). There are very few countries in the world that are considered MTBC free. *M. tuberculosis* can reside within macrophages in the host, often persisting for several years without causing any disease or symptoms for the infected individual, known as latent tuberculosis. It is estimated that globally there are over 2 billion people living with latent tuberculosis infection (Mendum *et al.,* 2015). Due to this, clinical signs can take months or even years to begin to appear.

Whilst *M. bovis* primarily infects animals, notably bovine ruminants, the disease has been reported to be transmitted to humans through the food chain by consumption of infected unpasteurised milk and even through meat (de la Rua-Domenech, 2006). This risk is typically greater in regions with high *M. bovis* rates twinned with lower food hygiene standards. In livestock herds, MTBC can spread through ingestion of contaminated milk from mother to calf, aerosol transmission or contaminated feed and water. Some, non-bovine, wild animals act as a reservoir of infection meaning that they are natural hosts to the pathogen and aid in the transmission of MTBC infection through populations. The freedom of wild animals acting as wildlife reservoirs for MTBC to move through environments, allows for a rapid transmission of disease to domesticated and farmed animals. Wild animals that are recognised as disease reservoirs for *M. bovis* infection include European badger in the United Kingdom, African buffalo in South Africa and brushtail possum in New Zealand (Palmer, 2013). Therefore, monitoring and surveillance of disease as

well as eliminating transmission from wildlife reservoirs to domesticated animals is imperative to aid in transmission control.

1.2.1. MTBC infection in animals

MTBC infects both wild and domesticated animals worldwide, with only a handful of countries globally holding an MTBC-free status. *M. bovis* is an important pathogen in cattle causing bovine tuberculosis. *M. bovis* is found globally and disease has severe economic implications in the agricultural industry due to causing reduced milk yields of livestock and weight loss in infected animals.

Due to the large range of animals which MTBC can infect, and the ability of MTBC to persist for extended periods of time within the environment (Allen *et al*., 2021), control of the disease can be complex. Symptoms of *M. bovis* infection in animals includes weight loss, enlarged lymph nodes, diarrhoea, and weakness.

M. bovis typically infects the respiratory tract of cattle where the pathogen is phagocytosed by host alveolar macrophages. Usually, macrophage host defences mean that upon phagocytosis, phagosome form around foreign bodies and a lysosome containing digestive enzymes fuses with the vesicle to create a phagolysosome. During a normal immune response, the cellular enzymes secreted during the formation of the phagolysosome can break down bacteria ingested by the macrophage, however, *M. bovis* can resist this degradation process. Persistence within macrophages leads to immune cell recruitment and granuloma formation. Granulomas are organized, inflammatory structures created as more macrophages, lymphocytes and other immune cells are recruited to the site of infection. The *M. bovis* containing granulomas eventually lead to necrotic tissue and lesions forming

at the infected site and eventually dissemination to other sites within the body. Dissemination happens in immunosuppressed animals and those animals in the later stages of infection. Lesions can appear in multiple organs including the liver, kidneys, and gastrointestinal tract.

Diagnosis of MTBC infection in ruminants is usually by single intradermal comparative cervical tuberculin test (SICCT), enzyme-linked immunosorbent assay (ELISA) and lateral flow tests for serum antibodies, all of which are indirect diagnostic techniques. SICCT tests, also known as delayed hypersensitivity tests, are the standard method of MTBC detection for ruminants including cattle, deer, and goats. The test is relatively simple and quick and involves an intradermal injection of tuberculin purified protein derivative followed by measuring the skin response in a specified time. However, the only available vaccine against MTBC, Bacille-Calmette-Guerin (BCG) vaccine, can sensitise animals reducing the efficacy of the tuberculin skin test (Srinivasan *et al.*, 2019). A further limitation of using the tuberculin skin test for diagnostics is that a positive reaction cannot discriminate between active and historic infection of the animal. False-negative results can be observed from recently infected and immunosuppressed animals therefore disease-free status of herds can only be achieved after several sequential negative test results. False-negative results can also be obtained from animals subjected to repeat exposure to the tuberculin test through desensitisation.

MTBC infection is a global concern with many countries implementing their own control programs to eradicate disease. Several countries with control programs in place have stringent testing and culling programs for MTBC positive animals. In

Europe, regulations are in place with the goal of eradicating MTBC. Despite efforts to eradicate disease, due to many wild animals serving as wildlife reservoirs, total eradication and control of MTBC can be difficult.

1.3. *Mycobacterium avium* complex

A *Mycobacterium* complex with ever growing clinical significance is the *Mycobacterium avium* complex (MAC). Members of this complex are all NTM mycobacteria therefore can cause disease in a range of hosts. MAC are divided into two species (*Mycobacterium avium* and *Mycobacterium intracellulare*). Allocation into species in MAC was preliminarily based on the Runyon classification system which specified organisms were to be categorised based on their growth rates, pigmentation and colony morphology (Koh *et al.*, 2002). With the progression of molecular techniques, molecular phylogeny is becoming more widely used to categorise members of the complex including sub-speciating members of the complex based on 16S and 23S ribosomal analysis into four subspecies. *Mycobacterium avium* are further sub-speciated into *Mycobacterium avium* subspecies *hominissuis* (MAH), *Mycobacterium avium* subspecies *paratuberculosis* (MAP)*, Mycobacterium avium* subspecies *silvaticum* (MAS) and *Mycobacterium avium* subspecies *avium* (MAA).

Species of mycobacteria categorised as belonging to MAC are opportunistic pathogens known to cause non-tuberculosis disease in animals and often humans too. There are many non-tuberculous mycobacterial species within the complex which cannot be differentiated from one another through traditional

microbiological techniques, such as making observations on colony morphology, or measuring time for colony growth on solid medium. All members of *M. avium* complex take between 10 and 20 days to form colonies on solid medium. As members of the complex cause a variety of diseases, require different treatments, and have different surveillance and management strategies, it is imperative to successfully isolate and speciate members of the complex from clinical samples. Collectively, MAC causes a range of diseases including chronic enteric disease in ruminants, tuberculosis-like infection in animals and disseminated infections in humans.

One of the subspecies of MAC with an ever-increasing global importance is *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Rathnaiah *et al.*, 2017). MAP has many specific characteristics which allows us to differentiate between the rest of the *Mycobacterium avium* complex. MAP is the slowest growing mycobacteria taking up to 16 weeks to grow on solid media, a characteristic which ultimately gives rise to the difficulty in diagnosing MAP infection. Furthermore, MAP exists in multiple forms known as subtypes (Agrawal *et al.*, 2021).

1.3.1. Mycobacterium avium subspecies paratuberculosis

MAP is a subspecies within the acid-fast, slow-growing *Mycobacterium avium* complex. MAP is primarily a veterinary opportunistic pathogen of ever-increasing global importance causing Johne's disease in ruminants, but also has potential links to Crohn's disease in humans (Rosenfeld and Bressler, 2010, Rathnaiah *et al.*, 2017). MAP is most reported to be isolated from ruminants such as cattle, sheep and goats, however the bacterium has also been reported in other

ruminant as well as non-ruminant species including deer, alpaca, stoat, pigs, horses and rabbits.

MAP is an obligate, intracellular pathogen that results in chronic diarrhoea, malnutrition, muscular wasting, and reduced milk yields. MAP can rapidly spread through herds of animals via the faecal-oral route.

1.3.2. Pathogenesis of MAP

For MAP infections acquired via the faecal-oral route, upon ingestion of MAP, the bacterium typically ends up in the ileum via blood from the mesenteric lymph nodes (Sweeney *et al.*, 2006). From there, MAP fibronectin attachment protein aids in linking MAP cells to M cells, located in the Peyer's patches, via fibronectin and β1 fibronectin receptors which allows M cells to help relocate MAP from the intestinal lumen to the submucosa (Sigurethardottir *et al.,* 2004). Upon release across the intestinal membrane, MAP cells are phagocytosed by macrophages. After phagocytosis, MAP can persist within macrophages as the phagosomes containing mycobacteria are unable to progress through the endocytic pathway and fuse with lysosomes, thus keeping macrophage phagosomes in an immature state (Tooker *et al.*, 2002, Hostetter *et al.*, 2003). Mycobacteria can arrest the development of phagosomes through manipulation of host IL-10 secretion, disruption of host cell Rab7 pathways and maintenance of host Coronin1 (Cor1) protein which ultimately reduces F-actin levels and thus reduced movement of phagosome to lysosome (O'Leary *et al.*, 2011, Keown *et al.*, 2012, Saha *et al.*, 2021). As the infection persists and more macrophages are recruited to the small intestines, they begin to cluster into structures known as granulomas. Granulomas

encourage the formation of necrotic lesions in the intestines resulting in chronic malabsorption of nutrients, protein losses and a condition known as chronic granulomatous enteritis. Collectively this results in the physical characteristics associated with Johne's disease (Chaubey *et al.*, 2016).

1.3.3. MAP as a cause of Johne's disease

Johne's disease was first described in 1895 (Johne and Frothingham, 1895). Johne's disease is a chronic, contagious, intestinal infection causing thickening of intestinal wall leading to malabsorption of nutrients and muscular wasting. The disease mainly infects hoofed mammals, also known as ruminants, that chew cud regurgitated from their rumen. Johne's disease primarily affects sheep, cattle, and goats. Despite this, Johne's disease has also been reported to infect non-ruminants such as non-human primates, stoats, rabbits, and foxes (Beard *et al.*, 2001a, Beard *et al.*, 2001b, Fechner *et al.*, 2017). Johne's disease can be transmitted via the faecal-oral route from swallowing infected manure and through calves ingesting bacteria passed through milk of infected adults or infection can occur *in utero*. Although it was previously believed that calves less than 6 months of age were more susceptible to MAP infection (Windsor and Whittington, 2010), more recent studies have suggested that calves of any age of up to 1 year are susceptible to infection (Mortier *et al.*, 2013). Despite calves being more susceptible to infection, disease is not often detectable for a few years, due to long sub-clinical incubation periods of the infection.

Johne's disease is said to have four stages of infection (USDA, 2024). These four stages are silent subclinical infection, subclinical shedders, clinical infection,

and terminal stage. The first stage of infection, silent subclinical infection, can persist for years before the disease progresses, with infected animals remaining asymptomatic during this time. Current tests to indicate infected animals are faecal cultures and ELISA tests however, during this subclinical stage of infection, there is not a high enough cell count of mycobacteria being shed in the faeces of the infected animals and therefore, infection can remain undetected by faecal culture. Furthermore, infected animals can remain undetected through ELISA testing, as there is not a high enough antibody titre in the blood during this stage of infection. Despite infection remaining undetected, mycobacteria are still actively shed through the faeces of infected animals, further spreading infection throughout the herd. Once infection progresses to the subclinical shedding stage of infection, mycobacteria are intermittently detectable from faecal culture testing, however, infected animals remain asymptomatic.

These first two subclinical stages of infection are typically when MAP is introduced to herds. Once infection progresses to clinical stages and disease, symptoms appear in animals. Infection usually progresses to this stage when animals are 3 to 5 years of age. Symptoms in the clinical stages of infection include intermittent diarrhoea, muscular wasting due to poor nutrient absorption, and decreased milk yields. Faecal culture tests at clinical stages of infection will show high cell counts of MAP whilst ELISA tests will likely show positive results at this stage too. The final, terminal stage of infection is when the disease is most prominent and identifiable. Animals appear emaciated due to extreme inflammation of the intestines and chronic diarrhoea, with many animals also developing bottle jaw (USDA, 2024). Bottle jaw causes swelling beneath the jawline

of animals caused by a persistent shortage of protein within the bloodstream of infected animals. For every animal infected at this late stage of infection, there remains another 15 to 25 likely to be infected and in the earlier, undetectable stages of infection within the herd (USDA, 2024).

The prevalence of this chronic, contagious infection in goats, sheep and deer is less characterised than with cattle, however, it is still a globally significant problem. Clinical signs of disease in both goats and sheep are harder to identify than with cattle, potentially leading to many goats and sheep going undiagnosed worldwide. Whilst the main indicator of disease is still weight loss through inefficient nutrient absorption, goats don't suffer from diarrhoea and only around 20 % of sheep display this symptom (Carrigan and Seaman, 1990, Idris *et al.,* 2021).

1.3.4. Agricultural and economic significance of MAP

Although Johne's disease is endemic in the United Kingdom (UK) (Carslake *et al.*, 2011), due to problems with accurate and sensitive diagnoses, as well as Johne's disease not being a notifiable disease, there is limited data on the prevalence of Johne's within the UK. Velasova *et al.* (2017) found the prevalence of MAP within dairy farms to be 68 % when using ELISA testing to sample bulk milk from 225 British farms. Consequently, this has a huge impact on the agricultural industry, resulting in premature culling, infertility and a reduction in meat and milk yields (Clark *et al.,* 2008). Further financial losses for farmers of diseased herds come from prevention and testing costs needed for diagnosis and monitoring. It is estimated that Johne's disease can cost dairy farmers £60.57 per infected cow per year due to reduced milk yields from cattle (Barratt *et al.*, 2018). In 2005, the total financial loss

in Great Britain was estimated to be £0.327-10 million as a result of dairy and beef cattle infected with Johne's disease (Bennett R. and IJpelaar, 2005), whilst a more recent estimation, predicts around £36.5 million is lost annually to Johne's disease (Rasmussen *et al.*, 2021). A case study investigating the economic impact of Johne's disease on dairy farms in Scotland, found that milk producers with infected herds suffered the most from Johne's disease and their loss per cow was two times greater than that of an uninfected producer's profit (Barratt *et al.*, 2018).

Identifying Johne's disease and the presence of MAP in animals is important for herd management and to reduce the disease prevalence. Diagnostic tests to detect MAP in animals need to be rapid due to the potential global economic losses annually and sensitive as MAP can be transmitted even during the subclinical phase of the infection (Chaubey *et al.*, 2016, Rathnaiah *et al.*, 2017).

1.3.5. Control and management of Johne's disease

There is currently no cure for Johne's disease and as vaccination against Johne's can only control disease and is not a preventative measure, management and surveillance are the implemented control strategies. Within the UK, although there are no stringent government policies with a requirement to test cattle for Johne's disease; many companies and individual government agencies set their own requirements and standards.

In the UK, government agencies implemented a control programme focused on screening, eradication, and biosecurity. Animal Health and Veterinary Laboratories Agency (AHVLA) have a protocol in place known as Herdsure® which is used to establish disease status in cattle and for control and monitoring of herds.

The three main steps of the protocol are to establish a Johne's disease status of herds, improve health status of herds with Johne's disease and monitor and maintenance of health status of herds with Johne's disease. Within this programme, biosecurity measures involve testing blood and faeces of cattle in herds before introducing any individuals to a new herd. Advice from the Scottish Government is for farmers to liaise with their veterinarian to implement health and welfare programmes to attempt to limit the spread of infection.

Arla, a large UK milk supplier to supermarkets, has stringent guidelines on the testing of cattle for Johne's disease. They have a requirement in place for milk producing herds to undergo antibody ELISA testing once a year. If prevalence of ELISA positive animals is more than 4 % the entire herd is required to undergo additional whole herd testing. Calving herds are also required to be tested before breeding when possible. To reduce the risk of false-positive results, which themselves have economic implications, herds that have undergone tuberculin skin testing have to wait a minimum of 60 days before being tested for MAP, to avoid any cross-reactivity affecting results.

In the US, preventative measures are recommended for MAP-negative herds including keeping herds closed and requirements for new animals introduced into these herds, to only come from other MAP-negative herds.

1.3.6. MAP and the link to human health

Parallels can be drawn between characteristic symptoms of Crohn's disease in humans and Johne's disease in ruminants (Mintz and Lukin, 2023). Both diseases are chronic, inflammatory granulomatous diseases affecting the gastro-intestinal

tract. The exact aetiology of Crohn's disease is still yet to be proven, however, ever since reports first emerged in 1913 regarding similarities between disease symptoms in humans compared to Johne's disease symptoms in cows, there has been speculation that MAP plays a role in contributing to Crohn's disease (Dalziel, 1913, McNees *et al.*, 2015, Aitken *et al.*, 2021).

Although there has been no firm link established between Johne's disease and Crohn's disease in humans, the Food Standards Agency (FSA) and the Department for Environment, Food and Rural Affairs (DEFRA) recommended that strategies are implemented to reduce the number of MAP infected animals that enter the food chain, with current measures focussing on milk. Furthermore, there is a constant stream of research being performed to ascertain or discredit this link between the two diseases. Many studies have isolated viable MAP the blood of Crohn's disease patients (Naser *et al.*, 2004, Mendoza *et al.*, 2010).

1.4. Diagnostic tests for detection of mycobacterial infection

Diagnosis of mycobacterial infections in animals is crucial in herd management and surveillance, as vaccination against mycobacterial infection is not currently widespread (for MTBC), or is unavailable (for MAC). Currently, there is only one vaccine available for the prevention of MTBC infection in the form of the live attenuated *M. bovis* strain Bacille-Calmette-Guerin (BCG). Although other potential vaccine candidates have been investigated, none have yet to provide better protection from MTBC than the BCG vaccine (Vordermeier *et al.*, 2016). As vaccination on a national level for domesticated livestock has challenges, rapid and

accurate diagnosis of mycobacterial infection is imperative for controlling disease. Diagnosis of mycobacterial infections remains problematic due to the fastidious, slow-growing nature of mycobacteria, as well as a lack of clinical symptoms from the infected animal. Due to the severity of disease both MTBC and MAC can cause; there is a huge importance in diagnosing mycobacterial infections quickly and accurately. Methods of detection can take a traditional approach using long-standing microbiological methods such as selective culture methods, or detection can take a modern approach by molecular methods such as polymerase chain reaction (PCR). Globally recognised diagnostic tests for identification of MAC infection in ruminants include culture from faecal samples and ELISA testing, whilst globally recognised standards for diagnosis of MTBC in animals is the intradermal tuberculin skin test.

Diagnostic tests for the detection of mycobacteria fall under two general categories: direct methods of detection and indirect methods of detection. Traditional diagnostic methods are typically direct methods of detection, whereas more modern molecular diagnostic methods are typically indirect methods of detection.

1.4.1. Direct methods of detection of mycobacterial infection

Direct methods of diagnosis detect the presence of the pathogen isolated from a host. Direct detection methods include acid-fast staining and microscopy, bacterial isolation through culture, and histopathology and antigen detection from tissue samples.

Culture of mycobacteria on Herrold's egg yolk agar is classed as the gold-standard and a direct method of detection (Clark *et al.*, 2008). Culture-dependant methods of diagnosis can use faeces, tissue, milk, sputum and blood as the sample matrix; however, faecal culture remains the main culture method in diagnostics. Once a suitable sample is obtained, samples are decontaminated, usually by a chemical decontamination method (Stephenson *et al.,* 2021). The most used chemical decontamination methods for faecal samples are sodium hydroxide (NaOH), hydrochloric acid (HCl), sulphuric acid (H_2SO_4), oxalic acid, benzalkonium chloride, trisodium phosphate and sodium chloride (NaCl) (Tell *et al.*, 2003, Sattar *et al.*, 2018). For slower growing members of the *Mycobacterium* genus, such as MAP, growth on solid media can take up to 16 weeks, in turn delaying the diagnosis of disease which can be detrimental to the livestock industry and gives time for the infection to rapidly spread throughout herds (Chaubey *et al.,* 2016). Once colonies are visible on culture medium, additional tests can be performed to confirm the identity of the *Mycobacterium* species. These tests can include Ziehl-Neelsen staining or PCR. Whilst faecal culture is useful for detection of clinical cases and can be used to determine prevalence of infection by direct colony counts, there are also many limitations to the method.

A big limitation of using faecal culture is the need to use decontaminants to remove any normal faecal microbiota found in the digestive system of the host, which reduces the sensitivity of the testing. Studies have shown that not all non-target organisms are successfully removed during chemical decontamination processes (Whittington, 2009). This can be problematic due to the slow-growing nature of mycobacteria; if the removal of other bacteria is unsuccessful, then these

contaminating organisms would be able to grow faster, thus outcompeting the mycobacteria for nutrients and resources on the growth medium. To overcome this issue and supress the growth of competing microbiota that are not killed through the chemical decontamination, antibiotics such as vancomycin and nalidixic acid can be used. Whilst the use of such antibiotics can be used to complement chemical decontamination processes and improve culture sensitivity, the antibiotics can also inhibit the growth of mycobacteria (Sattar *et al.*, 2018). Furthermore, long incubation periods can cause a delay in obtaining results.

Another limitation to the use of faecal culture as a diagnostic method for detection of MAP, is a lack of sensitivity due to low cell numbers of mycobacteria being shed in faeces of infected animals, especially during the subclinical phases of infection. This can often lead to false-negative results. Sensitivity of the faecal culture method for diagnosis of MAP infection in ruminants is only 30-50 % meaning this is an impractical method for diagnosing animals in subclinical stages of infection (Whitlock *et al.*, 2000).

Histopathology of tissue samples is another direct method of detection for diagnosing mycobacterial infections. For detection of MTBC, tissue samples are taken post-mortem from animals that test positive to SICCT. Upon post-mortem examination, any abnormal lymph nodes or organs displaying suspected tuberculous lesions are sampled. Organs that can potentially display these lesions are kidneys, adrenal glands, liver, spleen, and pancreas (Martín-Hernando *et al.,* 2007). If a SICCT positive animal has no visible lesions in these locations, then specific lymph nodes are targeted for sampling including lymph nodes, from the

head or respiratory tract. As with faecal culture, histopathological samples also require decontamination prior to further processing to avoid growth of any contaminating bacterium that may be present in the tissue sample. The waxy cell wall of mycobacteria means they can be resistant to extreme changes in pH, therefore chemical decontamination is the preferred choice. Diagnosis from histopathology of tissue samples can be achieved through direct microscopy of mycobacteria, culturing or through histopathological staining of the multi-nucleated cells associated with granulomas and necrotic tissue. Similarly to faecal culture, tissue sampling can often lead to false-negative results due to the interspersion of mycobacteria in the body.

1.4.2. Indirect methods of detection of mycobacterial infection

Indirect methods are used as an alternative to direct detection methods to overcome the limitations faecal culture and tissue sampling approaches present. Indirect methods of detection of mycobacteria involve detecting cell-mediated and humeral immune responses with tests. ELISA is an example of a blood-based *in vitro* test that detects anti-mycobacteria antibodies in the blood. Agar gel immune-diffusion assays (AGID) are another blood-based *in vitro* test using the serum from blood to detect antibodies produced in response to mycobacterial infection. ELISA tests are the recommended main testing method for use with the Johne's disease protocol (AHVLA, 2012).

ELISA assays are an immunological assay detecting soluble substances, for example, antibodies, peptides, hormones, or proteins from the sample, making them a commonly used indirect method of mycobacterial detection. ELISA assays

are typically performed in a 96-well plate, meaning they can be a high-throughput diagnostic assay. ELISA assays were first developed in the 1990s to allow for the identification of mycobacterial species including MTBC, MAC and *Mycobacterium kansasii* (Schoningh *et al.*, 1990).

AGID diagnostic tests have been used as early as 1984 and can be used for diagnosing Johne's disease in animals (Sherman *et al.*, 1984). Briefly, an agar plate is prepared with two holes, or wells, in the medium. Serum extracted from the test subject is placed in one of these wells, whilst a MAP antigen is placed in the second well. The serum and the antigen will diffuse into the agar. If the animal is MAP-positive, the serum will contain MAP antibodies, which will bind to the diffused MAP antigen. The antibodies in the serum and the MAP antigen will form a complex and precipitate in the agar. The precipitate appears as a thin white line in the agar between the two wells. Results from this test rely on subjective interpretation of the results by the laboratory user. In addition, it has been reported that AGID tests lack both sensitivity and specificity. In one study by Ferreira *et al.* (2002), AGID sensitivity was reported to be 57 % and specificity 92.5 %. In the same study, there were also two false-positive reactions. Due to these results, it was concluded that AGID testing was an unsatisfactory screening diagnostic method to detect infection at a subclinical level within a herd of cattle (Ferreira *et al.,* 2002). Similarly poor findings were recorded by Robbe-Austerman *et al.* (2006) who reported a specificity of 99.5 % and sensitivity of 8.3 % for the detection of MAP from sheep populations in the United States.

Although indirect serological methods which rely on the presence of antibodies in the blood have high specificity, sensitivity can be as low as culturing, as they too require the animal to be in the clinical stages of infection, as antibodies are only produced during this stage (Stabel, 1997, Clark *et al.*, 2008, Chaubey *et al.*, 2016). Despite a low sensitivity, serological tests are more rapid than faecal culture. Furthermore, ELISA testing can produce false-positive results when testing for Johne's disease, if animals have recently been tested for *M. tuberculosis* using the SICCT test. This SICCT used for detection of *M. tuberculosis* can elicit an antibody response which can be detected in ELISA tests used for Johne's disease (Kennedy *et al.,* 2014). False-positive ELISA tests from milk samples can also arise from cows that have recently calved. This usually happens up to 10 days after calving (AHVLA, 2012).

Issues with the current direct and indirect methods of detection are that they are time consuming and lack sensitivity and specificity, particularly in the critically important subclinical stage of the infection. Furthermore, conventional methods of diagnosis involving post-mortem examination and histopathological analysis of tubercle formation are unsuitable for quick, early diagnosis of live animals. Due to the incurable nature of Johne's disease, the severity of MTBC infection and the rate at which mycobacterial infections can spread through herds, a diagnostic test which is not only highly sensitive and specific, but also rapid is essential to detect the disease during asymptomatic stages of infection. Further limitations of using faecal culture and ELISA as methods of diagnosing MAP infection in goats, is that sensitivity of these tests is relatively poor and require the animal to be in the later, clinical stages of infection before a positive result is identified

through the tests (Buendia *et al.,* 2022). This can lead to false-negative results and under-reporting of disease.

PCR assays have been developed to increase the sensitivity of mycobacterial detection. PCR has been proven to detect more positive animals than ELISA or culture-based methods. Faecal samples are most commonly used to extract mycobacterial DNA template required for PCR detection, however due to the nature of intermittent and infrequent shedding of MAP in faeces, particularly during the early stages of infection, the sensitivity of this approach still is not high enough for optimal reduction of disease prevalence (Clark *et al.*, 2008).

During MTBC diagnosis, additional molecular analysis can be employed to genotype members of the complex such as spacer oligonucleotide typing (spoligotyping), mycobacterial interspersed repeat units-variable number tandem repeat (MIRU-VNTR) or whole genome sequencing. Spoligotyping can genotype members of MTBC through amplification of chromosomally encoded loci that contain a variable number of repeat sequences interspersed with nonrepetitive spacers. After hybridisation with multiple spacer oligonucleotides, strain dependant patterns of the amplified DNA are determined and presented as digital codes (Kamerbeek *et al.*, 1997). Strain-dependant digital codes are available in online databases for more efficient identification. However, these approaches can often be costly and time consuming and still require purified extraction of mycobacterial DNA.

1.5. Bacteriophage

To overcome the limitations posed with using traditional methods of diagnosis of mycobacterial infections, the specific and lytic nature of bacteriophage (phage) can be exploited. Bacteriophages are viruses that specifically target bacterial cells and can be used as an alternative to chemical or mechanical cell lysis methods. They are intracellular, obligate parasites that hijack the machinery of host cells to replicate. Bacteriophage can attach to the bacterial cell wall, and their nucleic acid can enter the cell. Upon entering the host cell, bacteriophage can enter latency by integrating their nucleic acids into the host genome, persisting in the cell for long periods of time (lysogeny), or the virus can produce progeny phage and lyse out of the cell. Both lysogenic and lytic bacteriophage have been isolated from mycobacteria, the latter being a focus in aiding in the detection of mycobacteria from animal host cells.

Within the mycobacteriophage genome, lytic phage possess a lytic cassette that encodes for an endolysin enzyme, which can target the peptidoglycan cell wall of the host bacterial cell, holin, a membrane-perforating protein and mycoarabinogalactan esterases to break down the outer mycolic acid arabinogalactan complex of the mycobacterial cell wall (Payne *et al.*, 2009, Fernandes and Sao-Jose, 2018). Holins are a diverse group of pore-forming, membrane proteins, essential for the lysis and release of progeny phage from bacterial cells. Holins are also essential in determining the fate of the bacterial cell, as without the activity of these proteins, the digestive esterase enzymes are unable to accumulate and break down the peptidoglycan within the mycobacterial cell wall (Bavda *et al.*, 2021). Mycobacteriophage D29 is an example of a lytic bacteriophage.

This bacteriophage is widely used in mycobacterial research due to the broad host range, as the phage is capable of infecting and killing pathogenic and non-pathogenic strains including *M. bovis*, *M. avium* subspecies *paratuberculosis, M. ulcerans* and *M. smegmatis* (Grant, 2021). Using a bacteriophage with lytic properties provides an alternative approach to challenge the limitations of sensitivity, specificity and detection of viable cells set out by the current diagnostic methods.

1.6. Novel rapid methods for detection of mycobacteria using Mycobacteriophage D29

1.6.1. Actiphage® Core Assay

Actiphage® assays were developed as an alternative in diagnostic testing to have greater sensitivity than faecal culture and ELISA based diagnostic tests. The Actiphage® Core assay was adapted from the FASTPlaqueTB™ assay (Biotec Laboratories Ltd; Ipswich, UK), which was originally developed to detect *M. tuberculosis* from sputum through analysis of plaque formation as a result of bacteriophage infecting viable cells from samples (McNerney *et al.*, 2004). Actiphage® Core assay has been successfully used for the detection of *M. bovis* and *M. avium* subspecies *paratuberculosis.* As mycobacteria can persist within host animal macrophages, peripheral blood mononuclear cells (PBMCs) and somatic cells found in milk are good targets for isolation of mycobacteria in animals (Stanley *et al.*, 2007, Botsaris *et al.*, 2013, Swift *et al.,* 2013). Briefly, mycobacteria are isolated from the white blood cells of animals and incubated with Actiphage® reagent

containing a broad host-range, lytic Mycobacteriophage. The incubation period is long enough to allow attachment and internalisation of bacteriophage into cells, but short enough so that lysis of bacterial cells does not occur. Any bacteriophage not internalised is inactivated with virucide and samples are plated onto a lawn of sensor cells. Sensor cells that make up this bacterial lawn are cells that are sensitive to the bacteriophage. Petri dishes of samples plated onto the bacterial lawn are incubated for sufficient time to allow for bacteriophage replication and lysis from any mycobacterial cells that may be present in the samples and then allow for any progeny bacteriophage released from lysed cells to attach, replicate and lyse the sensor cells. After incubation, the presence of plaques (zones of clearing) on the plates confirms the presence of mycobacteria from the sample. One plaque equates to one bacterial cell, thus confirming presence of mycobacteria from clinical samples. Due to the broad host range of the bacteriophage used in Actiphage® reagent, the presence of plaques alone is not sufficient to determine the species of mycobacteria present in the sample. Therefore, for the Actiphage® Core assay to be used for diagnostics, any plaques containing mycobacterial DNA must be picked, purified, and identified using a species-specific PCR.

1.6.2. Actiphage Rapid™ assay

Actiphage Rapid™ assay, descended from the Actiphage® Core assay, overcomes the need for additional processing and analysis of plaques produced by the Actiphage® Core assay, through development of an assay that can extract mycobacterial DNA ready to be used in PCR identification reactions. Actiphage Rapid™ assay was primarily optimised for use with cattle samples, but has also successfully been used to identify mycobacterial infection from deer, humans and

environmental samples (Swift *et al.*, 2020, Verma *et al.*, 2020, Kubala *et al.*, 2021, Beinhauerova and Slana, 2023). Mycobacteria can be extracted from PBMCs or somatic cells of infected animals, and DNA obtained by exploiting the lytic characteristic of a broad-host Mycobacteriophage.

Identification of mycobacteria is achieved through the detection of unique DNA sequences exclusive to the target mycobacteria using endpoint or real-time PCR (qPCR).

1.7 Molecular targets for identification of mycobacteria by PCR

Once Actiphage Rapid™ assay has successfully been used to isolate mycobacterial DNA from samples, Actiphage Rapid™ is complemented by polymerase chain reaction (PCR) to detect and identify mycobacterial infection. PCR, first described by Mullis *et al.* (1986), is a molecular identification technique that, when unique signature DNA sequences are targeted, can correctly identify organisms.

PCR targets for identification of mycobacteria can include single copy genes or multi-copy insertion sequences. Single copy genes are highly conserved, coding regions within the genome which allow for highly specific detection of mycobacterial DNA sequences, although due to their single copy nature, these PCR assays are often compromised on their sensitivity. In contrast, detection of multi-copy insertion sequences can give a PCR assay improved sensitivity, however, using this target detection method can lack specificity.

Insertion sequences are multi-copy, short transposable elements found in bacterial genomes. Insertion sequences don’t encode for any functional genes apart

from those required for the mobility around the genome (Siguier *et al.*, 2006). In mycobacteria, insertion sequences can influence gene expression and virulence through insertion and disruption of genes within the genome (Roychowdhury *et al.*, 2015). Insertion sequences can be species-specific making these ideal PCR targets for identification and diagnosis of disease.

1.7.1. IS1311 insertion sequence

The IS*1311* insertion sequence is found within members of MAC. This insertion sequence has a multi-copy presence within the MAC genome, appearing up to ten times throughout the genome, however, copy number can vary between different lineages of MAP (Mizzi *et al.,* 2024). The use of specific primers to detect IS*1311* is well documented (Marsh *et al*., 1999, Johansen *et al.*, 2005, Shankar *et al.*, 2010). As this insertion sequence is found in all members of the *M. avium* complex and contains single nucleotide polymorphisms (SNPs) exclusive to different members of the complex, further analysis can be applied to amplified PCR products to allow for differentiation of members within the complex.

First described by Whittington *et al.* (1998), the many enzyme restriction sites found within the 1317 bp IS*1311* insertion sequence, can be exploited and restriction enzyme analysis used to differentiate members within the complex. Restriction enzymes *Mse*I and *Hinf*I (or their isoschizomers) are most used to digest an amplified IS*1311* PCR product into various fragment sizes which, once resolved by gel electrophoresis, differing band patterns are indicative to the member of MAC that the DNA was isolated from. Restriction digest fragment lengths are shown in table 1.1.

Table 1.1. Restriction digest fragment lengths of IS*1311* from *M. avium* complex type strains exposed to *Hinf*I and *Mse*I

***M. AVIUM* COMPLEX TYPE**	**RESTRICTION DIGEST BAND PATTERN (BP)**
MAP BISON (B) TYPE	67, 218, 323
MAP CATTLE (C) TYPE	67, 218, 285, 323
MAP SHEEP (S) TYPE	285, 323
M. AVIUM* SUBSPECIES *AVIUM	134, 189, 285

Restriction fragment length polymorphism profiles for different members of M. avium complex after enzyme restriction digest of IS1311 insertion sequence using HinfI and MseI. Mycobacterium avium subspecies paratuberculosis can be resolved into three separate subtypes (bison, cattle, sheep) because of varying presence of HinfI and MseI restriction sites within the insertion sequences. All strains of Mycobacterium avium subspecies avium have the same restriction digest band pattern with this restriction enzyme analysis and there are no known further subtypes for this species.

1.7.2. IS900 insertion sequence

IS*900* is an insertion sequence exclusive to MAP, appearing up to 18 times within the genome. This 1451 bp long insertion sequence was first mapped and characterized by Green *et al.* (1989) and contains a 1200 bp gene encoding for a putative transposase known as *P43*. Due to the multi-copy nature of the IS*900* insertion sequence, primers designed to detect the insertion sequence are a more favourable target when detecting MAP, due to their increased sensitivity over their single copy gene counterparts.

This insertion sequence is well-characterised with many published primers designed to target the sequence. Early primer design for detection of IS*900* was based on very limited cattle (C) strain sequences (Millar *et al.*, 1995). The multi-copy insertion sequence IS*900* is the DNA target used to complement the Actiphage® assay in identification of MAP.

1.7.3. IS6110 insertion sequence

IS*6110* is one of the multi-copy insertion sequences found in MTBC. This 1355 bp insertion sequence has been used for years to detect MTBC (Thierry *et al.*, 1990). Frequency of appearance of this insertion sequence can vary between different strains of MTBC, with strains being categorised as high copy number strains, or low copy number strains. Low copy number strains have been shown to have a higher number of their IS*6110* mutated compared to high copy number strains and it has also been speculated that this also relates to less transposition ability (Comín *et al.*, 2022).

1.8. Polymerase chain rection

Polymerase chain reaction (PCR) is an important component of Actiphage® assays for them to be used as a sensitive and specific diagnostic tool. Since the invention of PCR in 1986, many variations of the PCR assay have been developed such as multiplex PCR, nested PCR and more recently digital-droplet PCR (ddPCR). Multiplex PCR allows for the detection of multiple targets within the same reaction, which is advantageous for diagnostic assays. Nested PCR can be used for more sensitive detection of DNA, by using PCR product from a preliminary round of PCR as DNA template for a second round. Digital-droplet PCR is a relatively new technique that allows for exact quantification of samples by separating these samples into tens of thousands of individual droplets using water-oil emulsion technologies and amplification of template DNA occurs in each one of these droplets.

Whilst PCR can have an improved sensitivity and specificity over other diagnostic approaches, PCR first requires an independent, effective DNA extraction step. Many DNA extraction procedures may also require decontamination to remove PCR inhibitors. After DNA extraction, during PCR, DNA quantity is increased exponentially through thermal cycling reactions until a detectable threshold is reached. At this point, amplification products can be detected through gel electrophoresis, also known as endpoint or conventional PCR, or through hybridisation with probes or intercalating dyes in real-time assays also known as quantitative (qPCR) assays. Actiphage® assays were designed to work originally with endpoint PCR (Stanley *et al.*, 2007) and more recently with real-time PCR (qPCR) (Kubala *et al*., 2021).

1.8.1. Endpoint PCR

PCR is a commonly used molecular technique which allows for the exponential amplification of target DNA sequences. Typically, the main steps involved in PCR amplification are initial denaturation, followed by a series of steps (denaturation, annealing and extension) which are repeated in a cyclic manner to allow for exponential amplification of DNA. The initial denaturation step, where the DNA sample is heated to around 94-98 °C, is performed first to ensure the hydrogen bonds which hold double-stranded DNA (dsDNA) helix together are denatured, giving way to two single-stranded DNA (ssDNA) molecules. This initial denaturation step can take up to 15 minutes to ensure that all secondary structures of the DNA are dissociated and available for primer binding. During every PCR cycle, there is a brief denaturation step to allow for the dissociation of bonds between dsDNA. This denaturation step is shorter than the initial denaturation as only the target sequence needs to be dissociated. This is followed by annealing whereby the temperature is lowered to allow for the specific binding of primers complimentary to the target ssDNA sequence. Once primers are bound to the DNA, the temperature is increased to 72 °C for the extension step where DNA polymerase can hybridise to the primers and extend the DNA fragments from the 5' end to the 3' end. Thermal cycling is repeated resulting in an exponential increase in amplified PCR products (amplicons). At the end of the thermal cycling PCR reaction, amplicons can be resolved using gel electrophoresis and visualised through staining of the gel. Template DNA for use in PCR reactions can be extracted via several methods which can include direct boiling of cells containing target DNA or by using extraction kits or assays. Actiphage assays can be used as such to extract DNA to be used for PCR.

1.8.2. Real-time PCR

Real-time PCR or quantitative PCR (qPCR) is a molecular biological technique that can monitor the amplification of targeted DNA in real time, quantitatively every cycle as opposed to at the end of the reaction process, such as with conventional PCR. qPCR is favourable in diagnostic testing, as qPCR facilitates high throughput detection, sensitivity, specificity, and quantification of target DNA. Furthermore, the ability to multiplex several targets in one reaction allows for the addition of internal amplification controls to monitor reactions for inhibition, which is essential for diagnostic qPCR assays.

The two most common variants of qPCR are the fluorogenic 5' nuclease assay, or the intercalating dye assay. Fluorogenic 5' nuclease assays, also known as hydrolysis probe assays, first developed by Holland *et al.* (1991) and later advanced into a real-time format first described by Heid *et al.* (1996), exploit the 5' to 3' nuclease activity of the *Taq* polymerase enzyme from *Thermus aquaticus* to allow specific detection of DNA products. These qPCR assays require a hydrolysis probe, dually labelled with two dyes known as the reporter dye and quencher dye. Reporter dyes have fluorescent properties as these are what allows qPCR amplification to be measured, whilst quencher dyes can be fluorescent (TAMRA™) or non-fluorescent (DABCYL and Black Hole Quencher™ (BHQ™)). The oligonucleotide probe is designed to only bind to the DNA sequence between the two primer sites. The reporter dye is synthesised to the 5' end of the probe, whilst the quencher sits at the 3' end. Before the probe hybridizes to any PCR amplicons, the reporter and quencher dye are in close proximity to each other meaning the quencher acts by absorbing the fluorescence of the reporter fluorophore, resulting

in no fluorescence emitted from the reporter. Due to the 5'-exonuclease properties of *Taq* polymerase, as it moves along the sequence, the enzyme degrades the hydrolysis probe thus releasing and separating the reporter from the quencher fluorophore. In turn, this means there is now no fluorescence resonance energy transfer between the two dyes, and the reporter is no longer supressed, causing its fluorescent signal to be detected. This fluorescent signal accumulates throughout the exponential stages of the qPCR assay allowing for a quantitative measure of fluorescence with every cycle (figure 1.2.).

In contrast, qPCR that depends on intercalating dyes to detect a change in fluorescence have no specificity to the target DNA product. Intercalating dye assays, most commonly the SYBR green assay, rely on a fluorescent dye that can intercalate between double-stranded DNA sequences to monitor increasing fluorescence with each qPCR cycle and amplicon production. This intercalating dye is a non-specific, non-discriminative dye that will bind to any double-stranded DNA within the reaction mix. Consequently, SYBR green qPCR assays can lead to a greater number of false positives than hydrolysis probe assays, if the dye intercalates between any non-specific contaminants or primer-dimers that may be present in the reaction mix. To overcome this issue, SYBR green assays often require the additional step of high-resolution melting (HRM) or melt curve analysis to determine whether fluorescence from the qPCR is because of the target DNA, or non-specific binding of the dye. When used in a diagnostic setting, although SYBR green assays are appealing due to being easier to design and set up, as well as typically having a lower running cost than hydrolysis probe assays, their major drawback is the risk of false-positive results if upstream processing methods cannot remove all non-target-

specific DNA from the biological sample it was extracted from or primers lack specificity. Due to this, when dealing with biological samples, TaqMan probe assays are favourable due to their greater specificity over SYBR green assays.

Figure 1.2. Schematic diagram of real-time hydrolysis probe assay

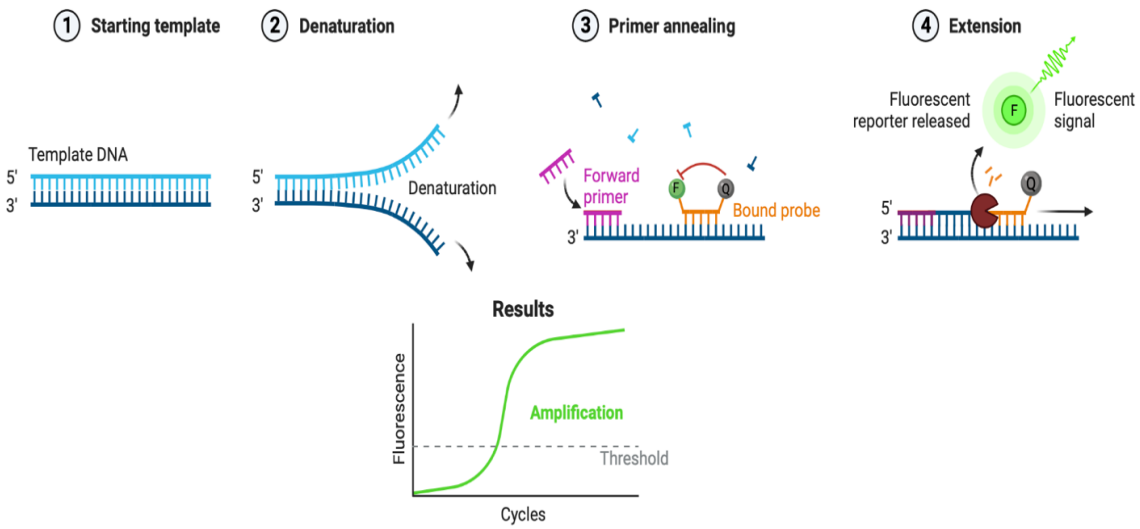


Image produced using biorender.com

*Schematic diagram of how a hydrolysis probe-based qPCR assay works. DNA template is extracted and prepared for qPCR (**1**). Then, the high temperature of the denaturation step causes a dissociation of the hydrogen bonds between the two strands of DNA (**2**). As the temperature cools to prepare for primer annealing, the fluorescently labelled oligonucleotide probe binds to the single stranded DNA followed by the primers (**3**). At this stage, the reporter dye (**F**) is being supressed by the quencher dye (**Q**) due to their proximity and fluorescence resonance energy transfer. During extension, 5' exonuclease activity of Taq polymerase degrades the probe, thus releasing the reporter dye, in turn allowing a fluorescent signal to be detected (**4**). This results in an increase in fluorescence detected after every cycle, and the number of cycles it takes for the fluorescent signal to rise above a baseline (threshold) level can be quantified.*

1.8.3. Real-time PCR melt curve analysis

With qPCR assays, there are many downstream analyses that can be performed to obtain further information on the PCR products. This further analysis can often be performed within the same reaction as the qPCR, often being added as additional steps within the qPCR cycling profile after the final extension step. One such analysis is melting the qPCR products and measuring the temperature of dissociation of the double-stranded DNA, known as melt curve analysis. Melt-curve analysis exploits the fact that fragments of differing sizes and with differing G-C content will have different melting temperatures. Melt-curve analysis is typically used when an intercalating dye-based qPCR has been performed. As an intercalating fluorescent dye binds to any double stranded DNA in a non-discriminatory manner, many non-specific products can also be detected, therefore, melt curve analysis is an essential step when performing dye-based qPCR assays to discriminate between off-target and on-target effects.

Post-amplification, the temperature of the qPCR machine is lowered typically to around 65 °C then slowly raised in small 0.5 °C increments up to 95 °C. As the temperature is raised, the dsDNA products dissociate which results in a decrease in fluorescence intensity. This decrease in fluorescence intensity is read by the qPCR machine, generating the characteristic peaks of a melt curve. Peaks can be analysed, and the melting temperature of dsDNA amplicons can be determined. This technique is often used to complement qPCR assays performed with an intercalating dye such as SYBR green assays. As the dye from such assays does not discriminate between dsDNA, melt-curves are a good way to check that any qPCR amplification is because of target amplification and not due to contamination, non-

specific amplification, or primer-dimer events through analysis of shape of the curves and through melting temperature of products.

Previously, melting analyses have successfully detected polymorphic variation in genome sequences, as specific as identifying down to a single nucleotide in *Botrytis cinerea* (Chatzidimopoulos *et al.*, 2014). In addition, melt curve analysis has also been used to identify antibiotic resistant *Mycobacterium tuberculosis,* arising from single nucleotide polymorphisms in PCR targets, with high specificity ranging from 88 % to 100 % succession rate (Rezaei *et al.,* 2017).

High resolution melting is like melt curve analysis however temperature increments tend to be narrower (around 0.2 °C) which allows for better discrimination in variations of nucleic acid sequences. This technique has previously been used to differentiate different *Mycobacterium* species using 16S rRNA qPCR methods (Issa *et al.*, 2014).

1.8.4. The use of qPCR software to analyse results

Throughout the process of optimising qPCR for diagnostic testing, qPCR efficiency assays are an important tool in evaluating the performance kinetics of the qPCR. To determine whether a qPCR is reproducible and there is accurate quantification of the sample, efficient qPCR assays should be able to produce a linear standard curve with a coefficient of determination (R^2) > 0.980 and have an efficiency within the range of 90 % and 110 %. This data is obtained from qPCR assays where serial dilutions of target DNA are run as template to produce a standard curve. Cq values recorded from this qPCR are plotted against the log of the starting quantity of template or the dilution factor for each target within the

dilution series. From this standard curve, the equation of the linear regression line ($Y = mX + C$), and the coefficient of determination are used to analyse the efficiency of the assay.

In addition to analysis of the linear regression line and coefficient of determination, qPCR assays comparing different reaction conditions require multiple other factors (dynamic range, R^2 value and precision) to be analysed. Dynamic range is the range between the highest and lowest dilutions of starting template that create an acceptable efficiency between 90 % and 110 % and a slope of -3.3 ± 10 %. qPCR assays with an efficiency lower than 90 % can be due to poor primer design, presence of PCR inhibitors, or inappropriate reagent concentrations and reaction conditions. qPCR assays with an efficiency higher than 110 % can be due to cross contamination, polymerase activators and primer dimers. Additional information that can be drawn from a standard curve is the R^2 value. This value gives information on how well we can correlate *X* (for example, cell concentration) and *Y* (Cq value) on the graph. An R^2 value of 1 indicates that there is a direct correlation between *X* and *Y* (Cq value) meaning we can accurately predict the cell concentration of unknown samples based on their Cq value. An R^2 value of 0 shows there is no correlation between the two variables and therefore we cannot accurately use the standard curve to interpret unknown samples. Typically, an R^2 value > 0.980 will provide good confidence in correlating the two variables.

A further evaluation point to consider is precision. There should be a small standard deviation in the ΔCq from the expected Cq difference between two samples in a dilution series. For example, in a 2-fold dilution series, ΔCq between

two successive dilutions should be 1, in a 10-fold dilution series, ΔCq between two successive dilutions should be 3.3. The standard deviation must be ≤0.167 to quantify 2-fold dilutions in more than 99.7 % of cases. The larger the standard deviation, the less accuracy there is in discriminating dilutions in the series.

1.9. Aims and objectives

The aim of the current research is to analyse the use of qPCR-based methods to complement Actiphage Rapid™ assay to aid in detection of mycobacteria from blood and milk samples of a range of animals including cattle, deer and goats. This research aims to understand the compatibility of both commercially available qPCR kits and qPCR kits manufactured in-house and to determine their potential for inclusion in the Actiphage Rapid™ assay diagnostic kit.

The use of Mycobacteriophage as a biological lysing agent to obtain mycobacterial DNA sequence data was also under investigation and to determine whether this too was compatible for diagnosis of mycobacterial infections.

The aims of this research are:

1. Determine whether Actiphage Rapid™ assay is compatible with qPCR for detection of mycobacteria from clinical samples
2. Develop an in-house qPCR kit for mycobacterial detection and evaluate performance against commercially available qPCR kits
3. Investigate the wider applications of Actiphage Rapid™ assay as a lysing agent

CHAPTER 2

MATERIALS, METHODS AND STANDARD PROCEDURES

2.1 GENERAL MEDIA AND REAGENTS

All media were prepared using reverse osmosis (RO) sterile water and autoclaved prior to use at 121 °C for 15 min unless stated otherwise.

2.1.1. General media

2.1.1.1. Phosphate buffered saline

Two phosphate buffered saline (PBS) tablets (Thermo Fisher Scientific; USA) were dissolved per 200 ml RO water and sterilised by autoclave.

2.1.2. Media Preparation for culture growth

All media were prepared using RO sterile water and autoclaved prior to use at 121 °C for 15 min unless stated otherwise.

2.1.2.1. Difco™ Middlebrook 7H9 broth

Difco™ Middlebrook 7H9 medium (Becton Dickinson; UK) was prepared by dissolving 9.3 g of medium in 1 L of RO water and sterilised by autoclave. Mycobacteria were sub-cultured and maintained every two months when grown in Middlebrook 7H9 broth. All cultures were incubated at 37 °C statically.

When culturing *Mycobacterium avium* subspecies *avium*, medium was supplemented with 1.65 µg ml^{-1} Mycobactin J (IDvet; France), 10 % ADC (v/v) (Becton Dickinson; UK) and 1 % glycerol (v/v) (Sigma-Aldrich; USA).

Mycobacterium avium subspecies *paratuberculosis* cultures were supplemented with 1.65 μg ml^{-1} Mycobactin J (IDvet; France) and 10 % OADC (v/v) (Rapid Labs; UK).

When culturing *M. smegmatis* and *Mycobacterium tuberculosis* complex (MTBC), medium was supplemented with 10 % (v/v) OADC (Rapid Labs; UK).

2.1.2.2. Difco™ Middlebrook 7H10 agar

Difco™ Middlebrook 7H10 agar (Becton Dickinson; UK) was prepared by dissolving 3.8 g in 200 ml RO water and sterilised by autoclave. Mycobacteria were sub-cultured and maintained every two months on Difco™ 7H10 agar supplemented with 10 % OADC (Rapid Labs; UK). All cultures were incubated statically at 37 °C.

2.1.3. Electrophoresis Buffer

2.1.3.1. TAE

50x stock of TAE was prepared by dissolving 242 g tris base in RO water and adding: 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA solution (pH 8.0) and adjusting the volume to 1 L. For use in electrophoresis gels and buffers, 50x stock was diluted to a 1x stock using RO water.

2.1.4 Bacterial strains

BACTERIA	STRAIN	REFERENCE
MYCOBACTERIUM SMEGMATIS	*MC^{2}155*	Snapper *et al.* (1990)
MYCOBACTERIUM SMEGMATIS	IAC	Handley-Hartill (2023), University of Nottingham
***MYCOBACTERIUM BOVIS* BCG**	Pasteur	Reference Strain, NCTC 5692
MYCOBACTERIUM AVIUM	avium	Field Isolate, UK
MYCOBACTERIUM AVIUM* SUBSPECIES *AVIUM	7210	Human isolate, Finland
	7212	Human isolate, Finland
MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS	ATCC 19851	American Type Culture Collection
	B4	Field Isolate, Northern Ireland
	K10	Li *et al.* (2005)
	M71/03	Moredun Institute, Scotland (Biet *et al.* 2012)
	M110/05	Moredun Institute, Scotland

	M112/05	Moredun Scotland	Institute,
	M113/05	Moredun Scotland	Institute,
	213G	Moredun Scotland	Institute,
	218	Moredun Scotland	Institute,
	235G	Moredun Scotland (Biet *et al.* 2012)	Institute,
	M266/04	Moredun Scotland	Institute,

2.1.5. Mycobacteriophage isolates

ISOLATE	REFERENCE
D29	PBD Biotech (Suffolk; UK)
TM4	PBD Biotech (Suffolk; UK)
PHAGE U1	Royal Veterinary College (London; UK). Unpublished isolate
PHAGE U2	Royal Veterinary College (London; UK). Unpublished isolate

PHAGE U3	Royal Veterinary College (London; UK). Unpublished isolate
PHAGE U4	Royal Veterinary College (London; UK). Unpublished isolate
PHAGE U5	Royal Veterinary College (London; UK). Unpublished isolate

2.2. PERIPHERAL BLOOD MONONUCLEAR CELL EXTRACTION

2.2.1. PBMC extraction media and reagents

2.2.1.1. Heparin

Heparin lithium salt (Sigma-Aldrich; USA) was resuspended in 12.54 ml sterile MQ water to achieve a concentration of 40 mg ml^{-1} (150 USP mg^{-1}). 2.5 ml of reconstituted heparin was filter sterilised through a 0.2 µm filter into sterile 500 ml Duran bottles for use in whole blood sampling.

2.2.1.2. ACK lysis buffer

To prepare a 10x ACK lysis buffer stock 82.6 g (154.4 mM) Ammonium chloride (Sigma-Aldrich; USA), 10 g (10 mM) Potassium bicarbonate (VWR; USA) and 0.37 g (97.3 µM) EDTA tetrasodium salt (Thermo Fisher Scientific; USA) were dissolved in 1 L sterile RO water. For use in PBMC extraction, the 10x stock was diluted to a 1x stock using sterile RO water.

2.2.1.3. Media Plus

Media Plus (MP) (Difco™ 7H9-based medium) was prepared by supplementing 7H9 broth (section 2.1.2.1.) with 10 % (v/v) OADC (Rapid Labs; UK) under aseptic conditions. 7H9 broth was sterilised by autoclave prior to addition of OADC, after OADC was added, Media Plus could not be autoclaved.

2.2.2. Blood sampling

Bovine, Ovine and Cervid whole blood was sampled from animals by independent third-party veterinarians across the United Kingdom. Blood was collected in either lithium heparin (LH) or sodium heparin (NH) coated vacutainers (Becton Dickinson; USA). Veterinarians were advised to collect a total of 10 ml whole blood from animals where possible.

For blood samples collected from an abattoir (Staffordshire; UK), heparin was prepared (section 2.2.1.1.) and 2.5 ml used per 500 ml Duran bottle. Duran bottles were sterilised by autoclave prior to addition of heparin. After addition of heparin, Duran bottles were manually rotated several times to coat entire surface of bottles with heparin. Bovine blood was collected in bottles and mixed thoroughly by inversion to ensure homogenous mixture of blood with heparin.

2.2.3. PBMC extraction methods

For both ACK lysis and Ficoll-Paque™ PLUS extraction methods, heparin treated whole blood was used. This was achieved by making heparinised containers in house (section 2.2.1.1.) or using lithium and sodium heparin treated vacutainers (Becton Dickinson; UK) to collect samples. Whole blood was supplied by either third

party veterinarians from practices across the UK or a single abattoir located in Staffordshire (UK).

2.2.3.1. ACK lysis

To extract PBMC from blood, first 2 ml of whole blood was transferred to a 50 ml conical based tube. 40 ml ACK lysis buffer (section 2.2.1.2.) was added to the whole blood and mixed for 30 s. The solution was incubated at room temperature for 5 min with occasional gentle mixing. After 5 min incubation period, the solution went from turbid to translucent. If this was not achieved an additional 2 min of gentle mixing was performed. The solution was centrifuged in a Heraeus Megafuge at 300 x g for 5 min at room temperature. The supernatant was discarded, and pellet resuspended in 5 ml PBS. The sample was centrifuged for a further 5 min at 300 x g and supernatant discarded. The pellets containing the PBMC were resuspended in 1 ml MP and stored overnight at room temperature. Samples were spun at 13,000 x g for 3 min, supernatant discarded and pellets ready for use in the Actiphage Rapid™ assay (section 2.4.4.3.).

2.2.3.2. Ficoll-Paque™ PLUS PBMC extraction

To extract PBMCs from blood, 3 ml of the blood separation medium Ficoll-Paque™ PLUS (GE healthcare Life Sciences; UK) was loaded into a Leucosep tube (Greiner BIO-ONE; UK). Tubes were centrifuged at 1,000 x g for 1 min to allow Ficoll to pass below the porous barrier. Next, 2.5 ml of PBS was added to the tubes. 2 ml of whole blood was then added to the tube and gently mixed with PBS by aspirating into the pipette and back into the tube. Samples were centrifuged in a Heraeus Megafuge with a swing-out rotor at 800 x g for 15 min at 20 °C with zero deceleration (brake

off). The upper plasma layer was removed by aspiration and discarded. The buffy coat layer below the plasma layer contained the PBMCs to be harvested (figure 2.1). PBMCs were transferred to a 15 ml conical based tube prefilled with 6 ml PBS. Samples were centrifuged at 200 x g for 10 min at 20 °C and supernatant discarded. The pellet containing the PBMC were resuspended in 1 ml MP and stored overnight at room temperature. Samples were spun at 13,000 x g for 3 min, supernatant discarded and pellets ready for use in the Actiphage Rapid™ assay (section 2.4.4.3.).

2.2.3.3. Somatic cell extraction from milk

Milk samples were left to warm up to room temperature for 1 hour before processing. To extract somatic cells from milk in preparation for both the Rapid- and Core Actiphage assays, 50 ml samples were centrifuged in a Heraeus Megafuge at 2,500 x g for 15 min at room temperature in 50 ml conical based tubes. After centrifugation the cream layer was removed using a sterile spoon and the milk aspirated. The remaining pellet was resuspended in 3 ml MP and samples centrifuged at 2,500 x g for 10 min at room temperature. Supernatant was discarded and pellet was resuspended in 1 ml MP ready for use in either the Actiphage Rapid™ (section 2.4.4.4) or Actiphage Core assay (section 2.4.6.).

Figure 2.1. Peripheral blood mononuclear cell extraction from whole blood using Ficoll-Paque PLUS

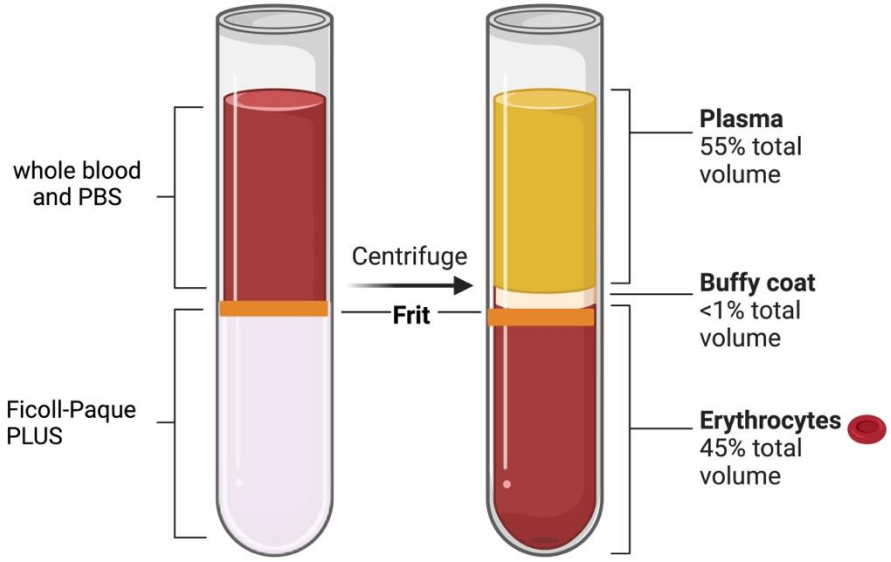


Image produced using BioRender

Ficoll-Paque™ PLUS is aliquoted into a Leucosep tube and centrifuged for 1 min to drop below the frit (depicted in orange on image). Whole blood is then mixed with PBS and lightly layered above the frit (shown in tube on left of figure). Samples are then centrifuged with zero deceleration to prevent the now separated blood from re-mixing. After centrifugation blood has separated on a density gradient into three distinct layers; plasma at the top, buffy coat (or PBMC layer) just above the frit (depicted in orange on image), and erythrocytes below the frit (as shown in tube on the right in the figure).

2.3. RESUSCITATION OF FREEZE-DRIED MYCOBACTERIA CELLS

MAP and MTBC cells that were freeze-dried for long term storage required resuscitation before use in Actiphage assays. Briefly, 1 ml of sterile Media Plus (warmed to room temperature), was added to each Wheaton vial containing mycobacteria to be resuscitated. Freeze-dried cells were mixed gently with the Media Plus by gentle pipetting and left to incubate statically at 37 °C for 1 h. After incubation cells were ready to be used for Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.).

2.4. ACTIPHAGE® ASSAYS

2.4.1. Actiphage® titre assay media and reagents

2.4.1.1. Actiphage® reagent

Freeze-dried mycobacteriophage D29 (PBD Biotech; UK) was reconstituted in 1 ml sterile MP (section 2.2.1.3.). This was gently mixed until a uniform suspension of phage was achieved.

2.4.1.2. Mycobacterium smegmatis culture

A single colony of *M. smegmatis* from an agar plate was inoculated into Media Plus (section 2.2.1.3.) and left to incubate at 37 °C shaking for 18 ± 3 h until the culture had grown to a concentration of approximately 1×10^8 cfu ml^{-1}.

2.4.1.3. Phage titre plates

To make phage titre plates, 6 ml MP (section 2.2.1.3.), 1 ml *M. smegmatis* culture (section 2.4.1.2.) and 6 ml molten 7H10 agar (section 2.1.2.2.) tempered to

40 °C were added to a sterile petri dish. Petri dishes were gently mixed to ensure a homogenous mixture and allowed to set.

2.4.2. Phage titre assay

2.4.2.1. Negative control

For the negative control, 1 ml sterile MP and no Actiphage® was used.

2.4.2.2. Phage titre

10-fold serial dilutions of reconstituted Actiphage® (section 2.4.1.1.) were performed using sterile MP as the diluent. Then five 10 µl volumes of each dilution of Actiphage® were spotted onto phage titre plates (section 2.4.1.3). Plates were left to dry before being incubated for 18 h ± 3 h at 37 °C. After incubation, plaques obtained on the dilution that gave approximately 10 plaques per spot were counted and phage titre (see equation below) calculated.

$$pfu\ ml^{-1} = \frac{number\ of\ plaques\ x\ dilution\ factor}{volume\ plated\ (ml)}$$

2.4.3. Actiphage Rapid™ assay media and reagents

2.4.3.1. Actiphage® reagent

Freeze-dried mycobacteriophage D29 (PBD Biotech; UK) was reconstituted in 1.1 ml sterile MP (section 2.2.1.3.). This was gently mixed until a uniform suspension of phage was achieved (approximately 1 x 10^9 pfu ml^{-1}).

2.4.4. Actiphage Rapid™ assay

2.4.4.1. Positive Control

Using actively growing culture of target *Mycobacterium*, a series of 10-fold dilutions were carried out aseptically into sterile MP (section 2.2.1.3.). MAP K10 was used for IS*900*, *F57* and IS*1311* PCR assay analysis, *M. bovis* BCG was used for IS*6110* PCR assay analysis and *M. avium* was also used for IS*1311* and IS*901* PCR assay analysis. 10 µl of diluted cells were added to 100 µl Actiphage® reagent (section 2.4.3.1). A final cell concentration of 1 x 10^3 cfu ml^{-1} was added per assay, and 10^8 phage particles were added per assay.

2.4.4.2. Negative Control

For Actiphage Rapid™ assays from whole blood samples 110 µl of reconstituted Actiphage® reagent (section 2.4.3.1.) was used. For Actiphage Rapid™ assays from milk samples 200 µl of reconstituted Actiphage® reagent was used as the negative control.

2.4.4.3. Actiphage Rapid™ assay from whole blood sample

Pellets from the blood sample preparation (section 2.2.3.1. or 2.2.3.2.) were resuspended in 110 µl of Actiphage® reagent (section 2.4.3.1.), mixed by pipetting and transferred to an Actiphage Rapid™ reaction tube with a 0.22 µm pore CA membrane filter. Actiphage assays were left to incubate for 3.5 h at 37 °C then centrifuged at 13,000 x g for 3 min. The filter column was discarded, and the DNA extract was purified and concentrated with a Zymo Clean & Concentrator kit (section 2.5.1.1.) or stored at -20 °C for future use.

2.4.4.4. Actiphage Rapid™ assay from milk sample

Samples suspended in 1 ml MP (section 2.2.1.3.) were transferred to a sterile 1.5 ml microfuge tube and centrifuged at 13,000 x g for 3 min. Supernatant was discarded and pellet was resuspended in 200 µl Actiphage reagent (section 2.4.3.1.). Samples were mixed by pipetting and transferred to an Actiphage reaction tube with a 0.22 µm pore and CA membrane filter. Assays were incubated statically at 37 °C for 3.5 h then centrifuged at 13,000 x g for 3 min. After centrifugation, the inner filter of the reaction tube was removed and the flowthrough containing DNA collected in the bottom section. DNA extract was purified with a Zymo Clean & Concentrator kit (section 2.5.1.1.) or stored at -20 °C for future use.

2.4.5. Actiphage® core assay media and reagents

2.4.5.1. Virusol

One tablet of Virusol (PBD Biotech; UK) containing ferrous ammonium sulphate as the virucidal agent, was dissolved in 4.5 ml of sterile RO water under aseptic conditions.

2.4.5.2. Actiphage® reagent

Freeze-dried mycobacteriophage D29 (PBD Biotech; UK) was reconstituted in 1 ml sterile MP (section 2.2.1.3.). This was gently mixed until a uniform suspension of phage was achieved (approximately 1 x 10^9 pfu ml^{-1}).

2.4.5.3. Sensor cells

A single colony of *M. smegmatis* (MC^2155) was incubated in MP (section 2.2.1.3.) at 37 °C for 18 ± 3 h until culture had reached a concentration of 1 x 10^8 cfu ml^{-1}.

2.4.6. Actiphage® Core assay

2.4.6.1. Positive control

Actively growing *M. smegmatis MC²155* culture (section 2.4.5.3) was ten-fold serially diluted into sterile MP to achieve a concentration of approximately 1 x 10^2 cfu ml^{-1}. An actively growing culture of *M. smegmatis* with an OD_{600} 0.2 is approximately 10^7 cells. 1 ml of the cells at a concentration of 10^2 cfu ml^{-1} were added to a reaction vessel with 100 µl of Actiphage reagent for the positive control (figure 2.2).

2.4.6.2. Negative Control

For the negative control, 1 ml of sterile MP was added to a reaction vessel with 100 µl of Actiphage reagent (figure 2.2).

Figure 2.2. Preparation of positive and negative controls for use in the Actiphage®

Core assay

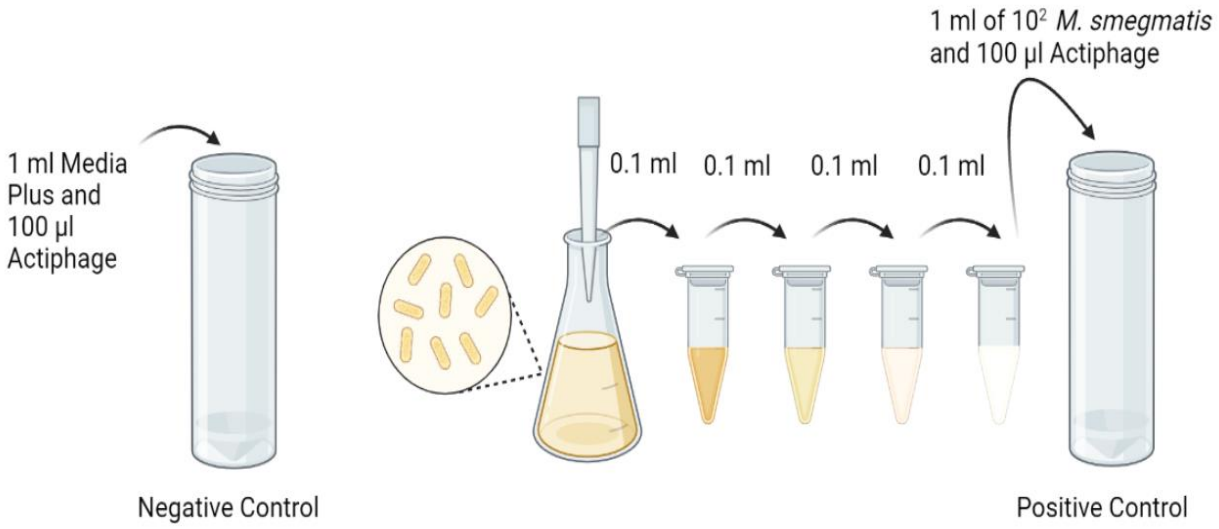


Image designed using BioRender.com

For the negative control, simply, 1 ml of Media Plus is added to a reaction vessel with 100 µl Actiphage® reagent at a concentration of 10^7 phage particles. For the positive control an actively growing culture of M. smegmatis was 10-fold serially diluted until the culture was at an approximate concentration of 1 x 10^2 cfu ml^{-1}. Once at the correct concentration 1 ml of these cells were added to a reaction vessel with 100 µl Actiphage® reagent.

2.4.6.3. Actiphage® Core assay

For the Actiphage® Core assay a sample size of 1 ml was used. In a reaction vessel to each sample and prepared control, 100 µl of Actiphage® reagent (section 2.4.5.2.), to achieve 10^7 phage particles per assay was added. All samples and controls were incubated statically at 37 °C for 1 h. After incubation, 150 µl of Virusol (section 2.4.5.1.) was added to each reaction vessel to inactivate any exogenous phage that had not infected the mycobacteria. The contents of reaction vessels were incubated at room temperature for 5 min under constant rotation using an orbital rotator to ensure all internal surfaces of the reaction vessel were covered. After rotation, 5 ml MP and 1 ml of 1 x 10^8 cfu ml^{-1} sensor cells (section 2.4.5.3.) were inoculated into the reaction vessels. The contents of each reaction vessel were poured into empty, pre-labelled sterile petri dishes and 6 ml molten 7H10 agar (tempered to 40 °C) was added. Samples were gently mixed and allowed to set at room temperature. Once set, petri dishes were incubated at 37 °C for 18-24 h (figure 2.3).

After incubation, a successful assay should have more than 20 plaques on the positive control plate and less than 20 plaques on the negative control plate (figure 2.4.). If this criterion were achieved, the number of plaques on sample plates were accepted. Plaques formed within the countable range (30-300) were enumerated. Plaques were picked with a 1.5 mm biopsy punch. Plaque DNA was then extracted with gel DNA recovery Kit (ZymoResearch; Cambridge Bioscience; UK) (section 2.5.1.2.).

For Actiphage Core enumeration assays, after the addition of Virusol and 5 min rotation, 150 µl of sample was removed and dispended into 1.35 ml MP in a 2 ml

microfuge tube to create a 10^{-1} dilution. This dilution series was repeated until a 10^{-5} dilution was achieved. 150 µl from the final dilution in the series was discarded. Each dilution was then dispensed into a petri dish with 5 ml MP and 1 ml of 1 x 10^{8} cfu ml^{-1} sensor cells (section 2.4.5.3.). Then, 6 ml molten (tempered to 40 °C) was added to each plate, gently mixed and plates were allowed to set at room temperature. Once set, petri dishes were incubated at 37 °C for 18-24 h. After incubation, plates with plaques within the countable range of 20-100 were counted and pfu ml^{-1} was calculated using the following equation:

$$pfu\ ml^{-1} = \frac{number\ of\ plaques\ x\ dilution\ factor}{sample\ volume\ (ml)}$$

Figure 2.3. Schematic diagram of Actiphage® Core assay

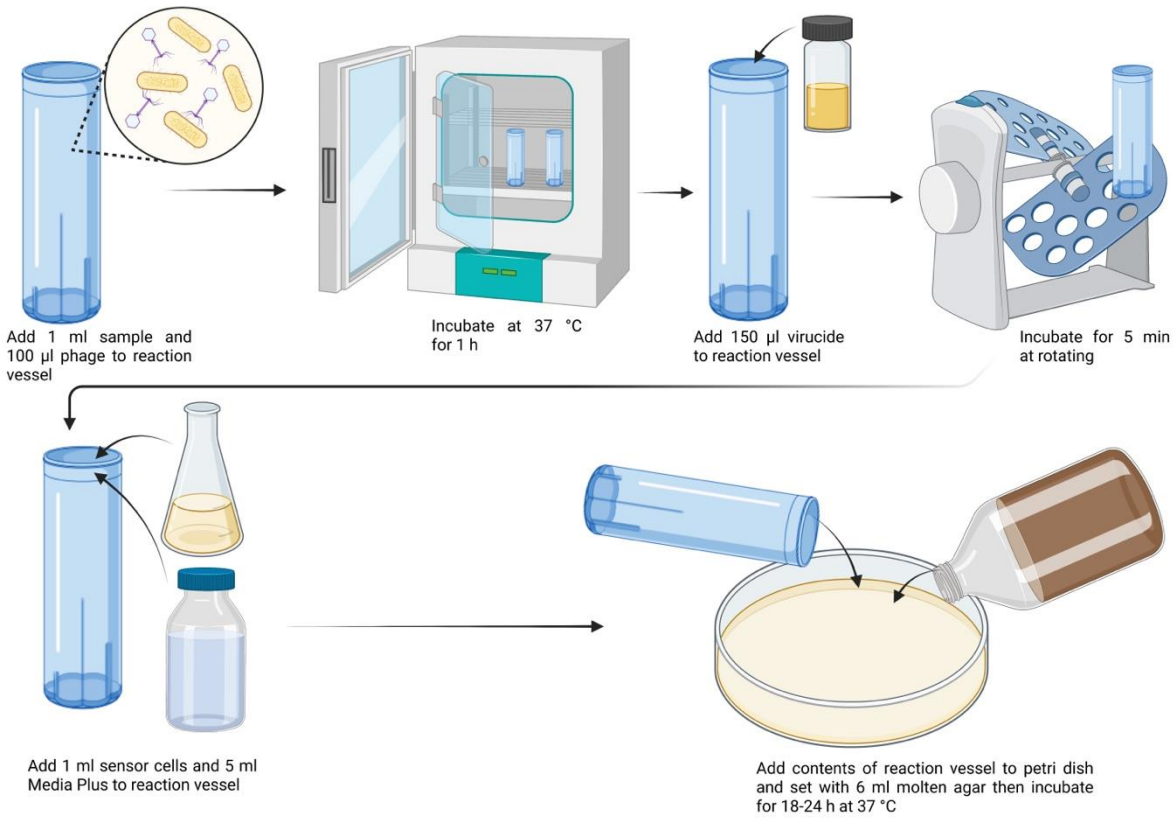


Image designed using BioRender.com

Briefly, 1 ml of sample, positive or negative control is dispensed into a reaction vessel along with 100 µl of 10^7 Actiphage® reagent. Reaction vessels are then incubated statically at 37 ℃ for 1 h after which 150 µl of virucide is added to every reaction. To ensure all exogenous phage which have not infected cells are killed by the virucidal agent, reaction vessels are incubated for 5 min at room temperature on a constant rotation using an orbital rotator. After incubation, 1 ml sensor cells (M. smegmatis) and 5 ml Media Plus are dispensed into reaction vessels then added to a sterile petri dish. Samples are set with 6 ml molten 7H10 agar and incubated for 18-24 h at 37 ℃. After incubation plaques can be counted and results recorded.

Figure 2.4. Visual representation of acceptable criteria for positive and negative controls for a successful Actiphage® Core assay

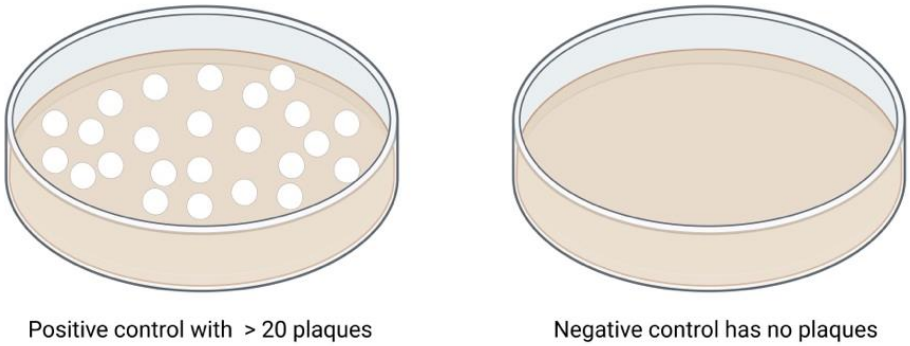


Image designed using BioRender.com

The acceptance criteria for a Actiphage® Core assay are a positive control plate with more than 20 plaques and a negative control plate with less than 20 plaques. Plaques are visible structures which appear as zones of clearing on a confluent bacterial lawn.

2.5. POLYMERASE CHAIN REACTION

2.5.1. DNA extraction and purification

2.5.1.1. Zymo DNA Clean & Concentrator™ -5 kit

All DNA obtained from Actiphage Rapid™ assays (section 2.4.4.) were purified using Zymo DNA Clean & Concentrator™-5 kit (ZymoResearch; Cambridge Bioscience; UK). To prepare buffers included in the kit for DNA purification, 24 ml of 100 % ethanol was added to the DNA wash buffer concentrate. For DNA purification and concentration, first, DNA binding buffer was added at a ratio of 2:1 to the DNA extract obtained from Actiphage Rapid™ assays. The sample was then transferred to a Zymo-Spin™ Column in a collection tube. The samples were centrifuged at 13,000 x g for 30 s and flow-through discarded. Then, 200 µl of DNA wash buffer was added to the spin column and centrifuged at 13,000 x g for 30 s. The wash step was repeated before spin columns were transferred to a sterile 1.5 ml microfuge tube. Then, 14 µl of sterile MQ water (warmed to 55 °C) was added directly to the matrix and samples were left to incubate at room temperature for 1 min. Samples were centrifuged at 13,000 x g for 30 s then the spin column was discarded. Purified DNA was used as template for PCR assays or stored at -20 °C for future use.

2.5.1.2. Zymo Gel DNA Recovery Kit

Zymo Gel DNA recovery kit (ZymoResearch; Cambridge Bioscience; UK) was used to extract and purify DNA obtained from plaques from the Actiphage Core assay (section 2.4.6.3.). To prepare buffers included in the kit for DNA recovery, 24 ml 100 % ethanol was added to 6 ml DNA wash buffer concentrate. First, five plaques from the same sample plate were picked and pooled in a 1.5 ml microfuge tube. Next,

three volumes of ADB were added to every volume of plaques (e.g., for 100 µl (mg) of plaques, 300 µl of ADB to be added). Samples were then incubated at 55 °C for 10 min until plaques had completely dissolved into the buffer. Dissolved plaques were then transferred into a Zymo-Spin™ Column in a collection tube. Samples were centrifuged at 13,000 x g for 30 s and flow-through discarded. Next, 200 µl DNA wash buffer was added to the column and centrifuged at 13,000 x g for 30 s. The wash step was repeated then spin columns transferred to sterile 1.5 ml microfuge tubes. 14 µl sterile MQ water (warmed to 55 °C) was added to the column matrix and centrifuged at 13,000 x g for 1 min to elute the DNA. Purified DNA was used as template for PCR assays or stored at -20 °C for future use.

2.5.1.3. Boiled lysate DNA extraction

For liquid cultures, 100 µl of culture was added to a 1.5 ml microfuge tube. Cultures were heated on a heating block at 95 °C for 15 min.

For cultures from solid media, one colony was transferred into 100 µl sterile distilled water within a 1.5 ml microfuge tube using a sterile loop. Cultures were heated on a heating block at 95 °C for 15 min.

Cultures were left to cool before being centrifuged at 13,000 x g for 3 min to remove cellular debris. The supernatant containing DNA was transferred to a sterile 1.5 ml microfuge tube. 1 µl of boiled lysate DNA was used as template for PCR assays or stored at -20 °C for future use.

2.5.1.4. Drop dialysis of DNA

To remove any organic compounds from DNA and minimise qPCR inhibition drop dialysis was used. Briefly, 50 ml double-distilled water was poured into a petri dish. A 25 mm Type-VS Millipore membrane (Merck; USA) with a mean pore size of 0.025 µm was placed gently to float on top of the water ensuring no air bubbles were trapped under the filter. Floating filters were left to sit on the water for approximately 5 min to ensure filters were wet completely. After 5 min, 5-10 µl of DNA was carefully pipetted onto the centre of the filter membrane ensuring that the pipette tip did not come in contact with or submerge the membrane. Once DNA was placed on filters, petri dishes were covered, and samples left to dialyse for 1 h at room temperature. After dialysis, DNA was retrieved carefully and transferred to a new sterile 1.5 ml microfuge tube ready for use in qPCR.

2.5.1.5. Serial dilutions of ATCC DNA

Genomic DNA (gDNA) from *Mycobacterium avium* subspecies *paratuberculosis* K10 (ATCC; USA) was used to develop standard curves for qPCR. When 10-fold serially diluted gDNA was required, 1 µl of quantitative gDNA was aliquoted into a sterile 0.2 ml PCR tube preloaded with 9 µl sterile, RNase free water (Qiagen; Germany). This would create a 10^{-1} dilution of gDNA. The dilution series was repeated down to a 10^{-5} dilution to achieve approximately ≥1 copy μl^{-1}. 1 µl of each dilution in the series was used as template for qPCR.

For qPCR assays where 2-fold diluted gDNA was required; 2 µl gDNA (≥1 x 10^5 copies μl^{-1}) was aliquoted into a 0.2 ml sterile PCR tube preloaded with 2 µl sterile, RNase free water to achieve a 2^{-1} dilution. The dilution series was repeated several

times down to a 2^{-7} dilution to achieve approximately ≥7.8 x 10^2 copies μl^{-1}). 1 µl of each dilution in the series was used as template for qPCR.

When 4-fold serially diluted gDNA was required for efficiency plots; 2 µl gDNA (≥1 x 10^5 copies μl^{-1}) was aliquoted into a 0.2 ml sterile PCR tube preloaded with 6 µl of sterile, RNase free water to achieve a 4^{-1} dilution of approximately ≥2.5 x 10^4 copies μl^{-1}. The dilution series was repeated to a 4^{-8} dilution to achieve approximately ≥1 x 10^0 copies μl^{-1}.

2.5.2. Endpoint PCR assays

2.5.2.1. IS900 PCR

The MAP IS*900* endpoint PCR uses the P90/P91 primer pair (table 2.1) as described by Millar *et al.* (1995). IS*900* is a MAP specific multi-copy insertion sequence. 5 µl of DNA template extracted from the Actiphage Rapid™ assay (section 2.4.4.) or the Actiphage Core assay (section 2.4.6.) or 1 µl DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction. In a 0.2 ml PCR tube, the 20 µl reaction mixture consisted of: 10 µl Qiagen HotStarTaq Plus Master Mix (Qiagen; Germany), 0.5 µM of each primer; P90 forward and P91 reverse, 2 µl CoralLoad Dye (Qiagen; Germany), made up to the final reaction volume of 20 µl with sterile molecular grade water.

Reactions were pulse centrifuged and placed in a UNO96 thermo-cycler (VWR; UK). Parameters for thermal cycling were as follows; initial denaturation step of 95

°C for 15 min followed by 37 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, followed by a final extension step of 72 °C for 4 min.

18 µl of PCR products were resolved on a 1.5 % (w/v) agarose TAE gel containing 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 70 V for 1 h. Gels were visualised under ultra-violet (UV) light using a Gel Doc system (BioRad; UK). The expected product size was 413 bp.

2.5.2.2. F57 PCR

F57 is a single copy gene found exclusively in MAP. PCR can target this gene utilising the F57a and F57b primer (table 2.1) as first described by Coetsier *et al.* (2000).

For each PCR reaction, 5 µl of DNA template extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) or 1 µl of DNA extracted using the boiled lysate method (section 2.5.1.3.) was used. In a 0.2 ml PCR tube, the following reagents were used; 1x Qiagen HotStarTaq Plus Master Mix (Qiagen; Germany), 0.5 µM of both F57a forward and F57b reverse primer and 2x CoralLoad Dye (Qiagen; Germany) made up to a final volume of 20 µl using sterile molecular grade water.

Reactions underwent a brief vortex followed by a pulse centrifuge before loading into an UNO96 thermo-cycler (VWR; UK). Parameters for thermocycling were as follows; one cycle of initial denaturation of 94 °C for 5 min followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 60 s, one cycle of a final extension at 72 °C for 7 min.

A volume of 18 µl of PCR product was resolved on a 1.5 % (w/v) agarose TAE gel containing 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 75 V for 1 h after which gels were visualised under UV light using Gel Doc system (BioRad; UK). The expected product size for *F57* PCR is 439 bp.

2.5.2.3. IS901 PCR

IS*901* is a multi-copy insertion sequence found in strains of *M. avium*. IS*901* PCR uses the MK7/MK8 primer pair (table 2.1) developed and first described by Kunze *et al.* (1991).

Briefly, 5 µl of DNA template extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) or 1 µl of DNA extracted using the boiled lysate method (section 2.5.1.3.) was used. Mycobacterial template was added to a 0.2 ml PCR tube along with; 1x One*Taq* Hot Start Quick-Load Master Mix with GC Buffer (NEB; UK) and 0.5 µM of both MK7 forward and MK8 reverse primer made up to 20 µl using RNase-free molecular grade water. Once all reagents and template were added to PCR tubes, all samples were subject to a brief vortex followed by a pulse centrifuge and placed in a UNO96 thermo-cycler (VWR; UK).

PCR amplification of template was performed as per the following cycling parameters; one cycle of initial denaturation at 95 °C for 2 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension of 68 °C for 45 s followed by a single step of final extension of 68 °C for 5 min.

18 µl of PCR products were resolved on a 1.5 % (w/v) agarose TAE gel stained with 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 80 V for 1 h. After 1 h, gels

were visualised under UV light using a Gel-Doc system (BioRad; UK). The expected product size was 378 bp.

2.5.2.4. IS1311 PCR-REA

MAC IS*1311* PCR-REA uses the M56/M119 primers (table 2.1) as described by Marsh *et al.* (1999). IS*1311* is a multi-copy insertion sequence present in MAC. Different members of the complex have slightly differing IS*1311* sequences allowing differentiation of the complex through restriction enzyme analysis.

Briefly, 5 µl of DNA template extracted from the Actiphage Rapid™ assay (section 2.4.4.), Actiphage Core assay (section 2.4.6) or 1 µl DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction. In a 0.2 ml PCR tube, the reaction mixture consisted of 1x One*Taq* Hot Start Quick-Load Master Mix with GC Buffer (NEB; UK), 0.5 µM of both forward and reverse primer; M56/M119 and template DNA made up to a final reaction volume of 25 µl using sterile molecular grade water.

Reactions were pulse centrifuged and placed in a UNO96 thermo-cycler (VWR; UK). Parameters for thermal cycling were as follows; initial denaturation at 94 °C for 2 min followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 68 °C for 1 min, followed by a final extension step at 68 °C for 5 min.

5 µl of PCR product was resolved on a 1.5 % (w/v) agarose TAE gel containing 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 80 V for 1 h. Gels were visualised under ultra-violet (UV) light using a Gel Doc system (BioRad; UK). The expected product size was 600 bp.

If a 600 bp product was observed, the remaining 20 µl of PCR product was subject to restriction digest. To each PCR reaction, 2 µl CutSmart buffer (NEB; UK), 1 µl *Mse*I (NEB; UK) and 1 µl *Hinf*I (NEB; UK) were added. Restriction digests were left to incubate at 37 °C for 3 h. 10 µl of digest was resolved on a 2.0 % (w/v) agarose TAE gel containing 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 80 V for 1 h. Gels were visualised under UV light using a Gel Doc system (BioRad; UK). The expected band sizes for each species and lineage are listed in table 1.1.

2.5.2.5. IS900 Nested PCR

Nested PCR exclusively targeting IS*900* insertion sequence of MAP used the primer pairs TJ1/TJ2 and TJ3/TJ4 (table 2.1) as first described by Bull *et al.* (2003). This PCR is made up of two sequential PCR reactions.

For the first reaction, DNA extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage® Core assay (section 2.4.6.) was used at a volume of 6 µl. DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction at a volume of 1 µl. PCR reagents and DNA template were added to a 0.2 ml PCR tube. Each PCR reaction consisted of, 12.5 µl Qiagen HotStarTaq Plus Master Mix (Qiagen; Germany), 2.5 µl CoralLoad Dye (Qiagen; Germany), 0.1 µM of both forward (TJ1) and reverse (TJ2) primers and DNA template made up to 25 µl with RNase-free water. Samples were pulse centrifuged placed in an UNO96 thermo-cycler (VWR; UK). Parameters for thermal cycling were as follows; 1 cycle of initial denaturation at 94 °C for 5 min followed by 10 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 3 min, after which a final extension step of 72 °C for 7 min was performed.

For the second PCR reaction, 9 µl of PCR product created by the first PCR was used as template. The template was added to a 0.2 ml PCR tube with 12.5 µl Qiagen HotStarTaq Plus Master Mix (Qiagen; Germany), 2.5 µl CoralLoad Dye (Qiagen; Germany), 0.2 µM of both forward (TJ3) and reverse (TJ4) primers made up to 25 µl with RNase-free water. Samples were briefly pulse centrifuged before being loaded into an UNO96 thermo-cycler (VWR; UK). Parameters for this second PCR reaction were as follows; 1 cycle of initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 3 min, after which a final extension step of 72 °C for 7 min was performed.

A volume of 20 µl of PCR product was resolved on a 1.5 % (w/v) agarose TAE gel, stained with 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK0) at 70 V for 1 h. After which the agarose gel was visualised under UV light using a Gel Doc system (BioRad; UK). The expected band size for a positive sample for nested PCR was 294 bp.

2.5.2.6. IS*6110 PCR*

IS*6110* endpoint PCR uses the primer pair T4/T5 (table 2.1) as described by Eisenach *et al.* (1990). IS*6110* is an insertion sequence unique to MTBC. DNA extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) was used at a volume of 5.5 µl. DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction at a volume of 1 µl. In a 0.2 ml PCR tube, 10 µl HotStarTaq Plus Master Mix (Qiagen; Germany); 0.5 µM of each primer T4 forward and T5 reverse; 2.5 % DMSO (v/v) (NEB; UK); 2 µl CoralLoad Dye

(Qiagen; Germany) was added to the DNA template and made up to a final volume of 20 µl with sterile molecular grade water.

Reactions were pulse centrifuged and placed in an UNO96 thermo-cycler (VWR; UK). Parameters for thermal cycling were as follows; an initial denaturation step of 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30s and a final extension step of 72 °C for 5 min.

A volume of 18 µl PCR product was resolved on a 1.5 % (w/v) agarose TAE gel containing 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 70 V for 1 h. Gels were visualised under UV light with a Gel Doc system (BioRad;UK). The expected product size was 123 bp.

2.5.2.7. RD4 flanking PCR

RD4 endpoint PCR uses the primer pair RD4 flanking R1/RD4 flanking F2 (table 2.1) as described by Taylor *et al.* (2007). This PCR is used to speciate members of the MTBC by detection of presence or absence of region of difference 4 (RD4). Primers flank this region therefore absence of RD4 results in a 142 bp band and presence of RD4 results in a 176 bp band.

DNA extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) was used at a volume of 6 µl. DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction at a volume of 1 µl. In a 0.2 ml PCR tube, 10 µl HotStarTaq Plus Master Mix (Qiagen; Germany); 0.5 µM of each primer RD4 flanking F2 forward and RD4 flanking R1 reverse; 2 µl CoralLoad Dye (Qiagen; Germany) was added to the DNA template and made up to a final volume of 20 µl with sterile molecular grade water.

Reactions were pulse centrifuged and placed in an UNO96 thermo-cycler (VWR; UK). Parameters for thermal cycling were as follows; an initial denaturation step of 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30s and a final extension step of 72 °C for 5 min.

A volume of 18 µl PCR product was resolved on a 1.5 % (w/v) agarose TAE gel containing 2.5 % (v/v) ethidium bromide (Sigma-Aldrich; UK) at 70 V for 1 h. Gels were visualised under UV light with a Gel Doc system (BioRad;UK). The expected product size was 142 bp to confirm absence of RD4 and 176 bp to confirm presence of RD4.

Table 2.1. Primers and fluorescent probes used for PCR and qPCR reactions

PRIMER NAME	PRIMER SEQUENCE (5'-3')	TARGET DNA	REFERENCE
P90	GAAGGGTGTTCGGGGCCGTCGCTTAGG	MAP IS*900*	(Whittington *et al.,* 1998)
P90SHORT	GAAGGGTGTTCGGGGCCGTCG	MAP IS*900*	Handley-Harthill, unpublished data
P91	GGCGTTGAGGTCGATCGCCCACGTGAC	MAP IS*900*	(Whittington *et al.,* 1998)
F57A	GGTCGCGTCATTCAGAATC	MAP *F57*	(Coetsier *et al.*, 2000)
F57B	TCTCAGACAGTGGCAGGTG	MAP *F57*	(Coetsier *et al.*, 2000)
TJ1	GCTGATGCGCTTGCTCAT	MAP IS*900*	(Bull *et al.*, 2003)
TJ2	CGGGAGTTTGGTAGCCAGTA	MAP IS*900*	(Bull *et al.*, 2003)
TJ3	CAGCGGCTGCTTTATATTCC	MAP IS*900*	(Bull *et al.*, 2003)

TJ4	GGCACGGCTCTTGTTGTAGT	MAP IS*900*	(Bull *et al.*, 2003)
MK7	GTCTGGGATTGGATGTCCTG	MAA IS*901*	(Kunze *et al.*, 1991)
MK8	CACCACGTGGTTAGCAATCC	MAA IS*901*	(Kunze *et al.*, 1991)
RD4 FLANKING F2	TGTGAATTCATACAAGCCGTAGTC	MTBC RD4	(Taylor *et al.*, 2007)
RD4 FLANKING R1	CCCGTAAGCGTTACTGAGAAATTGC	MTBC RD4	(Taylor *et al.*, 2007)
T4	CCTGCGAGCGTAGGCGTCGG	MTBC IS*6110*	(Eisenach *et al.*, 1990)
T5	CTCGTCCAGCGCCGCTTCGG	MTBC IS*6110*	(Eisenach *et al.,* 1990)
M56	GCGTGAGGCTCTGTGGTGAA	MAC IS*1311*	(Marsh *et al.*, 1999)
M119	ATGACGACCGCTTGGGAGAC	MAC IS*1311*	(Marsh *et al.,* 1999)
IS1311F	CGATTTGGAGTTGCGGATTC	MAC IS*1311*	n/a

IS1311R	ACCTCGCTTTTGGAGATGCC	MAC IS*1311*	n/a
PROBE NAME	**Probe Sequence (5'-3')**	**Target DNA**	**Reference**
MAP RT PROBE	FAM-TGCGCGTCGTCGTTAATAACC-BHQ1	MAP IS*900*	Unpublished Data (Handley-Harthill; UoN)
IS900RLP	FAM-CATGGTTATTAACGACGACGCGCAGC-BHQ1	MAP IS*900*	(Donaghy *et al.*, 2011)
IAC PROBE 2	FAM-TCGAAAGGGCAGATTGTGTGGACA-BHQ1	*M. smegmatis* IAC plasmid	Unpublished data (Handley-Harthill; UoN)

Primers and probes used for PCR and qPCR reactions. All sequences are displayed reading from 5' to 3'. Both fluorescent probes used FAM (excitation 470 nm, emission 510 nm) as the fluorophore and black hole quencher 1 (BHQ1) as the quencher.

2.5.3. Real-time PCR assays

2.5.3.1. BactoReal® Kit Mycobacterium avium ssp. paratuberculosis

This qPCR assay used the commercial BactoReal® Kit *Mycobacterium avium* ssp. *paratuberculosis* (Ingenetix; Austria) for detection of MAP. Reagents (stored at -20 °C) were thawed at room temperature followed by a brief vortex and pulse centrifuge prior to use. For preparation of master mix, 3 µl water, 10 µl DNA reaction mix (2x), 1 µl *Mycobacterium avium* ssp. *paratuberculosis* assay mix (primer and probe for detection of MAP on FAM acquisition channel) and 1 µl CR assay mix (primer, probe and target for detection of internal positive control on the Cy5 acquisition channel) were added to a 0.2 ml PCR tube or 0.1 ml strip tube dependant on qPCR rotor used. A total of 5 µl DNA template was used, extracted using Actiphage Rapid™ assay (section 2.4.4.).

The qPCR assay was run on a Rotor-Gene Q qPCR machine (Qiagen; Germany) according to the following parameters: 1 cycle 50 °C for 2 min followed by 1 cycle at 95 °C for 20 s then 45 cycles of 95 °C for 5 s and 60 °C for 1 min. Data acquisition was performed every cycle at 60 °C with sample target detection being performed in FAM (excitation 470 nm, emission 510 nm) acquisition channel and internal control acquired through Cy5 (excitation 625 nm, emission 660 nm).

2.5.3.2. Bio-T kit® Mycobacterium avium paratuberculosis

This qPCR assay utilised a commercial kit, Bio-T kit® *Mycobacterium avium paratuberculosis* (Biosellal; France) for the detection of MAP. The protocol was adapted to allow the inclusion of the exogenous internal positive control (IPC) when setting up the reaction in favour of adding the IPC at the DNA extraction step as per

the protocol. Reagents (stored at -20 °C) were thawed at room temperature followed by brief vortex and pulse centrifugation. Then 15 µl Master Mix and 0.2 µl IPC were transferred into a 0.2 ml PCR tube or 0.1 ml strip tube dependant on qPCR rotor used. For DNA extracted from Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) a volume of 4.8 µl template was used. For DNA extracted using the boiled lysate method (section 2.5.1.3.) a volume of 1 µl was used as DNA template.

The qPCR assay was performed on a Rotor-Gene Q (Qiagen; Germany) according to the following 2-step cycling parameters; 1 cycle of 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s then 60 °C for 30 s with data acquisition at this step using the fluorophores FAM (excitation 470 nm, emission 510 nm) for target detection and Cy5 (excitation 625 nm, emission 660 nm) for internal control.

2.5.3.3. Bio-T kit® MTBC

The commercial kit, Bio-T kit® MTBC (Biosellal; France) was used for the detection of MTBC. The protocol was adapted to allow the inclusion of the IPC when setting up the qPCR reaction as well as adjusting the master mix for a final volume of 20 µl as opposed to 15 µl as per protocol.

Reagents (stored at -20 °C) were thawed at room temperature followed by brief vortex and pulse centrifugation. Then 15 µl Master Mix and 0.2 µl IPC were transferred into a 0.2 ml PCR tube or 0.1 ml strip tube dependant on qPCR rotor used. For DNA extracted from Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) a volume of 4.8 µl template was used. For DNA

extracted using the boiled lysate method (section 2.5.1.3.) a volume of 1 µl was used as DNA template.

The qPCR assay was performed on a Rotor-Gene Q (Qiagen; Germany) according to the following 2-step cycling parameters; 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min with data acquisition at this step. Data acquisition was achieved using the fluorophores; FAM (excitation 470 nm, emission 510 nm) for target detection and Cy5 (excitation 625 nm, emission 660 nm) for internal control.

2.5.3.4. Empirical MTBC qPCR kit

A qPCR kit developed by PBD Biotech (Suffolk; UK) and produced by Empirical Bioscience (Michigan; USA) was used for the detection of MTBC.

Reagents (stored at -20 °C) were thawed at room temperature followed by a brief vortex and centrifugation at 13,000 x g for 10 s. Reagents and template were dispensed into 0.2 ml PCR tubes for use with the 36-well rotor or 0.1 ml strip tubes for use with the 72-well rotor. Each 20 µl reaction contained; 10 µl 2X InhibiTaq qPCR Master Mix, 1 µl 20X TB Primer/Probe Mix, 1 µl PBD internal amplification control (IAC) plasmid and 3 µl Nuclease free water. For DNA extracted from Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) a volume of 5 µl template was used. For DNA extracted using the boiled lysate method (section 2.5.1.3.) a volume of 1 µl was used as DNA template. Once reagents and template were added to the appropriate PCR tubes, all samples were loaded onto the Rotor-Gene Q (Qiagen; Germany).

The PCR was run on a 2-step cycling protocol according to the following parameters; initial denaturation of 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 64 °C for 30 s. Data acquisition was obtained during the annealing/extension step through the use of the FAM (excitation 470 nm, emission 510 nm) and Cy5 (excitation 625 nm, emission 660 nm) fluorophores for target detection and internal control detection respectively

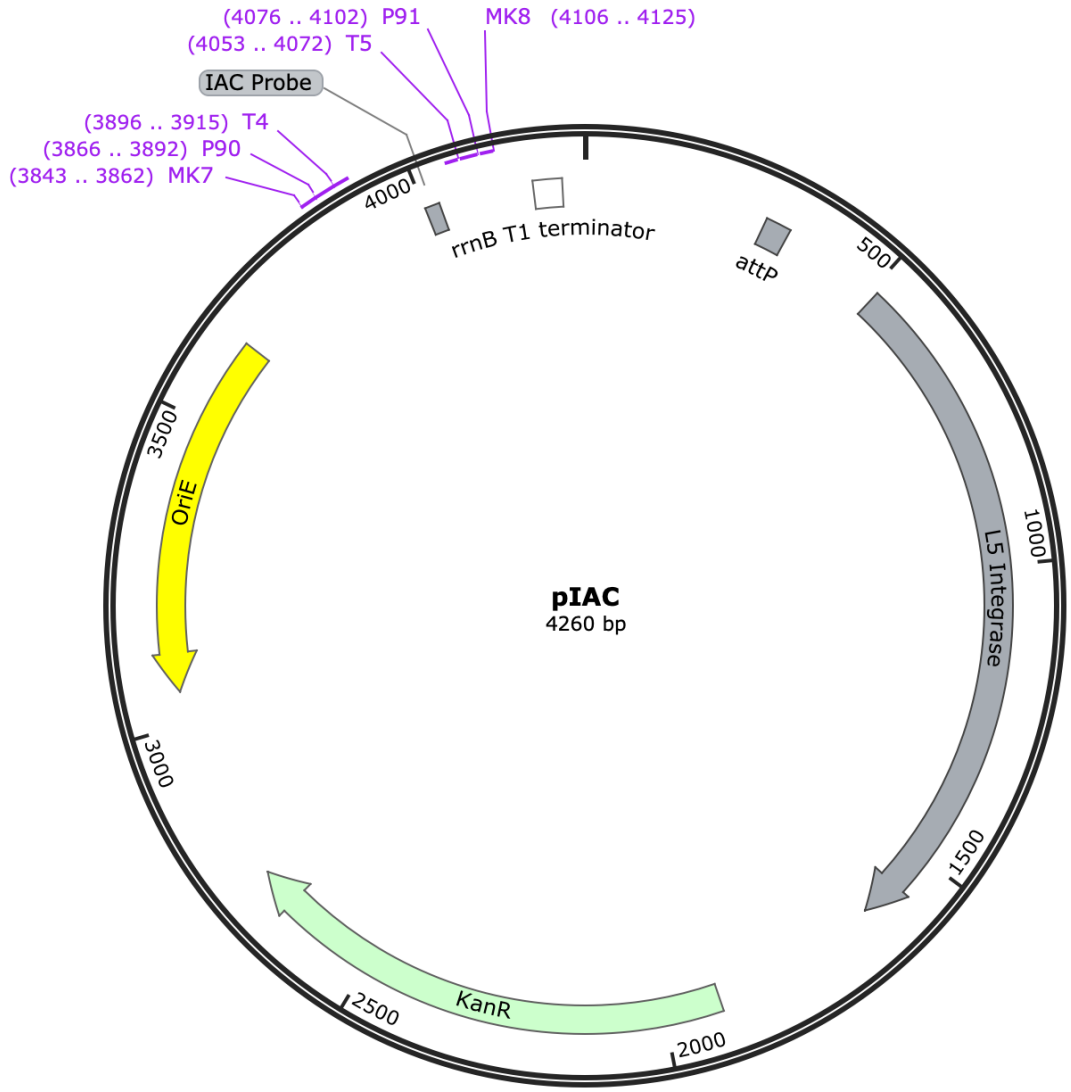
2.5.3.5. IS900 qPCR

IS*900* qPCR utilised the primer pair P90/91 (table 2.1) and the IS900RLP probe to identify 413 bp sequence of MAP. DNA extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) was used at a volume of 5 µl. DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction at a volume of 1 µl.

Briefly, 1x Luna Universal Probe qPCR Master Mix (NEB; USA), 0.5 µM P90 forward primer, 0.5 µM P91 reverse primer, 0.25 µM molecular probe and either 5 µl or 1 µl of DNA template dependant on extraction method were added to a 0.2 ml PCR tube made up to a final volume of 20 µl with sterile, RNase free water. In experiments when an internal amplification control (IAC) was incorporated in the reaction, 1 µl of IAC plasmid (figure 2.5), 0.25 µM IAC probe 2 (table 2.1) and 0.5 µM forward and reverse primers was also included in the master mix and volume of RNase free water was adjusted to reflect this. Forward and reverse primers varied depending on whether a competitive or non-competitive qPCR format was followed, however for all reactions primers were either P90/P91 primer pair or the T4/T5 primer pair.

qPCR was performed on a Rotor-Gene Q (Qiagen; Germany). The PCR was run on a 2-step cycling protocol according to the following parameters: initial denaturation of 95 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension step at 64 °C for 1 min 30 s. Data acquisition was obtained during the annealing/extension step using a FAM fluorophore (excitation 470 nm, emission 510 nm). For experiments where an internal amplification control was included in the qPCR reactions, fluorescent acquisition was also obtained using a HEX fluorophore (excitation 533, emission 559).

Figure 2.5. Plasmid map of internal amplification control used in in-house qPCR



Plasmid DNA known as pIAC (Handley-Hartill, 2023) was used as the qPCR internal amplification control. pIAC was designed to include primer recognition sites which are commonly used for detection of Mycobacterium tuberculosis complex (T4/T5 primer pair), Mycobacterium avium subspecies paratuberculosis (P90/91 primer pair) and Mycobacterium avium subspecies avium (MK7/MK8 primer pair) to allow for both competitive and non-competitive internal amplification control assays to be used.

2.5.3.6. IS1311 SYBR Green qPCR and high-resolution melting

IS*1311* SYBR green qPCR used the IS*1311*F/R primer pair (table 2.1) to identify a 192 bp region of IS*1311* which contained sites of single nucleotide polymorphisms, for detection of MAC. DNA extracted from the Actiphage Rapid™ assay (section 2.4.4.) or Actiphage Core assay (section 2.4.6.) was used at a volume of 5 µl. DNA extracted using boiled lysate method (section 2.5.1.3.) was used in each PCR reaction at a volume of 1 µl.

Briefly, master mix (stored at -20 ˚C) was thawed and mixed thoroughly. 1x PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher; USA), 0.5 µM IS*1311* forward primer, 0.5 µM IS*1311* reverse primer, either 5 µl or 1 µl DNA template dependant on DNA extraction method used were added to a 0.2 ml PCR tube and made up to 20 µl with sterile RNase-free water.

qPCR was performed on a Rotor-Gene Q (Qiagen; Germany). The PCR was run on a 2-step cycling protocol followed by high resolution melting according to the following parameters: initial denaturation of 95 ˚C for 2 min followed by 40 cycles of 95 ˚C for 15 s and annealing/extension step at 60 ˚C for 1 min. Data acquisition was obtained during annealing/extension step using SYBR green intercalating dye on the FAM channel (excitation 470 nm, emission 510 nm). Immediately after the 40 cycles, the temperature was raised to 65 ˚C and held for 1 min 30 s before the temperature being raised by 0.1 ˚C every 1 s until a final temperature of 95 ˚C was reached. Data acquisition was continuous during this high-resolution step on the FAM acquisition channel (excitation 470 nm, emission 510 nm).

2.6 DATA ANALYSIS

2.6.1. Q-Rex data analysis

All qPCR experiments performed on Rotor-Gene Q (Qiagen; Germany) were subject to analysis on the Q-Rex software (Qiagen; Germany). For all experiments, the absolute quantification plug-in was used and Cq threshold was calculated automatically by the software. Under the normalization tab on the software, 'dynamic tube' and 'use noise slope correction' were selected. For experiments using high resolution melting the 'high resolution melting' plug-in was also used.

2.6.2. Statistical analysis

All statistical analysis including creating qPCR efficiency plots was done using GraphPad Prism 10 software (Boston; USA).

CHAPTER 3

ESTABLISHING THE USE OF QPCR WITHIN THE ACTIPHAGE RAPID™ ASSAY PROTOCOL

PART A DETECTION OF *M. AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN DEER

PART B EVALUATION OF IN-HOUSE PRODUCTS

PART 3A

3A.1 INTRODUCTION

The Actiphage® Core assay was a development of the FASTPlaque assay where the presence of mycobacteria was determined based on the number of plaques formed on an *M. smegmatis* lawn. In the Actiphage® Core assay, identity of the mycobacterial cell that was detected was determined by amplification of signature sequences of specific mycobacterial pathogens using DNA extracted from plaques as PCR template for endpoint PCR (Stanley *et al.,* 2007). Whilst this method was found to be successful in detecting mycobacterial infection in a range of animals, the method was time-consuming, taking two days from receipt of sample to confirmation of results and required culture of mycobacteria to generate the lawns for plaque formation. This assay was later developed into a format that reduced the workflow from two days to one and removed the need for culture plates, with this most recent assay being known as the Actiphage Rapid™ assay. The Actiphage Rapid™ assay proved to be a quicker, more high-throughput advancement on the Actiphage® Core assay which maintained a similar level of sensitivity (Swift *et al.*, 2020). However, experiments to develop this assay format still used endpoint PCR for the final detection event (Swift *et al.*, 2020, Verma *et al.*, 2020)

Endpoint PCR for clinical diagnostics of other bacterial infections has routinely been outperformed by qPCR due to its accuracy, rapidity and the ability to be incorporated into high throughput testing protocols (Meddeb *et al.*, 2016, Zhou *et al.*, 2018). For direct diagnosis from clinical samples, 16S specific qPCR has shown

to be significantly more sensitive (76 % detection rate) compared to endpoint PCR (41 % detection rate) whilst no significant drop in specificity was observed when detecting a 530 bp fragment that covers the first three variable regions of the 16S gene (Meddeb *et al*., 2016). Due to these advantages, there are many commercially available, pre-optimised hydrolysis probe-based qPCR kits for diagnostics of mycobacterial diseases in animals. Typically, these kits contain all components required for the qPCR assay, including a master mix, probes, primers, internal amplification controls and exogenous PCR positive controls. All that is required from the end user is to provide their desired DNA template. The advantage of these kits is that in comparison to conventional diagnostic methods, they are less labour intensive and can be performed by less skilled individuals.

For diagnosis of Johne's disease, there are several readily available qPCR kits which use hydrolysis probe-based assays, however most of these kits have been predominantly optimised for detection of mycobacterial DNA isolated from faeces, tissue, milk, organs and sputum (see table 3.1 for details on commercially available qPCR kits for detection of MAP) with DNA isolated using chemical or physical DNA extraction methods. As the Actiphage Rapid™ assay is designed to detect mycobacteria from whole blood samples it was unknown whether these commercial kits, optimised for detection of DNA from faecal and tissue samples, were compatible with the DNA samples prepared using the mycobacteriophage lysis method and may contain substances from blood known to inhibit PCR amplification. For instance, Sidstedt *et al.* (2018) found that haemoglobin directly affects DNA polymerase activity and can act as a fluorescence quencher of free reporter dye

molecules during thermal cycling, whilst also reporting that immunoglobulin G (IgG) was found to bind to single-stranded genomic DNA, inhibiting DNA polymerisation.

Although these qPCR kits include estimates of sensitivity, these are generally high since the extraction methods they use have been optimised to often require much higher number of cells in the initial sample. BactoReal kit (ingenetix; Austria) has a limit of detection of 43 target copies per reaction, whilst Bio-T kit® MAP (Biosellal; France) limit of detection is 500 genome equivalents (GE) per gram of faeces. Extraction from faeces requires several vigorous sampling processing steps to separate the bacteria from the faecal matter and therefore there are large losses of material during the process. Hence there was no clear evidence that these kits would be sensitive enough to detect the low levels of cells that can be present in a blood sample.

Due to these unknown factors, PBD Biotech Ltd. wanted to know whether a commercially available qPCR kit could be used in combination with the Actiphage Rapid™ assay method for detection of mycobacteria infection from the blood of animals. Farmed deer were used to determine this since the company were receiving this type of sample at the time for commercial testing using standard end point PCR methods. It was perceived that using clinical samples, containing mycobacteria present due to natural infection, was a better model than spiking lab-grown cells into commercially sourced animal blood. It was hoped that the outcome of this work would result in an optimised Actiphage Rapid™ assay qPCR-based approach for detection of mycobacteria from blood samples which could

significantly improve sensitivity and efficiency of mycobacterial DNA isolation and detection from animals.

The aims of Chapter 3A were:

1. Determine whether Actiphage Rapid™ assay was compatible with qPCR
2. Evaluate the use of a commercial qPCR kit and Actiphage Rapid™ assay to detect mycobacteria from Cervidae clinical blood samples

Table 3.1. Commercially available qPCR kits for the detection of MAP

BRAND NAME	KIT NAME	DNA ISOLATION SITE	HOST
ADIAGENE	Adiavet ParaTb real time	Faeces, tissue, milk	Bovine, ovine, caprine
BIOSELLAL	Bio-T kit *Mycobacterium avium paratuberculosis*	Faeces	Ruminants
INGENETIX	BactoReal Kit *Mycobacterium avium paratuberculosis*	Faeces, tissue, milk	Not specified
APPLIED BIOSYSTEMS	VetMAX *Mycobacterium paratuberculosis* 2.0 Kit	Faeces, organs	Cattle, goat, sheep, wild ruminants
BIOINGENTECH	HumqPCR-realtime™ *M. avium*	Blood, serum, faeces, respiratory fluid, digestive system, tissue	Not specified
INDICAL BIOSCIENCE	Bactotype MAP PCR kit	Faeces, tissue	ruminants

Analysis of the characteristics of commercial qPCR kits for the detection of MAP available at the time of the study. The proposed use (i.e. MAP DNA isolation sites) are recorded as well as host animals reported by each company. Only one of these kits from BioIngenTech has been recommended for use with blood samples although host species was not specified.

3A.2 RESULTS

3A.2.1 Review of characteristics of commercially available kits

An internet search to look for *M. avium* subspecies *paratuberculosis* qPCR kits was performed to determine which commercial kit was to be used for experiments. A shortlist of qPCR kits was generated (table 3.1.). Any qPCR kits that did not list recommended DNA isolation site based off the internet search were not included on the list. As Actiphage® assays have protocols to test from milk and blood, qPCR kits that list milk or blood as a DNA isolation site were included. As not many qPCR kits have blood as a recommended DNA isolation site, qPCR kits that listed DNA isolation site as faeces or tissue were also considered. From the short list, practical considerations to determine which kit to use included delivery availability at time of sampling and companies interested in a collaboration with PBD Biotech to set up a commercial deal. Two companies interested in a collaboration were Ingenetix (Austria) and Biosellal (France). Initially, it was decided to use the Ingenetix qPCR kit as this kit had milk listed as a recommended DNA isolation site, in comparison to the Biosellal kit which only listed faeces as the recommended DNA isolation site.

3A.2.2. Use of BactoReal® qPCR kit for detection of mycobacteria from blood of farmed deer

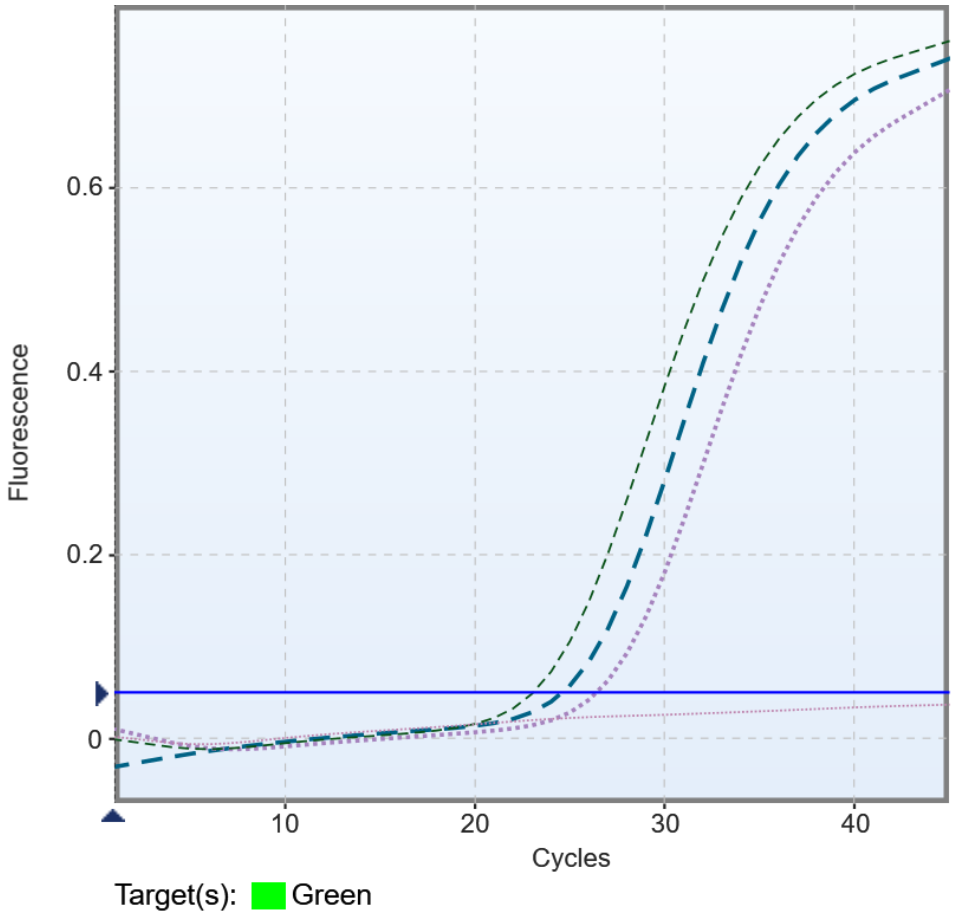
BactoReal® Kit *Mycobacterium avium* ssp. *paratuberculosis* (Ingenetix; Austria) was initially used to determine whether the detection of MAP from the blood of farmed deer could be achieved. Thirty-eight individual blood samples from farmed deer of unknown Johne's disease status were subject to PBMC extraction

(section 2.2.3.1.) and then the Actiphage Rapid™ assay was performed (section 2.4.4.) before qPCR was used to detect the sample DNA recovered from the assay (section 2.5.3.1.).

The Actiphage® positive control sample (approximately 10^2 MAP cells, strain K10 ATCC BAA-968) was prepared by performing 10-fold serial dilutions until approximately 10^2 cells was achieved based on optical density of the initial culture. The positive control gave a Cq value of 24.62 and the non-template negative qPCR control (water) produced an undetected (ND) signal.

In contrast the Actiphage Rapid™ assay negative control (MP alone containing no MAP cells) produced a Cq value of 26.43 which was only ~2 Cq values later than the positive control and indicated that some contamination may have occurred (figure 3A.1). The Cq values for all 38 clinical deer samples had a Cq value of ≥ 27.17 and therefore would be scored as negative based on the negative control value but based on the Cq value of the positive control, would be predicted to represent the detection of ~50 cells (based on a ΔCq of 1 which is a 2-fold decrease in DNA concentration). Since the value for the clinical samples was later than the negative control it did indicate that some contamination had occurred.

Figure 3A.1 Normalised qPCR data for detection of MAP from blood of farmed deer using BactoReal® kit



The blood of 38 farmed deer was subject to Actiphage Rapid™ assay and detection of MAP was performed by qPCR using BactoReal® Kit (Ingenetix; Austria). Water NTC remained undetected (...), MP negative Actiphage® assay control (...) had a Cq of 26.43 and MAP positive Actiphage® assay control (---) had a Cq of 24.62). Exogenous positive control (EPC) (---) had a Cq of 23.07. All samples (not shown on figure) had Cq values later than the negative assay control (Cq ≥27.17). Blue horizontal line (__) indicates the cycle threshold which determines Cq value for all qPCR assay samples.

3A.2.3. Use of Bio-T® Kit for detection of mycobacteria from blood of farmed deer

At this point, a total of 315 blood samples from individual deer of unknown Johne's disease status were received in the lab for testing over a period of 5 weeks. As BactoReal qPCR kit was unsuitable for troubleshooting due to the use of a single primer (section 3A.2.2.) Bio-T Kit® MAP (Biosellal; France) was used to determine whether this qPCR kit was suitable for use with Actiphage Rapid™ assay for routine detection of MAP from blood of deer.

DNA was extracted using Actiphage Rapid™ assay (section 2.4.4.) and then the DNA tested using Bio-T Kit® MAP (Biosellal; France, see section 2.5.3.2.) to determine the presence of MAP. Due to the large number of samples and different dates when samples were taken from deer and received for processing, Actiphage Rapid™ assays were performed on five occasions and nine qPCR runs were performed, which meant that there may have been variation seen between the batches of samples.

A summary of Cq values for negative and positive controls for all runs is shown in Table 3A.2. Both MP and water negative controls for all qPCR runs generated a Cq value <40 indicating amplification of DNA. On qPCR run 1 and 2, which used the same Actiphage Rapid™ assay controls, the MP negative control (which should contain no MAP template DNA) again had a Cq value within 2 cycles of the MAP Actiphage Rapid™ assay positive control (see Table 3A.2). For all other qPCR runs (3-9) despite both MP and water negative controls producing Cq values, these negative controls had a later Cq value than the positive controls (MAP and EPC). For run 3-9 the difference between MP negative control and MAP positive

control (ΔCq) ranged from 3.4 to 13.79 cycles. If DNA had been successfully extracted from 10^2 MAP cells, then it would be expected that there was approximately a Cq value difference of 6 cycles between a positive and a negative control sample (based on a reduction of 1 Cq value for each 50 % reduction in DNA concentration). Only runs 7, 8 and 9 met this criterion, but runs 3-7 had ΔCq values between the positive and negative controls of between 3.4 and 4.85, suggesting that either the assay was not efficiently extracting the DNA from the positive control samples, or some contamination was occurring.

Table 3A.2 Summary of negative and positive control Cq values from Actiphage Rapid™ assay of farmed deer

RUN	MAP (PC[1]) CQ	MP CQ (NC[2])	ΔCQ (NC-PC)	WATER (NTC[3]) CQ	EPC (PC[4]) CQ
1	20.48	22.58	**2.1**	35.19	19.98
2	22.81	23.63	**0.82**	30.33	19.27
3	26.53	30.05	3.52	39.10	28.62
4	24.85	29.70	4.85	30.33	19.27
5	20.88	24.28	3.4	33.01	17.19
6	21.95	26.50	4.55	29.04	18.81
7	20.88	30.75	**9.87**	34.35	18.11
8	18.73	32.52	**13.79**	36.18	18.23
9	19.76	31.27	**11.67**	31.57	19.06

[1] PC = Actiphage assay positive control

[2] NC = Media Plus (MP) Actiphage assay negative control

[3] NTC = qPCR assay non-template negative control (NTC)

[4] EPC = exogenous positive control (EPC)

Cq values were obtained from nine different qPCR runs using Bio-T MAP® kit (Biosellal; France). Colour coding indicates where the same DNA template was used. MAP cells (10^2) were used as the Actiphage assay positive control (PC). Media Plus (MP) was used as the Actiphage assay negative control (NC). Nuclease-free sterile water was used as the qPCR assay non-template negative control (NTC) and an exogenous positive control (EPC) provided with the Bio-T MAP® kit was used as the qPCR assay positive control (PC).

3A.2.3.1. Visualisation of qPCR products by gel electrophoresis

Run 1 and 2 (section 3A.2.3.), which were from the same sample set, had early Cq values for the MP negative assay control (Cq 22.58 and Cq 23.63). This Cq value was also very similar to the Cq value of the MAP positive control (Cq 20.48 and Cq 22.81) therefore through qPCR analysis alone it could not be determined which samples were MAP negative and which were MAP positive. Through qPCR analysis it could also not be determined whether the early Cq value for the MP was because of contamination in the negative control. Therefore, qPCR products were selected to be visualised by gel electrophoresis for further analysis.

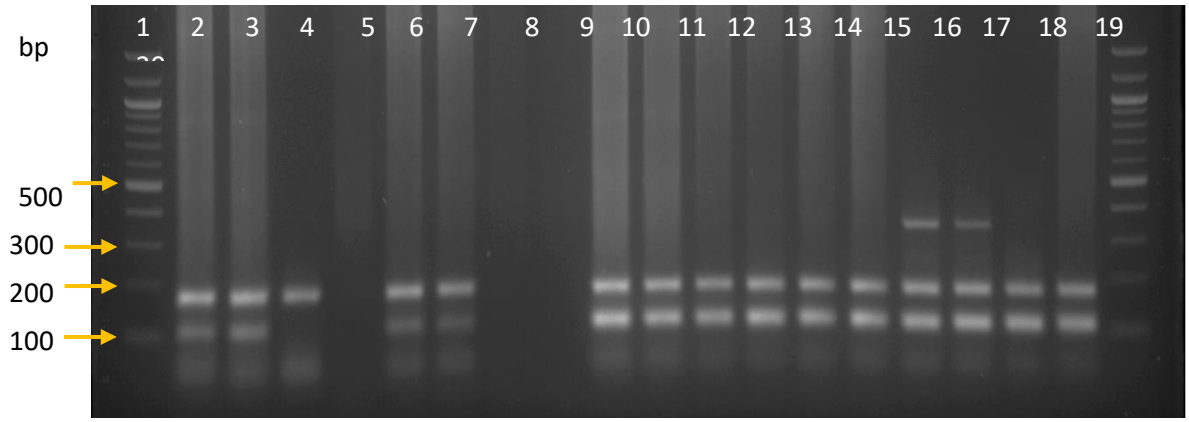
Within the test results for the 315 clinical samples, 25 showed inhibition observed through lack of amplification of the internal positive control on the Cy5 acquisition channel and remained not detectable (ND) on the target acquisition channel (FAM) therefore were determined to not be true MAP-negative samples.

Samples were selected at random to provide a dataset which displayed a range of Cq values (Cq < 25, n = 6, Cq > 25, n = 5, Cq ND, n = 3). Both MP and MAP Actiphage Rapid™ assay controls were also included on the agarose gel. All products were run on the same 2 % (w/v) agarose gel and visualised under UV light.

The three samples that were ND by qPCR on both the FAM and Cy5 acquisition channels did not show any indication of amplified products on the agarose gel (Figure 3A.2, lanes 5, 8 and 9) further suggesting qPCR inhibition contributed to a lack of detection. The kit positive control sample produced a band at approximately 100 bp and a band approximately 190 bp. The MAP Actiphage Rapid™ positive control which contains a known amount of MAP DNA, had a band

at approximately 350 bp in addition to the two other bands seen across all other non-inhibited samples. Similarly, MP negative assay control also followed this band pattern with a 100 bp, 190 bp and a 350 bp product. All deer samples, irrespective of Cq value, had band patterns like that of the kit positive control (a band at approximately 100 bp and a band approximately 190 bp). This suggests that bands at 100 bp and 190 bp are by-products of qPCR amplification derived from qPCR kit components and are not specific to MAP DNA. However, the 350 bp product is MAP specific product. Although this does also mean MP does show signs of contamination which caused the early Cq value and the 5 samples with a Cq <25 which based off qPCR results were presumptive positive, did not have this 350 bp product.

Figure 3A.2 Analysis of Bio-T® kit qPCR products by gel electrophoresis



Blood samples of farmed deer were subject to Actiphage Rapid™ assay and detection of MAP DNA determined by Bio-T MAP® qPCR kit (Biosellal; France). qPCR products from a random selection of samples that showed inhibition (n = 3), had a Cq > 25 (n = 5) and had a Cq < 25 (n = 7) were separated on a 2 % (w/v) agarose gel by gel electrophoresis and visualised under UV light. Actiphage Rapid™ assay positive (MAP) and negative (MP) controls were also visualised. Actiphage Rapid™ assay positive and negative controls had the same band pattern with three bands at approximately 100 bp, 190 bp and 350 bp (lanes 16 and 17).

Lane 1: 100 bp ladder (New England Biolabs; USA), Lane 2-4 and 6-7: samples with Cq >25, Lane 5, 8-9: samples ND, Lane 10-15: samples with Cq <25, lane 16: Media Plus negative control, lane 17: MAP positive control, lane 18: Bio-T Kit exogenous positive control, lane 19: sample 59, lane 20: 100 bp ladder (New England Biolabs; USA).

3A.2.3.2. Drop dialysis of inhibited samples to improve qPCR detection

Interpretation of the results in section 3A.2.3. and 3A.2.3.1., suggested 25 samples had qPCR inhibition through analysis of the internal amplification control on the Cy5 data acquisition channel and visualisation of samples by gel electrophoresis. It is well known that salts and other organic compounds can cause PCR inhibition (Schrader *et al.*, 2012) therefore to determine whether impurities within the template DNA could be removed and therefore improve detection of target DNA, drop dialysis was performed as per section 2.5.1.4. Briefly, between 5 µl and 10 µl DNA was dialysed on a type-VS membrane filter, floating on double-distilled water for 1 h before being recovered and used as DNA template for qPCR.

All samples from deer blood that had previously shown qPCR inhibition (section 3A.2.3.) showed improved detection and a lack of inhibition after dialysis (table 3A.3). Negative control samples were not subject to dialysis; however, the same negative controls were used in qPCR as previously used (section 3A.2.3.). The negative assay control (MP) and negative qPCR control (water) produced Cq values of 23.69 and 34.91, respectively. Analysis of the negative control samples that produced high Cq values showed that there was no MAP-specific band present, which indicated that there was no lab contamination by MAP DNA within these samples.

Table 3A.3 Cq values of samples detected by MAP qPCR after dialysis of template DNA

SAMPLE NUMBER	CQ (FAM) BEFORE DIALYSIS	CQ (FAM) AFTER DIALYSIS	CQ (CY5) AFTER DIALYSIS
3	ND	31.79	26.76
5	ND	32.32	24.09
7	ND	32.60	23.59
8	ND	28.69	22.93
9	ND	32.50	23.85
10	ND	32.10	23.40
11	ND	31.86	26.47
12	ND	32.62	24.71
17	ND	29.82	23.37
18	ND	31.24	23.51
20	ND	31.36	23.84
21	ND	31.40	23.27
22	ND	30.88	26.67
23	ND	33.80	24.06
26	ND	28.10	26.87
35	ND	29.74	23.31
36	ND	32.95	23.24
44	ND	27.57	23.56

47	ND	29.33	23.58
48	ND	31.83	23.38
128	ND	26.81	24.50
135	ND	28.15	22.73
136	ND	28.01	23.97
139	ND	28.35	24.80
140	ND	22.91	21.49

qPCR results for DNA extracted from PBMCs of deer using Bio-T® Kit MAP for detection of MAP. Table shows Cq values for samples before and after dialysis. All samples were inhibited (ND) by qPCR before dialysis. All samples showed an improvement in detection of MAP (Cq FAM) and reduced levels of inhibition (Cq Cy5) after dialysis.

3A.2.4. Determining the effectiveness of peripheral blood mononuclear cell lysis

When performing the Actiphage Rapid™ assay using blood samples, after PBMC isolation, they are suspended in Media Plus to lyse the cells and release any mycobacteria that may be residing within them into the culture media. To investigate whether the additional step of using sterile distilled water (SDW) to aid in the lysis of peripheral blood mononuclear cells (PBMCs) prior to Actiphage Rapid™ assay to improve detection of MAP and MTBC, blood samples from animals belonging to Bovine and Cervidae families were tested.

A total of 96 clinical whole blood samples were tested. PBMCs were either extracted using Ficoll-Paque PLUS (section 2.2.3.2.) or ACK (section 2.2.3.1.) PBMC isolation methods. A summary of samples used is shown in table 3A.4. After isolation, PBMC pellets were suspended in Media Plus and left to incubate for 1 h at room temperature (standard lysis method) or 250 µl SDW, left to incubate at room temperature for 20 min before addition of 750 µl MP and left to incubate for a further 1 h at room temperature (extended lysis method).

After PBMC lysis, Actiphage Rapid™ assay was performed as per section 2.4.4. Presence of mycobacteria was confirmed by qPCR using either the Bio-T kit *M. avium paratuberculosis* (n = 78) or Bio-T kit MTBC (n = 18), according to the suspected organism to be present in these clinical samples (the reason for choosing these kits is explained in part B of this chapter). Six samples being tested for MAP using extended lysis method failed to produce a Cq value. These samples showed no qPCR inhibition on the internal control channel, suggesting the lack of Cq value cannot be attributed to PCR inhibition.

When using extended lysis method to lyse PBMCs, 80 % of animals gave a positive result in comparison to 65 % when using standard lysis method. Paired t-test analysis of all 96 samples shows that there is no significant difference between the two methods of lysing white blood cells $p > 0.05$ ($p = 0.5471$). For 67 % of samples tested, an earlier Cq value was seen when using the extended lysis method PBMC lysis method.

Out of the seven samples taken from deer, only one produced an earlier Cq value using extended lysis method than the standard lysis method.

When considering only those samples tested for MAP (n = 78), paired t test analysis shows no significant difference between the two PBMC lysis methods $p = 0.1056$ ($p > 0.05$) similarly when considering only those samples tested for MTBC (n = 18), t test analysis shows there was no significant difference between the two PBMC lysis methods $p = 0.6839$ ($p > 0.05$).

Table 3A.4 Summary of samples used for PBMC lysis experiments

SAMPLE	NUMBER	PBMC EXTRACTION	MYCOBACTERIAL TARGET
DEER	7	ACK	MAP
CATTLE	71	Ficoll	MAP
CATTLE	18	Ficoll	MTBC

A total of 7 PBMC samples from deer extracted using ACK isolation method to detect M. avium subspecies paratuberculosis, 71 PBMC samples from cattle extracted using Ficoll to detect M. avium subspecies paratuberculosis and 18 PBMC samples from cattle extracted using Ficoll to detect Mycobacterium tuberculosis complex were used in experiments to whether the addition of water to Media Plus could aid in PBMC lysis.

3A.3 DISCUSSION

The use of qPCR with the Bio-T Kit® (Biosellal; France) was able to detect positive samples when using Actiphage Rapid™ assay as a DNA extraction method 290 times out of 315 without any further need of manipulation of DNA template. Despite this, out of 315 samples tested, 25 samples showed signs of qPCR inhibition and for these samples they could not be determined as positive or negative without any further analysis.

In qPCR, negative controls, including the MP Actiphage Rapid™ negative control are expected to have no amplification. This was not seen with any qPCR assays using Bio-T MAP Kit® as there was consistently Cq values assigned to MP negative controls. Visualisation of qPCR products by gel electrophoresis enabled greater insight into MP Cq values and allowed for conclusions to be drawn about whether MP had a Cq value because of off-target effects including contamination of target MAP DNA or whether there were qPCR bridging causing the Cq values.

In terms of qPCR, bridging refers to primer-dimer formation and non-specific interactions and amplification. In the case of probe-based qPCR assays, binding of probes to these unspecific primer-dimers will cause off-target fluorescence and amplification of undesired products. Alternatively, mis-priming to non-target DNA sequences found within the MP can also cause this unintentional amplification. Previous studies have also found that the longer reagents and template are left on the bench at room temperature before qPCR the increased chance of random artifacts being created within negative controls which can cause unspecific amplification (Ruiz-Villalba *et al.*, 2017). Samples used in the present experiments

were often processed in large quantities (between 70-120 samples at a time), therefore when manually pipetting individual qPCR tubes the length of time at which reagents were sitting at room temperature were at least 1.5 h. This could also have contributed to any unspecific amplification within negative control samples; however, experiments were not performed to determine whether this was a cause of Cq values for negative controls therefore this reasoning is only speculative.

The negative Actiphage Rapid™ assay negative control contains MP (the growth media for mycobacteria) and Actiphage® reagent. Due to the method of propagation of Actiphage® reagent, the reagent contains trace amounts of *M. smegmatis* DNA. This *M. smegmatis* DNA as well as any DNA from the mycobacteriophage will carry through to the DNA purification step and be added unintentionally as template to the qPCR reaction. As the primers used in the Bio-T MAP Kit® are not publicly available, it cannot be determined whether these primers share some homology to *M. smegmatis* or Mycobacteriophage D29 DNA sequences. Nevertheless, unspecific binding of primers and low primer specificity to the target DNA cannot be ruled out as a possibility to explain amplification of negative Actiphage Rapid™ assay controls.

Whilst contamination of negative controls can always be an issue when dealing with PCR reactions, we have established that with the Bio-T MAP Kit® we are able to determine whether the Cq value from the MP is due to contamination as it is possible to visualise qPCR products for this specific qPCR kit by gel electrophoresis (section 3A.2.3.1.).

Due to the issues of unspecific binding, a late Cq value (>35) can often indicate lack of amplification of target DNA and thus any samples, including negative controls with a late Cq value can be determined to be a negative sample. This commonly accepted rule is because an input of 10 copies of template in a qPCR with optimal efficiency and factoring in competition between primers will produce a Cq value by around cycle 35. Therefore, any samples or controls that have a Cq value later than this can be attributed to off-target effects or unspecific binding (Ruiz-Villalba *et al.,* 2021). This rule typically works for negative controls that do not include any DNA of species related to the target organism, however as Actiphage Rapid™ negative control contains traces of *M. smegmatis* DNA that could contribute to off-target fluorescence, a different boundary needs to be established. Therefore, for this qPCR assay to account for any off-target effects causing unspecific amplification within samples, any negative control that gives a Cq value >30 can be used to establish a base line to identify positive test results. Any qPCR assays where the negative control gives a Cq <30, products would need further analysis by gel electrophoresis to determine whether there is any contamination within the samples.

Additionally, to factor in variation that can be observed between different qPCR runs (section 3A.2.3.) and to differentiate a positive sample from a negative sample a ΔCq analysis method also needs to be implemented to standardise the results, where 'MPCq – SampleCq' can be used. If through ΔCq analysis, a sample has a ΔCq > 2 this sample can be classed as positive. Samples with a ΔCq > 5 are classified as a strong positive, whilst those with a ΔCq 1.5-2 are counted as presumptive or weak positive samples. The issue with this approach however is that

we are assuming that there is minimal contamination within the negative control and assuming that any Cq values observed are attributed to background noise. This can also mean that weak positive samples containing very low cell numbers of mycobacteria are going undetected.

In summary:

1. Actiphage Rapid™ assay is compatible with commercially available qPCR kits
2. Variation between qPCR runs means that a ΔCq analysis method needs to be implemented
3. Media Plus negative control can produce a Cq value however, agarose gel electrophoresis analysis can help elucidate whether this is attributed to contamination or unspecific amplification

CHAPTER 3

ESTABLISHING THE USE OF QPCR WITHIN THE ACTIPHAGE RAPID™ ASSAY PROTOCOL

PART A DETECTION OF *M. AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN DEER

PART B EVALUATION OF IN-HOUSE PRODUCTS

PART 3B

3B.1 INTRODUCTION

At the start of this project, the Actiphage Rapid™ assay had been optimised and successfully used to detect mycobacteria from clinical blood samples using conventional PCR (Swift *et al.*, 2020). The work described in Chapter 3A showed that qPCR is also compatible with Actiphage Rapid™ assay using commercially available qPCR kits. Despite this optimisation of protocols, troubleshooting was found to be difficult due to the lack of information made available by the companies that supply the qPCR kits. For instance, what specific sequences within the mycobacterial genome being targeted, or the sequences of the primers used, and what target is being used as the internal positive controls is not disclosed.

To develop Actiphage Rapid™ assay as a sensitive and rapid diagnostic kit, it was decided by the company that designing and implementing an in-house qPCR kit would provide more flexibility when troubleshooting and allow more control over the design of primers, probes and target sequences which would facilitate analysis of results during assay optimisation. As hydrolysis probe-based qPCR assays have increased specificity in comparison to intercalating dye-based qPCR assays, probe-based assays are favoured when developing diagnostic tests. Therefore, the work described in this chapter aimed to explore whether a new hydrolysis probe-based qPCR assay can be designed and optimised to be compatible with Actiphage Rapid™ assay for detection of mycobacteria.

When designing hydrolysis probe assays, the annealing temperature (T_m) of the probe as well as the forward and reverse primer needs to be carefully

considered. In hydrolysis probe qPCR assays, it is important for the probe to anneal to the target DNA before the primers. This is imperative, because if the fluorescent probe binds after or at the same time as the primers then the *Taq* polymerase may start synthesising new DNA strands without any bound probe. Consequently, no increase in fluorescence will be detected due to no probe degradation that cycle. Due to this, when designing TaqMan probe assays it is recommended that the probe has a T_m 5–10 °C higher than the T_m of the primers to avoid inaccurate results. Furthermore, when selecting appropriate reporter and quencher dyes, an important consideration is to ensure that the absorbance spectrum of the quencher dye matches the emission spectrum of the reporter dye (information on commonly used reporter and quencher dyes in table 4.1.). All these variables had to be considered when designing and optimising a qPCR assay for use with Actiphage Rapid™ assay.

Other components of qPCR assay design include the inclusion of internal amplification controls (IAC). Internal controls are non-target DNA present within the qPCR master mix, which are co-amplified with the target sequence. Although not compulsory, they allow for assay performance information to be recorded, detailing any qPCR inhibition occurring that could lead to false-negative test results. Inclusion of IAC in qPCR assays can be either in a competitive or a non-competitive format. In the former, both target DNA and IAC are amplified using a common primer pair. Drawbacks of this method when used in diagnostic testing is that the inclusion of IAC in a master mix in competitive format can lower the amplification efficiency of the PCR thus resulting in a lower detection limit for the target. This is a problem when the aim of the diagnostic tool is to detect low levels of target DNA in a sample

matrix, such that low-levels of infections (i.e. a low microbial presence in a clinical sample) may remain undetected leading to false-negative test results. Whilst designing an IAC of larger size than the target DNA can eliminate some competition because reaction kinetics should favour the smaller PCR product (Sachadyn and Kur, 1998), competition of an IAC larger than the target DNA can still influence PCR sensitivity (Brightwell *et al.*, 1998, Hoorfar *et al.,* 2004).

An alternative method, which may help overcome these issues posed with IAC in a competitive format is the use of IAC in a non-competitive format with the target molecule. In this instance, both IAC and target DNA are amplified using a different primer and probe set. This format eliminates the competition for the same primers however, the use of two different primer pairs in the same reaction mix can sometimes hinder amplification of the target DNA if differences within the primer sequences mean that qPCR parameters are more favourable to one primer pair than the other. For use in diagnostic qPCR, Hoorfar *et al.* (2004) recommended several criteria for optimal IAC use including using a competitive format with target DNA and IAC sharing the same primer-binding sites, using plasmid DNA as the IAC, and adding IAC to PCR master mix to allow for equal distribution of IAC in all PCR tubes.

Building on the success achieved using conventional end-point PCR methods with the Actiphage Rapid™ assay, it was decided to use the same primer sequences to develop an in-house qPCR assay in collaboration with Empirical Bioscience (USA) who provided the master mix for experiments detecting both MAP and MTBC, as well as the primers and probes used for detection of MTBC. The master mix

contained InhibiTaq™, a *Taq* polymerase optimised for use with hydrolysis probed based qPCR assays.

For detection of MAP IS*900* by qPCR, a modified version of the P90 primer first described by Millar *et al.* (1995) was used. The original P90 and P91 primer pair were designed based on the limited number of MAP sequences available at the time of the publication, however these sequences were mainly from MAP strains isolated from cattle. As more sequence data became available, it became clear that MAP from non-cattle hosts lacked full homology to the P90 forward primer thus reducing PCR sensitivity. This was apparent when attempts were made to detect mycobacterial DNA in blood samples originating from animals of ovine and caprine origins (Handley-Hartill, 2023). Recently, Bannantine *et al.* (2023) also highlighted these inaccuracies and binding inefficiencies within the P90 primer sequence. As such, the P90 primer was modified, removing the final six base pairs from the 3' end of the oligonucleotide to increase the specificity of the primer to non-cattle targets. This modified primer was named P90short (Handley-Hartill, 2023).

The work described in this chapter explores optimisation and analysis of qPCR assays and their use in complementing the Actiphage Rapid™ assay to allow for sensitive and specific detection of mycobacteria. Experiments were performed to optimise hydrolysis probe-based qPCR assays to allow for detection of MAP and compare these to a commercially available kit by Biosellal (France). Furthermore, individual components of the Actiphage Rapid™ assay were investigated to understand the scope of qPCR variability and how this may affect the diagnostic kit.

The aims of this chapter are:

1. To design and optimise probe-based qPCR assays for detection of MAP
2. Compare performance of qPCR kits designed in-house with commercially available qPCR kits
3. Analyse individual components of Actiphage Rapid™ assay to understand their contribution to qPCR variability

Table 3B.1. Commonly used reporter dyes and compatible quencher dyes

DYE	MAXIMUM EXCITATION (NM)	MAXIMUM EMISSION (NM)	COMPATIBLE QUENCHER
FAM™	495	520	BHQ™-1, TAMRA™
JOE™	529	555	BHQ™-1, TAMRA™
TET™	521	536	BHQ™-1, TAMRA™
HEX™	535	556	BHQ™-1, TAMRA™
TAMRA™	557	583	BHQ™-2
CYANINE 3 (CY3)	549	566	BHQ™-2
QUASAR®570	548	566	BHQ™-2
ROX™	586	610	BHQ™-2
CYANINE 5 (CY5)	646	669	BHQ™-3
QUASAR 670	647	670	BHQ™-3

List of commonly used reporter dyes, their excitation and emission wavelengths (nm) and the appropriate compatible quencher to be used to allow for optimal performance in qPCR (Merck).

3B.2 RESULTS

3B.2.1. Development and optimisation of MAP specific qPCR

A qPCR assay to detect MAP using a master mix provided by Empirical Bioscience (USA) in collaboration with PBD Biotech (UK) was optimised and analysed to determine suitability for use with Actiphage Rapid™ assay. The suitability of fluorescent probe, inclusion of an internal amplification control and compatibility with samples extracted from a PBMC matrix were all areas of investigation.

The internal amplification control was a plasmid specifically created (Handley-Hartill, 2023) to contain binding regions for P90/P91 primers (normally used to identify a region of IS*900* in MAP), T4/T5 primers (normally used to identify a region of IS*6110* in MTBC) and MK7/MK8 primers (normally used to identify a region of IS*901* in MAA) (figure 3B.1). The construct containing these primer sites was designed so that the PCR products produced using these primers was of a different size to those when the primers are used to amplify signature sequences in the target organism genome. Hence, the IAC construct is capable of being used as an amplification control for assays in both a competitive and a non-competitive format.

Figure 3B.1 Region of interest sequence of pMV306-IAC plasmid

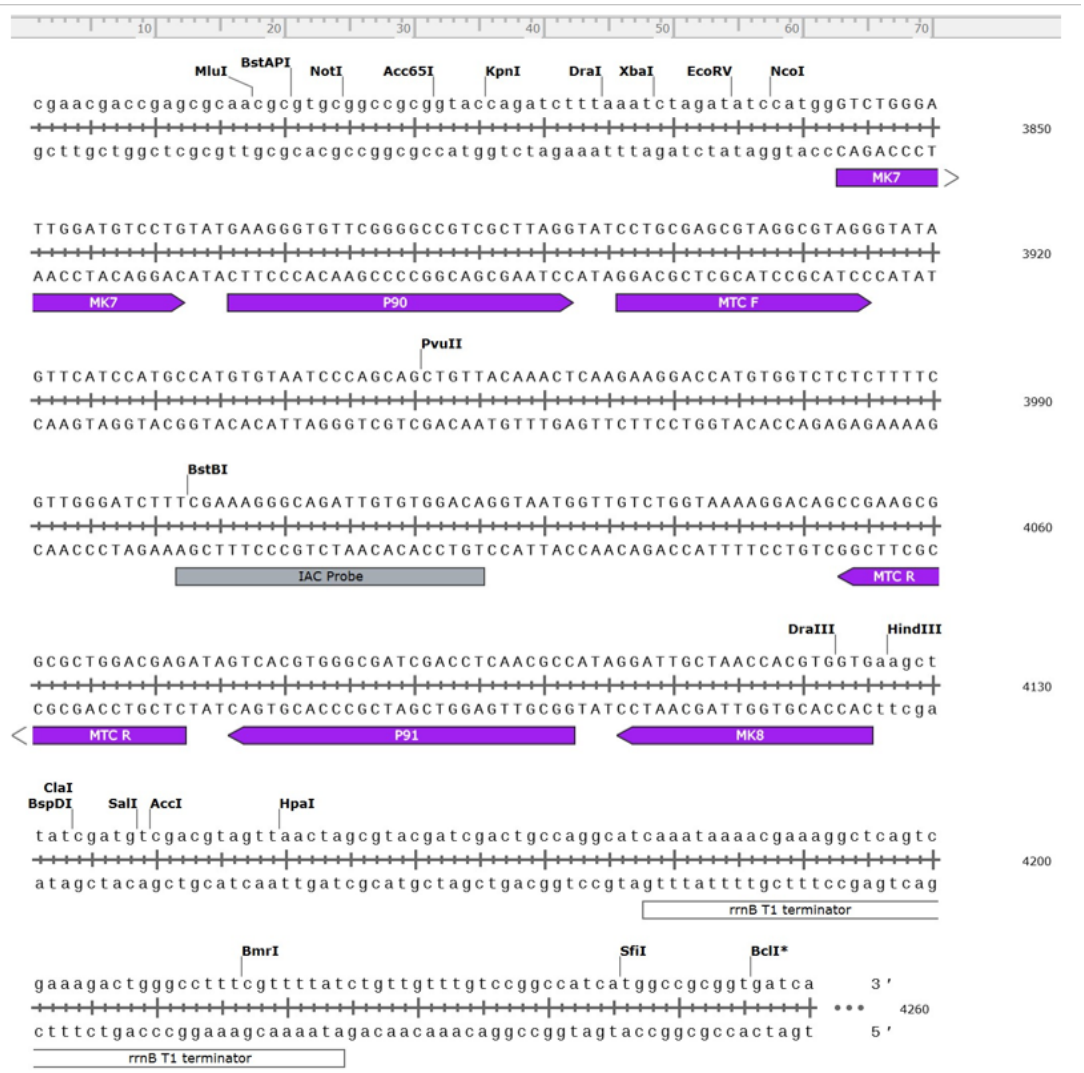


Figure taken from (Handley-Hartill, 2023)

Section of pMV306-IAC (IAC) plasmid that contains primer sites for recognition of P90/P91 primer pair, MK7/MK8 primer pair and MTCF/MTCR (also known as T4/T5) primer pair. Due to the presence of these primer sites, this plasmid can be used as an internal amplification control in either a competitive or non-competitive format for the detection of IS900 insertion sequence from MAP, IS901 insertion sequence from MAA and IS6110 insertion sequence from MTBC.

3B.2.1.1. Suitability of qPCR probe for detection of MAP extracted from Actiphage Rapid™ assay

The first qPCR component to be investigated and optimised was the fluorescent probe. Rather than designing this from scratch, a literature search identified several qPCR probes that had been previously optimised for use in qPCR detection assays for MAP. Potential probes were determined to be suitable if they targeted a region of IS*900* between the P90short forward primer site and the P91 reverse primer site (figure 3B.2). A probe known as IS900RLP (see Table 2.1), as first described by Donaghy *et al.* (2011) was identified for having specific binding to the conserved region of IS*900* and analysed for any homology to non-IS*900* sequences using Basic Local Alignment Search Tool (BLAST) (National Institute of Health; USA).

To determine the optimal probe concentration for use with P90short and P91 primer pair, qPCR efficiency assays were performed where the final concentration of probe was adjusted to either 0.25 µM or 0.1 µM. An internal amplification control (IAC) was not included in this qPCR therefore, at this point it was not possible to determine whether efficiency and Cq values were affected by inhibition.

For DNA template, a culture of MAP was enumerated using the Actiphage® Core assay. Briefly, MAP cells are incubated with Actiphage® reagent for 1 h to allow bacteriophage infection into host. After incubation, virucide is added and incubated for 5 min under constant rotation to ensure all internal surfaces of the reaction vessel are covered to kill off any Actiphage® that has not been internalised. Then, 10-fold serial dilutions are prepared, diluting the MAP cells before pouring

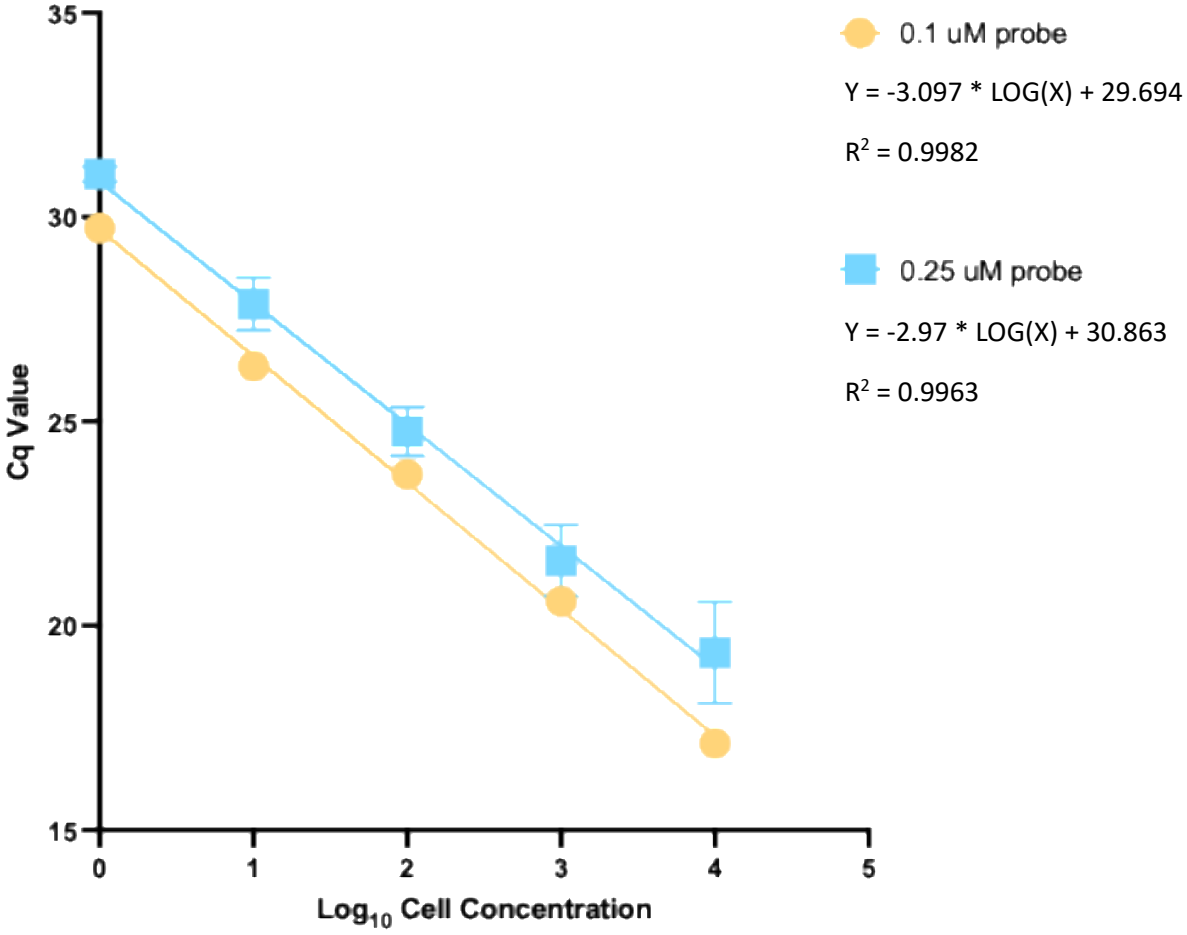
onto a soft agar containing MP growth supplement, *M. smegmatis* sensor cells and molten 7H10 agar. These plates are mixed gently to ensure homogenous mixture before being allowed to set and incubated at 37 ˚C for 18 h. After incubation plaque forming units can be counted and based off dilution used, PFU and enumeration can be determined. Once MAP cells are enumerated, 10-fold serial dilutions were prepared to achieve a range from 10^5 to 10^1 cells per assay. MAP DNA was extracted by Actiphage Rapid™ assay. Due to the slow growing nature of MAP (up to 16 weeks for visible colonies to appear on growth plates), enumeration by viable count methods were unfeasible, therefore using Actiphage® Core assay to enumerate cell concentrations was the only practical way to generate the DNA samples needed for assay development.

qPCR efficiency was calculated at 110.3 % when using IS900RLP probe at a final concentration of 0.1 µM compared to an efficiency of 117.1 % when using IS900RLP probe at a final concentration within the reaction of 0.25 µM (Figure 3B.3). Paired t test analysis showed that there was a significant difference between the two concentrations of probe used ($p < 0.05$, $p = 0.0030$). When using 0.1 µM of fluorescent probe, the calculated qPCR efficiency was just outside of the acceptable range of 90-110 % (Rogers-Broadway and Karteris, 2015), (probe efficiency 110.3 %) but was considered suitable for development of the assay.

Figure 3B.2 IS*900* region of interest covered by P90short and P90 primer pair

Region of interest to determine qPCR probe suitability for use with qPCR using P90short and P91 primers. IS900RLP (Donaghy et al., 2011)was selected as qPCR probe to be used for experiments

Figure 3B.3 Standard curve showing qPCR reaction efficiency with different probe concentrations



Efficiency plot of MAP DNA comparing two different concentrations of fluorescent probe (IS900RLP). MAP-genomic DNA was 10-fold serially diluted, and probe added at a final concentration of 0.25 µM (■) or 0.1 µM (●). The Y axis intercept values included below the respective probe concentration in the data labels were used for the efficiency calculation using the following equation $E = \left(10^{\left(\frac{-1}{slope}\right)} - 1\right) \times 100.$

3B.2.1.2. Analysis of addition of non-competitive internal amplification control to qPCR assay

To determine whether the inclusion of an internal amplification control (IAC) had inhibitory effects on the detection of MAP by qPCR, IAC was incorporated into the qPCR in a non-competitive format. MAP DNA enumerated and extracted as previously mentioned (section 3B.2.1.1.) was added as template to qPCR reactions with P90short and P91 used as the primer pair for detection (final concentration of 0.5 µM) and IS900RLP (final concentration of 0.25 µM) used as fluorescent probe for detection. To create a non-competitive assay format for detection of IAC, the T4/T5 primer pair (final concentration of 0.5 µM) which are specific for the IS*6110* sequence in MTBC and IAC probe 2 (final concentration of 0.25 µM) were also added to the master mix. All primer and probe information are listed in Table 3B.2. MP negative control was used as standard as per Actiphage Rapid™ assay and included as template for the qPCR reaction and RNase-free water used in the qPCR reaction as non-template negative qPCR control.

Negative Actiphage Rapid™ assay control (Media Plus with Actiphage® reagent) and negative non-template qPCR control (RNase-free water) performed as expected and no DNA was detected (ND) by qPCR. In a fully optimised qPCR, R^2 value should be more than 0.980 and efficiency should be 90-110 % meaning that qPCR assays where IAC was not included in the master mix fell into the acceptable criteria of an optimised qPCR assay (R^2 = 0.9906, efficiency 103.2 %). In comparison, qPCR assays where IAC was included in the master mix did not reach the criteria of being an optimised assay (R^2 = 0.9013, efficiency 114.9 %) (Figure 3B.4).

Furthermore, there was a significant difference in Cq values between master mix with IAC and master mix without IAC ($p < 0.05$, $p = 0.0010$). On average, MAP was detected 2.04 cycles later when IAC was included in the master mix of the qPCR assay compared to when there was no IAC in the master mix. The average difference in Cq (ΔCq) between 10-fold serially diluted DNA template was ΔCq = 2.94 when IAC was included in the qPCR master mix in comparison to ΔCq = 3.19 when there was no IAC included in the qPCR master mix. In theory, the difference between Cq values for 10-fold serial dilutions in qPCR should be 3.33 cycles therefore, ΔCq when there was no IAC in the master mix was closer to this ideal standard than when IAC was included in the reaction.

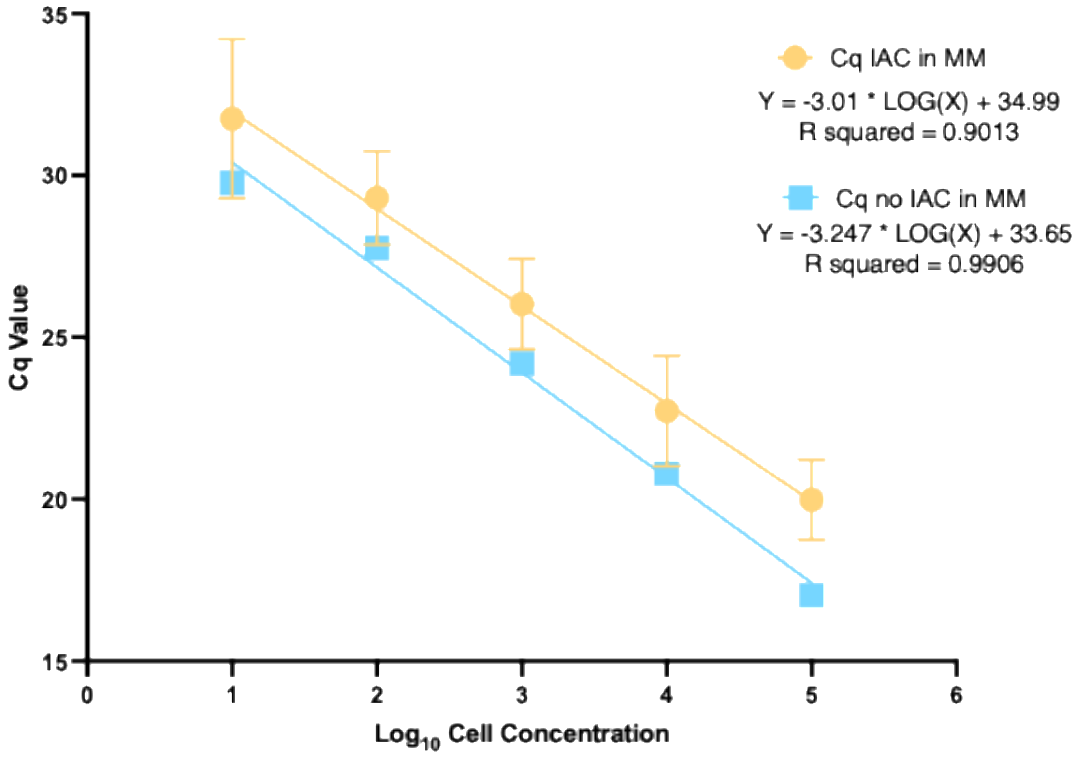
The conclusion from these experiments were that for the detection of the MAP genomic DNA the qPCR assays were more efficient and there was less qPCR inhibition when an internal amplification control was not incorporated into the master mix. This finding meant that the company decided to contract Empirical Biosciences to design a new PCR master mix.

Table 3B.2 Primers and probes used for qPCR experiments

TARGET SEQUENCE	PRIMER NAMES	FORWARD PRIMER 5'-3'	AMPLICON LENGTH (BP)
IS*900*	P90short	GAAGGGTGTTCGGGGCCGTCG	413
IS*900*	P91	GGCGTTGAGGTCGATCGCCCACGTGAC	413
IS*900*	IS900RLP	FAM-CATGGTTATTAACGACGACGCGCAGC-BHQ1	n/a
IS*6110*	T4	CCTGCGAGCGTAGGCGTCGG	123
IS*6110*	T5	CTCGTCCAGCGCCGCTTCGG	123
IAC PLASMID	IAC probe 2	HEX-TCGAAAGGGCAGATTGTGTGGACA-BHQ1	n/a

Forward (P90short) and reverse (P91) primers and probe (IS900RLP) sequences used for current experiments to detect a section of IS900 from MAP. T4 (forward primer), T5 (reverse primer) and IAC probe 2 were used for the detection of IAC plasmid DNA.

Figure 3B.4 Efficiency plot of MAP qPCR using Empirical master mix, with and without an internal amplification control



qPCR was performed using the Empirical master mix provided by PBD Biotech Ltd. Reactions were prepared with and without the presence of absence of a non-competitive IAC template. MAP cells were enumerated prior to Actiphage Rapid™ assay and 10-fold serially diluted for use with the Actiphage Rapid™ assay. Graph shows efficiency plot of MAP qPCR with (●) and without (■) the inclusion of the IAC in the master mix. Y intercept and R^2 values for both conditions are displayed in the legend below their respective labels.

4.2.1.3. qPCR detection of freeze-dried MAP cells spiked into peripheral blood mononuclear cells

Kubala (2024), a PhD student sponsored previously by PBD Biotech Ltd. developed a method to freeze dry mycobacterial cells which were still viable after resuscitation. This was done with the aim to include quantified amounts of freeze-dried mycobacteria in diagnostic kits to be used as a positive control. Although work was done on viability and storage of these freeze-dried cells, whether the cells could be used as a qPCR kit positive control remained unexplored. Therefore, when optimising qPCR assays, freeze-dried cells were resuscitated and used as template after DNA extracted with Actiphage Rapid™ assay.

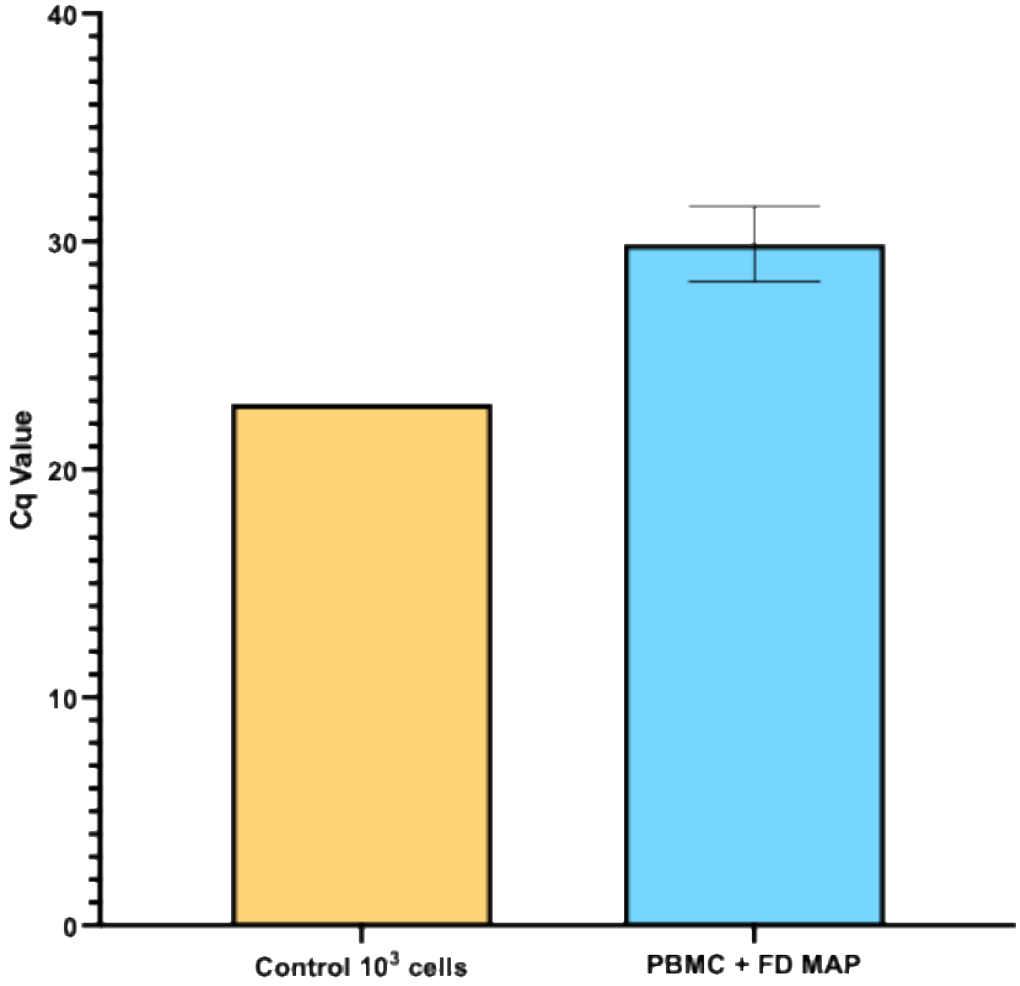
Using the IS900RLP probe, experiments were performed to determine whether MAP qPCR using Empirical master mix could be used to detect MAP cells. To ensure that the sample matrix best reflected what is normally present in the DNA extracted from clinical blood samples, it was also decided to extract these from PBMCs. To perform these experiments, freeze-dried MAP cells were resuscitated in 1 ml MP to achieve 10^4 cfu ml^{-1} then incubated at 37 °C for 1 h. 10^2 MAP cells were spiked into PBMCs harvested from whole blood of bovine origin obtained from an abattoir (section 2.2.2.) and the samples processed according to the method described for performing the Actiphage Rapid™ assay (section 2.4.4.). Briefly, after PBMC lysis, samples were centrifuged at 13,000 x g for 3 min, pellets resuspended in Actiphage® reagent and incubated at 37 °C for 3.5 h. After incubation, any DNA extracted from the assay was purified and used as template for qPCR reactions. Media Plus with Actiphage® reagent was used as a negative (no cells) Actiphage®

assay control and actively growing, viable MAP K10 culture at a concentration of 10^3 cells and Actiphage® reagent were used as a positive Actiphage® assay control.

Negative controls performed as expected and there was no amplification from either the negative Actiphage® assay control (no cells) and the non-template qPCR control (water). No internal amplification control was used in the qPCR assay for this experiment as this was previously shown to negatively affect qPCR efficiency, so PCR inhibition could not be monitored and the effect of the inclusion of an internal amplification control was not determined at this point.

The range of Cq values for freeze-dried MAP spiked into PBMCs (n = 3) was Cq 28.66-31.78 (mean 29.89, SD = 1.661) compared to Cq 22.88 for the positive control of 10^3 MAP cells (see Figure 3B.5). There was a mean difference of 7.01 ± 1.918 cycles between the positive control and the spiked samples. As there was a 10-fold difference between the number of cells added to the positive control and to the samples, all things being equal, a 10-fold difference in cell number would have a ΔCq between control and samples of 3.3 cycles. The difference in Cq values for this data is over 2-fold greater than this indicating there are inhibitory components from the blood that are affecting qPCR assay efficiency.

Figure 3B.5 Comparison of Cq values for MAP cells spiked into PBMCs with MAP cells in culture media alone



Empirical qPCR was performed to determine the effect of PBMCs on qPCR. Graph shows Cq values for 10^3 MAP cells in culture media (●) and 10^2 MAP cells spiked into PBMCs (●).

3B.2.2. Evaluation of MTBC qPCR using Empirical master mix

Probe concentration, effect of using an internal positive control and effect of PBMCs on detection of MAP using qPCR kits (Empirical Bioscience; USA) were all studied in section 3B.2.1. As the priority of the company shifted from detection of MAP in animals to the detection of MTBC in human and animal samples, the previous work on MAP optimisation with Empirical master mix was ended and focus moved to evaluating these new qPCR kits. The Empirical qPCR kit comes prepackaged with primers and probe for detection of MTBC. These primers and probe were therefore used for the following experiments to evaluate the use of MTBC qPCR kit (Empirical Bioscience, USA) to aid in detection of MTBC DNA extracted using Actiphage Rapid™ assay.

3B.2.2.1. Effect of different primer concentrations on detection of MTBC using Empirical qPCR kits

qPCR kits for detection of MTBC were manufactured by Empirical Bioscience. Two different batches of qPCR kit were tested, with the two kits containing primers of different concentrations. The first qPCR kit gave a final primer concentration of 1 µM per qPCR reaction, and the second qPCR kit gave a final primer concentration of 0.5 µM per qPCR reaction. As before, comparative experiments were performed to determine whether the difference in primer concentrations would affect detection of MTBC. Briefly, PBMCs obtained from bovine blood from an abattoir (section 2.2.2.) were subject to Actiphage Rapid™ assay. DNA extracted from this assay was subject to IS*6110* endpoint PCR to screen PBMCs for the presence of MTBC and only used for experiments if determined to be IS6*110*-negative. 5 µl of MTBC negative

DNA extractions were mixed with 1 µl of 1.4 ng μl^{-1} *M. bovis* BCG DNA which roughly equates to 2 x 10^5 genome copies, prepared using boiled lysate method as per section 2.5.1.3. As previous experiments with MAP suggested blood components have an inhibitory effect on qPCR, *M. bovis* BCG was spiked into PBMC DNA extracts to replicate a clinical sample and assess the extent of any qPCR inhibition.

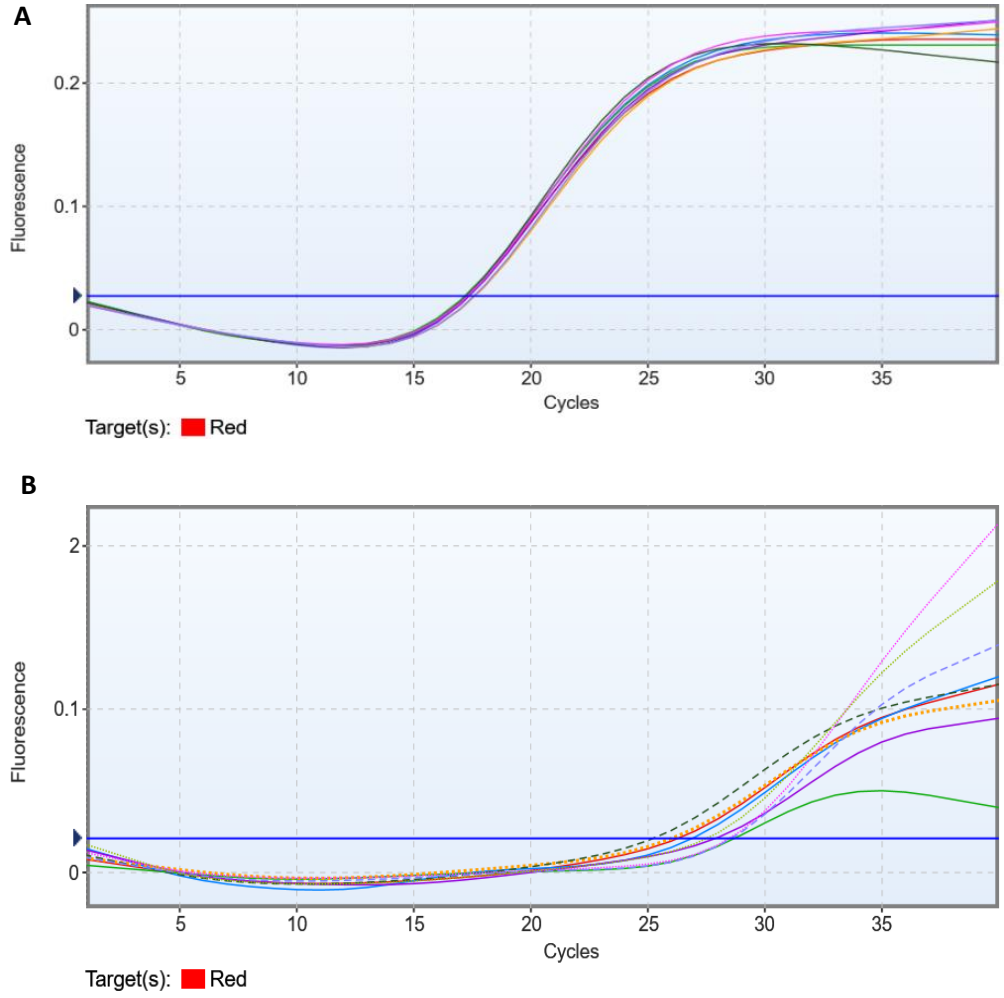
The IAC internal positive control was included in the master mix, and it was found that for both concentrations of primer (final concentration of 1 µM or final concentration of 0.5 µM) there was no qPCR inhibition observed as all samples produced Cq values on the Cy5 data acquisition channel. However, for the qPCR reaction using an increased primer concentration of 1 µM, the Cy5 fluorescence did not perform as well, with later Cq values for all samples compared to the Cq values for samples using 0.5 µM of primers, suggesting there was some PCR inhibition or competition (Figure 3B.6).

Although using 0.5 µM of primers showed no qPCR inhibition on the Cy5 acquisition channel for every sample, only the positive control (BCG DNA not spiked into PBMCs) produced a Cq value on the FAM acquisition channel. Cy5 acquisition channel measures the internal amplification, whilst FAM acquisition channel measures target DNA. This suggests that IAC primers, which are used for the internal control on the Cy5 channel, were outcompeting the low concentration of forward and reverse primers used for the target sequence.

The higher primer concentration improved detection of *M. bovis* BCG DNA when spiked into PBMC DNA. All three replicates (PBMC DNA spiked with 1 µl *M.*

bovis BCG) generated a signal above the threshold, using a final concentration of 1 µM forward and reverse primer, compared to no Cq values produced for the triplicate samples using a final concentration of 0.5 µM forward and reverse primers (Table 3B.3).

Figure 3B.6 Effect of primer concentration on detection of MTBC DNA



*Normalised data plots produced post-thermal-cycling by Q-Rex software show the amplification of the Cy5 (red) channel and a final concentration of primers of 0.5 µM (**Panel A**) or 1 µM (**Panel B**). All samples and controls produced sigmoidal curves. The horizontal blue line (—) indicates the threshold level set over which the Cq value of samples is determined.*

Table 3B.3 Cq values of *M. bovis* BCG DNA spiked into PBMCs using different concentrations of forward and reverse primers

	FAM		CY5	
SAMPLE NAME	**Cq Value (0.5 µM primers)**	**Cq Value (1 µM primers)**	**Cq Value (0.5 µM primers)**	**Cq Value (1 µM primers)**
PBMCS + *M. BOVIS* BCG (1.4 NG)	ND	29.16	17.27	26.30
PBMCS + *M. BOVIS* BCG (1.4 NG)	ND	27.96	17.29	26.90
PBMCS + *M. BOVIS* BCG (1.4 NG)	ND	28.38	17.18	28.70
MEDIA PLUS (NEGATIVE CONTROL)	ND	ND	17.63	26.02
BCG (POSITIVE CONTROL)	20.90	ND	17.26	25.24
WATER NTC	ND	ND	17.31	28.50

qPCR results for M. bovis DNA spiked into PBMCs. qPCR was performed with either a final concentration of 0.5 µM or 1 µM primers. Fluorescent detection on the FAM acquisition channel detects target DNA, whilst fluorescent detection on the Cy5 acquisition channel detects internal amplification control. Samples where there was no detectable fluorescence above the threshold level remained undetected (ND) by qPCR.

3B.2.2.2. Analysis of addition of internal amplification control to qPCR assay

From the results in section 3B.2.1.2 it was established that the addition of an internal amplification control inhibited qPCR reactions when MAP sequences were targeted. Results in section 3B.2.2.1 also demonstrated that internal controls may have an influence on Empirical qPCR kits when detecting MTBC. Experiments were now performed to determine whether inhibitory effects would be observed using MTBC specific qPCR with Empirical reaction mix. For these experiments, IAC was added in a competitive format, unlike in section 3B.2.1.2. where IAC was added in a non-competitive format. To do this *M. bovis* BCG DNA was diluted in ten-fold steps from 1.6 ng to 1.6 x 10^{-7} ng μl^{-1} to be used as template for qPCR reactions. Samples were tested in duplicate where IAC was included in the qPCR master mix of one set of samples and excluded from the second set.

Two samples, 1.6 ng μl^{-1} DNA template and no IAC in the master mix and 1.6 x 10^{-4} ng μl^{-1} DNA template and IAC in the master mix, were undetected (ND) by qPCR and were therefore omitted from the analyses. Efficiency of the qPCR in the absence of IAC in the master mix was calculated at 101.8 % in comparison to 282.0 % when IAC was included in the master mix. This suggests there was more inhibition of amplification in the presence of IAC. Excessive amounts of DNA can contribute to qPCR inhibition and amplification efficiency over 100 %, therefore the addition of IAC DNA to the reaction mixture along with target DNA template could cause an over-efficient qPCR reaction.

Analysis of the ΔCq values between the samples with and without the IAC showed that three samples had a ΔCq <2 (1.6 ng μl^{-1}, 0.16 ng μl^{-1} and 1.6 x 10^{-6} ng

μl^{-1}) and three samples had a ΔCq >2 (1.6×10^{-3} ng μl^{-1}, 1.6×10^{-5} ng μl^{-1}, 1.6×10^{-7} ng μl^{-1}) (table 3B.34). There was no pattern as to when ΔCq <2 and this appeared randomly distributed amongst the different DNA concentrations.

Table 3B.4 Detection of *M. bovis* DNA by qPCR in the presence or absence of an internal amplification control

DNA CONCENTRATION (NG)	IAC IN MM	NO IAC IN MM	ΔCQ (NO IAC – WITH IAC)
16	12.62	ND	n/a
1.6	16.26	16.23	-0.03
0.16	20.72	21.02	0.30
0.016	8.36	23.46	n/a
0.0016	24.6	27.5	2.90
0.00016	ND	31.41	n/a
0.000016	30.34	33.81	3.47
0.0000016	29.47	30.33	0.86
0.00000016	25.77	30.25	4.48

M. bovis BCG DNA was serially diluted from 16 ng μl^{-1} to 1.6 x 10^{-7} ng μl^{-1} and 1 µl of each dilution was used as template DNA for qPCR. An internal amplification control (IAC) was either included in a competitive format or excluded from the qPCR master mix (MM). ND = undetected.

3B.2.3. Adaptation of Bio-T kit® *Mycobacterium avium paratuberculosis* qPCR kit to include IPC during qPCR reaction set-up

Commercially available qPCR kits by Biosellal (France) were shown to be able to successfully detect MAP DNA present in deer whole blood when processed using the Actiphage assay (Chapter 3A). Therefore, it was decided to use these qPCR kits to compare results gained using the qPCR kits designed by Empirical Bioscience to determine whether the Empirical qPCR kits which were designed specifically for PBD Ltd. were comparable to commercially available qPCR kits.

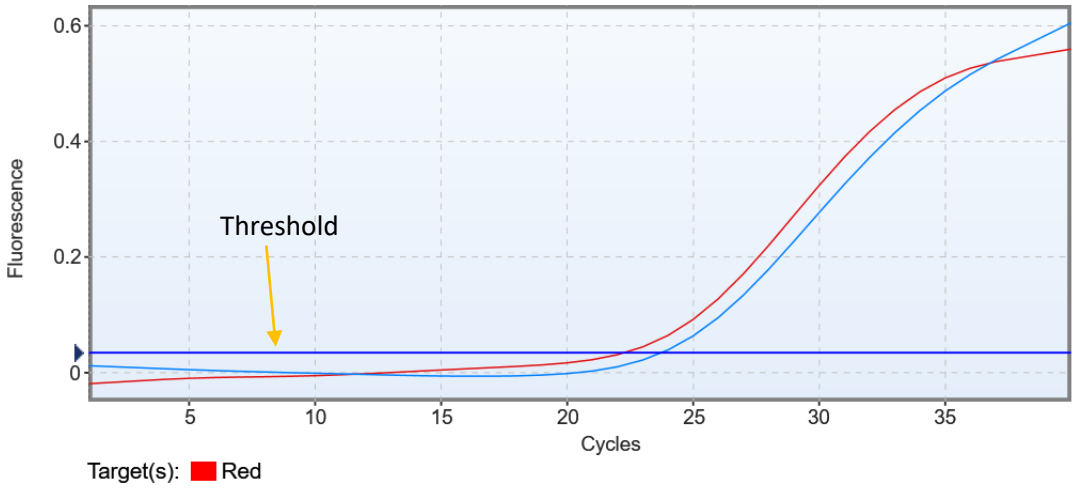
3B.2.3.1. Investigating concentration of exogenous amplification control

Before Bio-T kit® *Mycobacterium avium paratuberculosis* qPCR kit (Biosellal; France) was used to compare against the in-house qPCR, a modification of the instructions for use (IFU) was made. As per Biosellal IFU, exogenous amplification control (IPC) should be added during DNA extraction from faecal samples. To attempt to better align the protocol for the commercial kit with the in-house qPCR where IPC is added during qPCR reaction preparation, first the IFU was modified to determine whether addition of IPC used in Bio-T kit®MAP qPCR kits during preparation of the qPCR reactions had a negative effect on the qPCR detection of IPC. To do this, two different concentrations of IPC were added during the qPCR reaction preparation using MAP DNA samples extracted from whole blood of cattle (section 2.2.3.2) and then processed using the Actiphage Rapid™ assay procedure (section 2.4.4). Either 4.8 µl DNA template and 0.2 µl IPC were added to the qPCR reaction or 4.5 µl DNA template with 0.5 µl IPC was added. Although slightly different volumes of DNA template were used (difference of 0.3 µl between

samples), as this experiment was only interested in the performance of the IPC and fluorescence from the Cy5 acquisition channel that detects IPC, and not detection of target DNA, this difference in volume potentially causing a difference in fluorescence on the FAM acquisition channel for target detection was not an issue. Other than this modification, master mix and cycling parameters were performed as per the manufacturer's instructions (section 2.5.3.2.).

Analysis of the Cy5 data acquisition channel which detects the internal positive control from the Biosellal qPCR kit showed that there was little difference in Cq value between using 0.2 µl of IPC (Cq = 22.30) and using 0.5 µl of IPC (Cq = 23.76) (figure 3B.7), therefore a lower concentration of IPC in the master mix did not have a detrimental effect on detection of internal positive control.

Figure 3B.7 Normalised qPCR data plot for detection of MAP DNA from PBMCs with 0.5 µl or 0.2 µl IPC included in master mix



Normalised data plot of qPCR amplification of MAP DNA with different concentrations of exogenous amplification control (IPC) included within the qPCR master mix. MAP DNA extracted from PBMCs using Ficoll gradient separation and Actiphage Rapid™ assay was used as template in qPCR reactions. Included in the master mix was either 0.2 µl IPC (-) or 0.5 µl IPC (-). qPCR was run for 40 cycles, with RFU after normalisation of data reaching maximum of 0.6 RFU. 0.2 µl IPC gave the earliest detectable signal above threshold levels (—).

3B.2.3.2. Introducing exogenous positive control at different points of the assay

Results from section 3B.2.3.1. demonstrated that IPC could be added to the assay during qPCR reaction preparation without having an inhibitory effect on detection of samples. Therefore, experiments were performed to determine the effect of introducing the IPC at the point of qPCR reaction preparation as opposed to introducing IPC during DNA purification and concentration as stated in the Bio-T kit® MAP IFU.

Due to limited availability of animal blood at time of sampling, the only blood samples available were those that were being used for routine clinical diagnostics. Therefore, bovine whole blood samples (n = 3) were tested in duplicate for this experiment. As these blood samples were clinical samples, their MAP status was unknown, although like the previous section, these experiments are focused on the detection of IPC and the interactions of the internal control with PBMC extracts rather than target sample. Bovine whole blood was processed to extract PBMCs using Ficoll gradient method (section 2.2.3.2.) and Actiphage Rapid™ assay performed (section 2.4.4.). After the 3.5 hr Actiphage Rapid™ assay incubation, 5 µl of IPC was added to one set of samples prior to DNA purification and concentration (section 2.5.1.1.). The second sample set underwent the DNA purification and concentration in the absence of IPC.

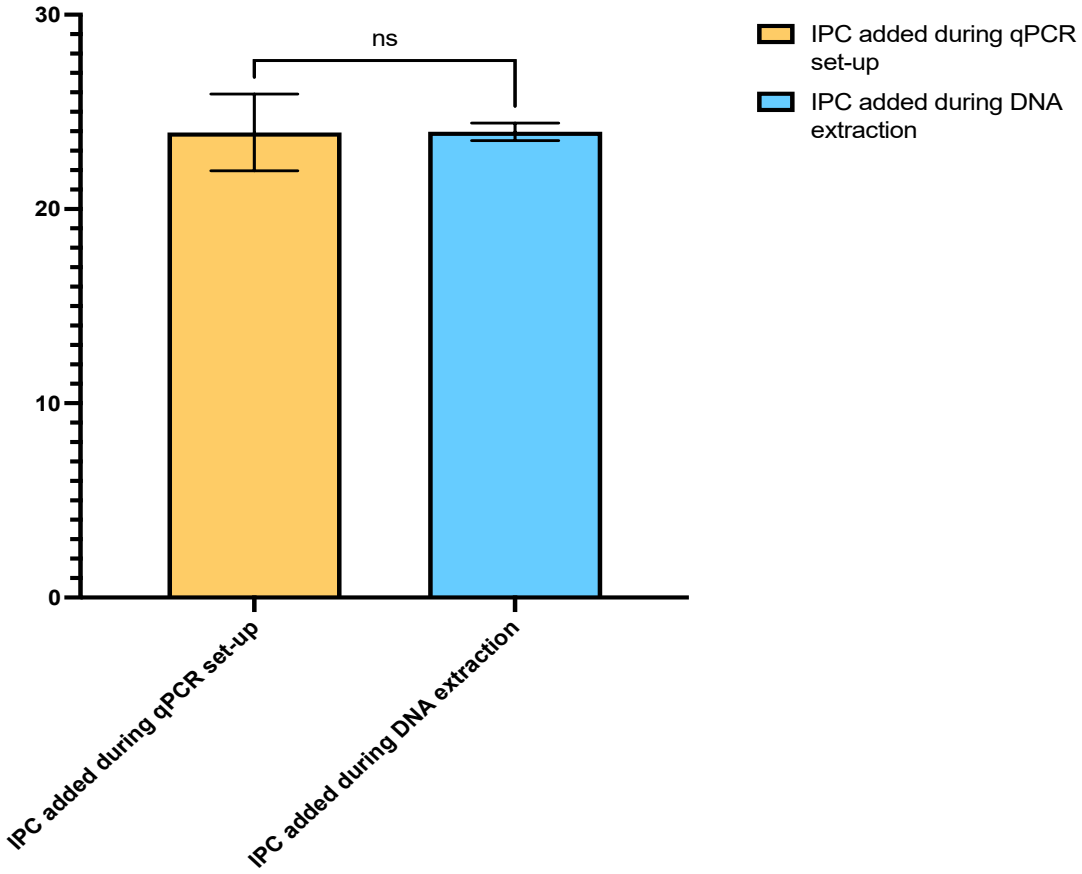
For samples where IPC was not introduced during DNA purification, 0.2 µl IPC was added to 4.8 µl qPCR template and 15 µl master mix in a 0.2 ml PCR tube. For samples where IPC was introduced during DNA purification, 5 µl of template was used with 15 µl of master mix for qPCR. Again, despite a slight difference in

volume of template DNA used (difference of 0.2 µl), the focus of this experiment was on IPC on Cy5 acquisition channel rather than detection of samples on FAM acquisition channel.

Analysis of Cq values for samples on the Cy5 acquisition channel showed the mean Cq value when IPC was added during qPCR reaction preparation was 23.94 (SD = 1.98) in comparison to 23.97 (SD = 0.46) when IPC was added during the DNA purification stage. Paired t test analysis showed that there was no significant difference between detection of IPC when comparing addition of IPC during DNA purification and addition of IPC during qPCR reaction preparation ($p > 0.05$, $p = 0.9803$) (Figure 3B.8).

All three samples where IPC was added prior to DNA purification gave earlier Cq values (mean = 22.67, SD = 0.8529) on the target acquisition channel (FAM) and a signal was detected above the threshold at least 1.58 cycles earlier compared to when IPC was added during qPCR preparation (mean = 24.76, SD = 0.3593). This suggests that although there is no difference in detection of IPC on Cy5 acquisition channel, for target channel detection (FAM), IPC may exhibit some inhibitory effects when added during qPCR reaction preparation. It was decided to use 0.2 µl of IPC added during qPCR reaction set up for future experiments for comparisons against Empirical qPCR kits as there was no difference in amplification of internal control when using a smaller volume or when adding the control during qPCR reaction set up.

Figure 3B.8 Effect on Cy5 data acquisition when IPC was added at different time points during the assay



PBMC extraction and Actiphage Rapid™ assay was performed on three samples of whole bovine blood. Samples were tested in duplicate with IPC being added to one set of samples during DNA purification and IPC being added to the second set during qPCR reaction preparation. Bar graph shows the mean difference in Cq value between samples where IPC was introduced during DNA extraction and purification (•) or during qPCR reaction set-up (•) on Cy5 data acquisition channel.

3B.2.4. Evaluation of Empirical and Biosellal qPCR kits

With methods established for using both the Empirical (Empirical Bioscience; USA) and Biosellal (Biosellal; France) qPCR kits for detection of mycobacteria DNA extracted from Actiphage Rapid™ assay (section 3B.2.1., 3B.2.2. and 3B.2.3.), experiments were next performed to compare the performance of both qPCR kits.

3B.2.4.1. Comparison of qPCR kits by amplification efficiency qPCR assay using serially diluted gDNA

Amplification efficiency qPCR assays were performed using quantified MAP K10 genomic DNA (gDNA) (catalogue number BAA-968D, ATCC; USA) as DNA template to compare qPCR amplification efficiency of these two kits. For these experiments 2-fold and 4-fold serial dilutions were chosen because this generates more data points over the range of concentrations used and so allows a more accurate determination of efficiency than when using 10-fold dilutions of the target DNA. For the 2-fold dilution series, the range of concentrations of gDNA template used for qPCR assays was ≥ 5 x 10^4 copies μl^{-1} to ≥ 1.56 x 10^3 copies μl^{-1}. The experiment was also repeated with 4-fold serially diluted K10 gDNA using the range of concentrations from ≥ 6.25 x 10^3 copies μl^{-1} to ≥ 1.5 copies μl^{-1}.

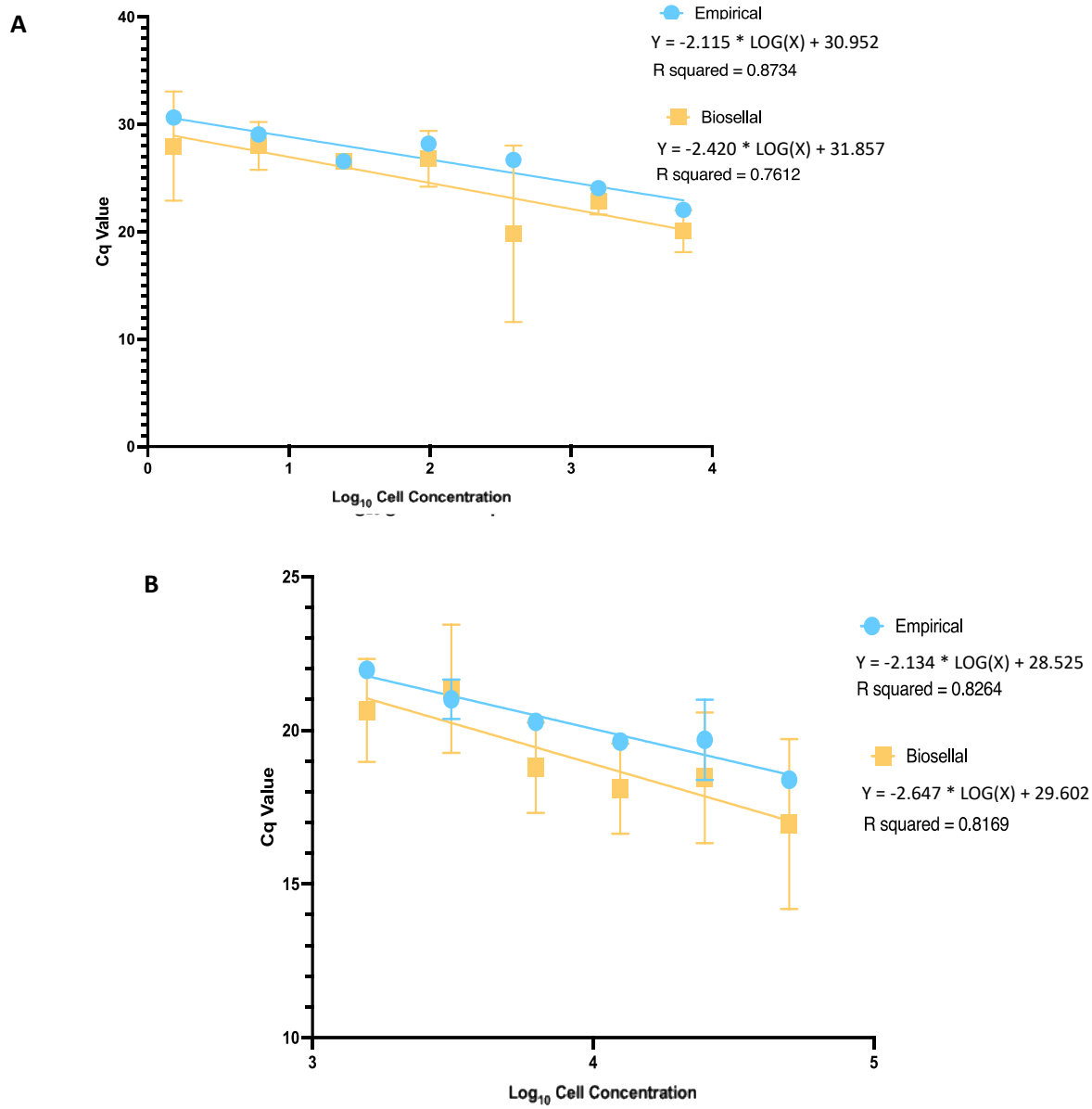
The results of both experiments showed that the Biosellal qPCR had a better amplification efficiency compared to Empirical qPCR kit. Biosellal qPCR efficiency for the 2-fold dilution series was 138.66 % (R^2 = 0.8169) and 158.95 % (R^2 = 0.7612) for the 4-fold dilution series in comparison to 194.17 % (R^2 = 0.8264) for 2-fold dilutions and 197.04 % (R^2 = 0.8734) for 4-fold serially diluted gDNA detected using Empirical qPCR kit (Figure 3B.9). Even though the Biosellal qPCR outperformed the Empirical

qPCR in terms of amplification efficiency, efficiency for all assays was above the threshold required to meet the standards of an efficient qPCR reaction (90-110 % efficiency is the criteria that must be reached). Furthermore, none of the R^2 values were above 0.990 therefore there is a lack of confidence in correlating diluted DNA and Cq value.

Šidák's multiple comparison test for multiple pairwise comparison between detection of gDNA using Biosellal qPCR kit and Empirical qPCR kit at each concentration showed there was no significant difference between the two qPCR kits at each dilution ($p > 0.05$ for every concentration) when 2-fold serially diluted gDNA was used as template (figure 3B.10).

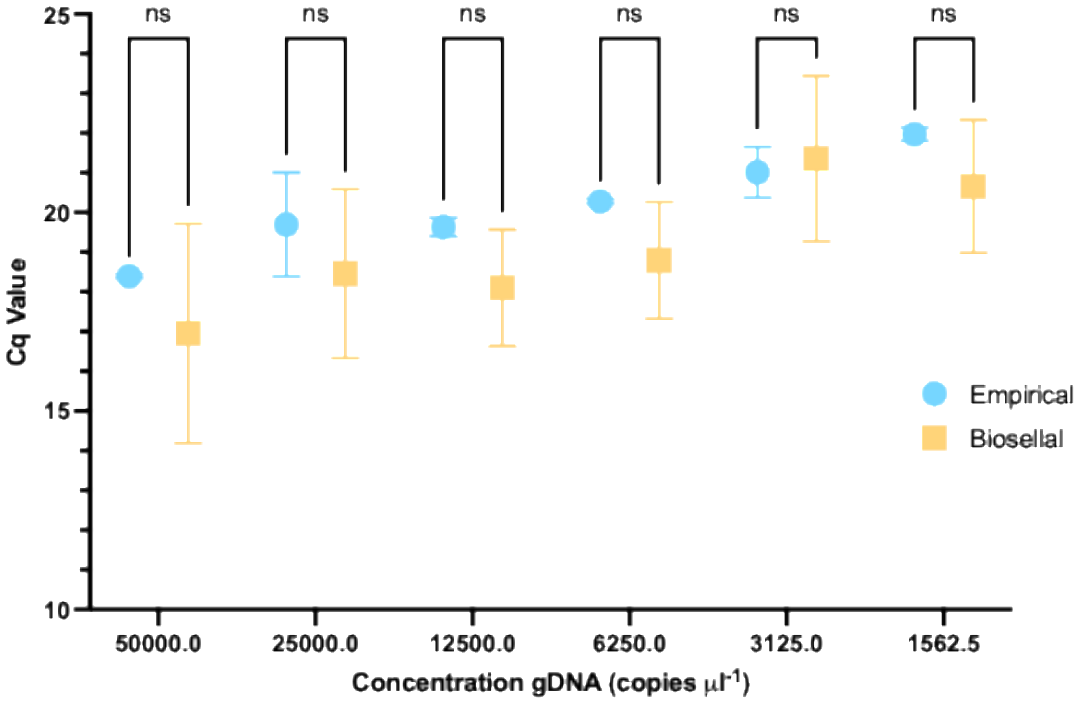
Detection of 4-fold serially diluted MAP gDNA by Empirical qPCR was not tested in duplicate therefore ΔCq analysis was performed instead of Šidák's multiple comparison test. The ΔCq analysis showed that mean ΔCq between dilutions using Empirical qPCR kit was 1.43 cycles (SD = 1.569) and mean ΔCq between dilutions using Biosellal qPCR kit was 1.32 cycles (SD = 3.381). Welch's t test analysis of ΔCq values showed there was no significant difference between the two qPCR kits ($p > 0.05$, $p = 0.9427$) (Figure 3B.11).

Figure 3B.9. Efficiency plots of 4-fold and 2-fold serially diluted MAP gDNA detected using either Biosellal or Empirical qPCR kit



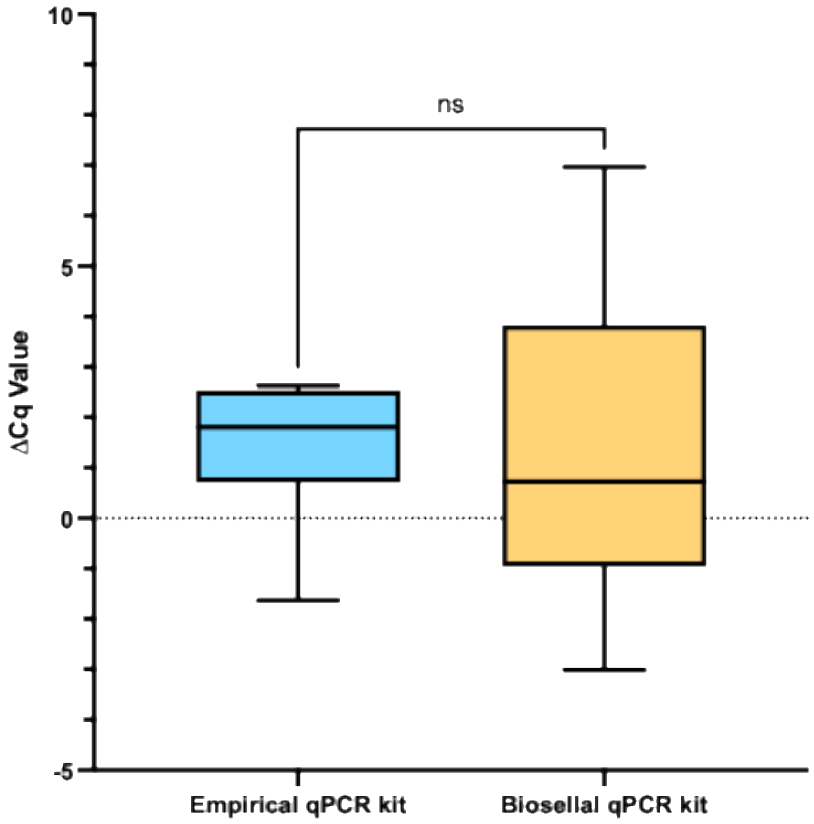
*Efficiency plots of 4-fold (**Panel A**) and 2-fold (**Panel B**) of serially diluted MAP gDNA detected using either Biosellal (■) or Empirical (●) qPCR kit. Genomic copies are plotted on a log_{10} scale on the x axis, whilst Cq value is plotted on the y axis. Gradient (Y) used to calculate qPCR efficiency and R^2 values used for analysis are shown under their respective sample labels.*

Figure 3B.10. Comparison of Cq value for 2-fold serially diluted MAP gDNA detected using either Biosellal or Empirical qPCR kit



Šidák's multiple comparison test was used to compare detection of 2-fold serially diluted MAP gDNA using either Biosellal (■) or Empirical (●) qPCR kit. "ns" indicates no significant difference between the results gained using the two kits at each concentration. Standard deviation error bars are shown for both qPCR kits (⊥).

Figure 3B.11. ΔCq analysis of 4-fold serially diluted MAP gDNA detected by qPCR using either Biosellal or Empirical qPCR kit



Box and Whisker plot shows the range and mean ΔCq values for detection of MAP gDNA using Empirical (■) or Bioselall (■) qPCR kit. Standard deviation error bars are shown for both qPCR kits (⊥). "ns" indicates no significant difference (ns) between ΔCq values for detection using Biosellal or Empirical qPCR kit using Welch's t test.

3B.2.4.2. Comparison of Empirical and Biosellal qPCR kits for detection of MAP from PBMCs

Section 3B.2.4.1. showed that Biosellal and Empirical qPCR kits are comparable and there was no significant difference when being used to detect pure gDNA standards. To determine whether results would also be comparable between the two qPCR kits to aid in the detection of MAP DNA isolated from PBMCs and extracted using Actiphage Rapid™ assay, experiments were performed to compare Empirical qPCR kit to Biosellal qPCR kit using bovine whole blood samples.

A blood sample was collected in a heparin vacutainer by a third-party veterinarian, and PBMCs extracted using both Ficoll-Paque PLUS (section 2.2.3.2.) and ACK (section 2.2.3.1.) methods. These two methods were used because the ACK method had been successfully used when testing the deer blood samples and therefore was included in case issues with sample matrix inhibition were related to the Ficoll method of PBMC purification. The MAP status of the cattle from which the blood originated was not confirmed by PCR prior to experiments, but the animals did not come from a herd known to be infected with MAP, and levels of MAP DNA being added far exceeded the levels detected in naturally infected samples and therefore should not interfere with the assay results. 10^2 MAP cells were prepared as previously mentioned by enumeration using Actiphage® Core assay and diluting to achieve a concentration of 10^3 cells ml^{-1}. 100 µl of MAP cells mixed with PBMC extracts and Actiphage Rapid™ assay was performed in duplicate for each PBMC extraction method. Detection of MAP performed using either Biosellal or Empirical qPCR kit.

No IAC was added to the PCR reaction when using Empirical master mix therefore no Cq values for internal control acquisition channel were generated. On the same qPCR, 10-fold serially diluted MAP DNA was used to create a standard curve. The efficiency of Empirical qPCR reaction was calculated to be 109 % (data not shown) indicating that there was no PCR inhibition. Internal positive control (IPC) was included in the Biosellal master mix, and no inhibition was observed on the internal positive control acquisition channel (Cy5) for the Biosellal kit.

On average, the Cq value for Empirical MAP assay positive control rose above the critical threshold level 3.95 cycles later than the Biosellal MAP positive control. Out of the four samples tested, two produced Cq values using Biosellal kits (Cq 34.15 and Cq 31.43) whereas none of the samples produced Cq values for the Empirical qPCR. Both replicates of Media Plus negative control on the Biosellal qPCR run produced Cq values (Cq 34.39 and Cq 29.90). In comparison, only one of the two Media Plus controls for the Empirical qPCR run had a Cq value (Cq 29.96) (table 3B.5.). This was the same negative control that gave the earliest Cq value when using the Biosellal kit.

Table 3B.5. Comparison of Cq values for the detection of MAP from bovine PBMCs using Actiphage Rapid™ assay and Empirical or Biosellal qPCR kit

	CQ VALUE			
SAMPLE	**Empirical (FAM)**	**Empirical (Cy5)**	**Biosellal (FAM)**	**Biosellal (Cy5)**
10^2 MAP CELLS (PAC)	30.23	n/a	25.61	26.85
10^2 MAP CELLS (PAC)	28.81	n/a	25.52	25.95
MEDIA PLUS (NAC)	ND	n/a	34.39	26.05
MEDIA PLUS (NAC)	29.96	n/a	29.90	26.41
FICOLL EXTRACTION 1	ND	n/a	ND	26.31
FICOLL EXTRACTION 2	ND	n/a	34.15	23.83
ACK EXTRACTION 1	ND	n/a	ND	26.14
ACK EXTRACTION 2	ND	n/a	31.43	28.81

PBMC extraction using ACK and Ficoll-Paque PLUS extraction methods were used to obtain PBMCs from bovine blood. Extraction methods were tested in duplicate. Actiphage Rapid™ assay was performed on extracts. 5 µl of purified DNA was used as template for qPCR reactions using either Empirical or Biosellal qPCR kit. As standard, 10^2 MAP cells were used as the positive Actiphage assay control (PAC) and Media Plus in Actiphage reagent was used as the negative Actiphage assay control (NAC). FAM acquisition channel was used for both qPCR assays for target detection and Cy5 acquisition channel was used for detection of internal control for Biosellal assay. No internal control was used for Empirical qPCR assay (n/a). Samples that did not create a signal above the threshold level were determined to be not detected (ND).

3B.2.5. Investigating Actiphage Rapid™ assay components and their effect on qPCR variability

Although when using purified, concentrated gDNA as template there was no difference between the performance of Empirical and Biosellal qPCR kits (section 3B.2.4.1), when template DNA was extracted from bovine PBMCs there was found to be a difference in the performance of the two qPCR kits (section 3B.2.4.2.). Therefore, Actiphage Rapid™ assay components including Actiphage reagent and PBMC lysis methods were investigated to determine whether components of the Actiphage Rapid™ assay were having any inhibitory effects on the qPCR reactions.

3B.2.5.1. Actiphage® batch variability and effect on detection of mycobacteria by qPCR

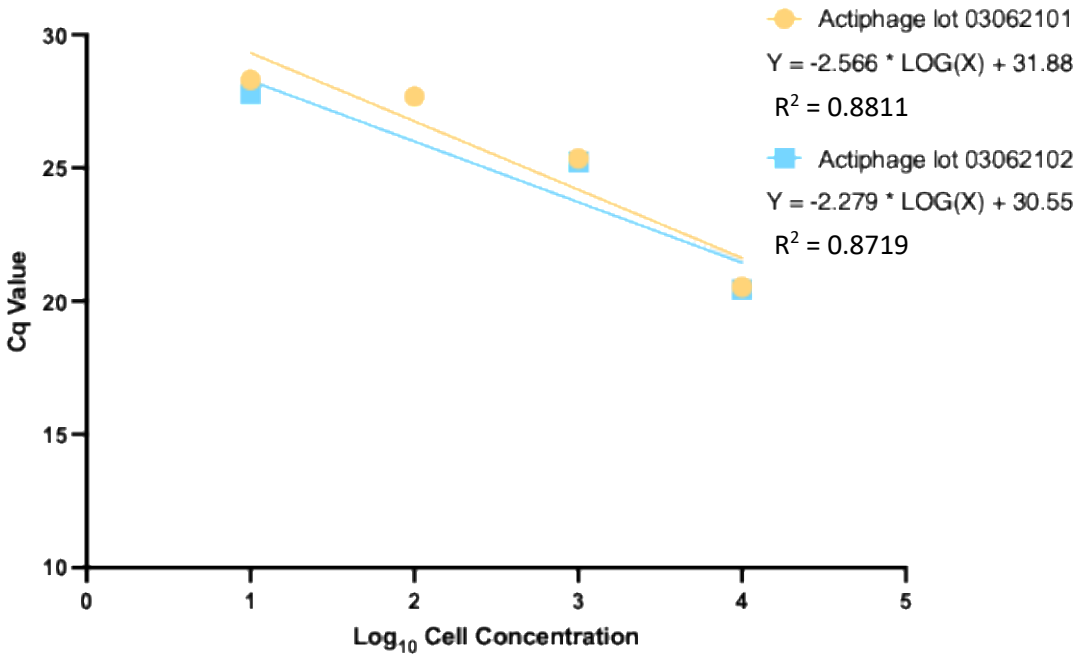
Two different batches of Actiphage® were used in Actiphage Rapid™ assays to determine whether Actiphage® batch variability was having an impact on qPCR detection of mycobacteria. Both batches of Actiphage® (lot number 03062101 and lot number 03062102) were produced as freeze-dried phage by PBD Biotech Ltd. Prior to use in Actiphage Rapid™ assay, *M. bovis* BCG cells were enumerated (section 2.4.6.) using both batches of Actiphage® reagent and cell concentration determined to be 4.3×10^5 cfu ml^{-1} by batch 03062101 and 7.9×10^5 cfu ml^{-1} by batch 03062102, indicating that there may be some small variation in the characteristics of these different batches of product.

These *M. bovis* BCG cells were 10-fold serially diluted from 1×10^5 cfu ml^{-1} down to 1×10^1 cfu ml^{-1} for use in Actiphage Rapid™ assay (section 2.4.4.). After

cleaning and concentrating the mycobacterial DNA, qPCR was performed using Bio-T Kit® MTBC (Biosellal; France) as per section 2.5.3.3.

The results indicated that there were some potential errors during the qPCR preparation of some samples (PCR inhibition detected on Cy5 channel and possible omission of template - samples scored “as not detected”). However, there were sufficient samples remaining to allow an efficiency plot to be generated and qPCR efficiencies were calculated to be 289.7 % for phage lot number 03062101 and 338.8 % for phage lot number 03062102 (Figure 3B.12), both of which were not within the acceptable range of 90-110 %. Nevertheless, when comparing the Cq value at each cell dilution with the two batches of Actiphage®, paired t test analysis shows there was no significant difference between the two batches of Actiphage® ($p > 0.05$, $p = 0.2123$). This suggests that some material in the phage samples could be affecting the qPCR signal.

Figure 3B.12. Efficiency plot of *M. bovis* BCG used in Actiphage Rapid™ assay using different batches of Actiphage®



A culture of M. bovis BCG was enumerated and then 10-fold serially diluted before being used in an Acitphage Rapid™ assay. Two different batches of Actiphage® were tested and M. bovis BCG DNA was detected by Bio-T Kit® MTBC (Biosellal; France). The data sets used to calculate efficiency for batch 03062101 (●) and batch 03062102 (■) are displayed beneath their respective data labels.

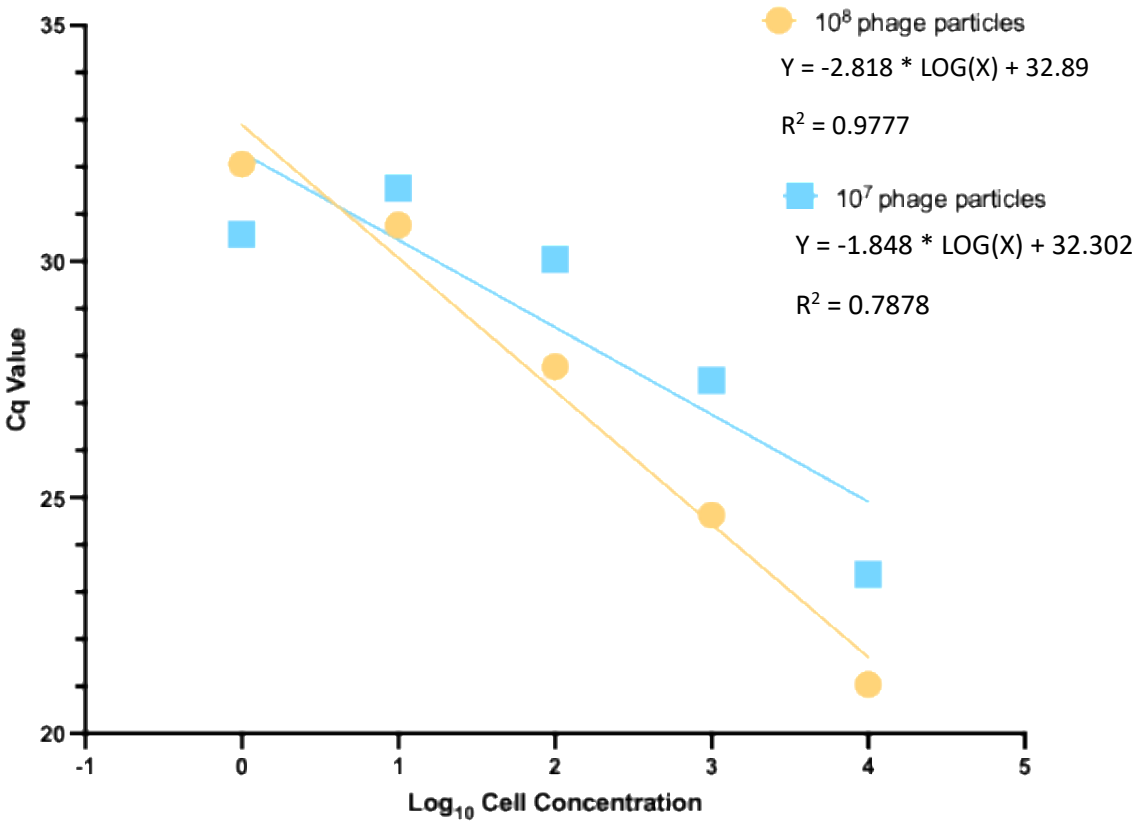
3B.2.5.2. qPCR detection of M. bovis BCG with different Actiphage particles per Actiphage Rapid™ assay

Although different batches of Actiphage® showed to have variability on qPCR reaction efficiency, overall, there was little variability between batches of Actiphage® and their ability to detect *M. bovis* BCG DNA (section 3B.2.5.1). The next step in analysis of Actiphage Rapid™ assay components and their effect on qPCR variability was to determine whether total Actiphage® particles per assay would have an impact on detection of DNA. As a standard 10^7 Actiphage particles are added per Actiphage Rapid™ assay therefore this was compared against adding 10-fold more Actiphage® particles per assay to determine the effect of varying Actiphage® particles per assay on the limit of detection.

Either 10^7 or 10^8 Actiphage® particles were added to ten-fold serially diluted *M. bovis* BCG cells (10^4 cells to 10^0 cells) and Actiphage Rapid™ assays performed (section 2.4.4.). After DNA purification, detection of *M. bovis* BCG DNA was achieved by using Bio-T Kit® MTBC (Biosellal; France).

All efficiency values were calculated to be above the acceptable range (90-110 %). When 10^8 phage particles were added per assay the qPCR efficiency was calculated at 126.0%, compared to 247.6% when 10^7 phage particles were added per assay (Figure 3B.13). When using 10^8 Actiphage particles per assay, *M. bovis* BCG was detected earlier compared to when using 10^7 Actiphage particles per assay. The average ΔCq between 10^8 or 10^7 Actiphage particles per assay was 1.35 cycles.

Figure 3B.13 Efficiency plot comparing qPCR detection of *M. bovis* BCG DNA using different total number of Actiphage® particles per assay



Efficiency plot showing the effect of varying Actiphage® particles per Actiphage Rapid™ assay using the Bio-T Kit MTBC (Biosellal; France) for qPCR detection of M. bovis BCG DNA. Actiphage® reagent was added at 10^8 particles (●) or 10^7 particles (■) per assay. Y values shown for each data are the gradient of the line used to calculate qPCR efficiency.

3B.2.6. Investigating Cq value of negative assay control

When using commercial qPCR kits, it was found that the Actiphage Rapid™ assay negative control (Media Plus) can generate a signal and Cq value. A data analysis method (ΔCq method) was established to overcome this problem when attempting to diagnose MAP infection in deer (Chapter 3A). However, the company wished to understand this better, so experiments were performed to understand the source of this signal and determine whether detection of Media Plus were because of processing errors or if they were false-positive results due to some sort of contamination.

3B.2.6.1. Effect of PBMCs on Media Plus negative control

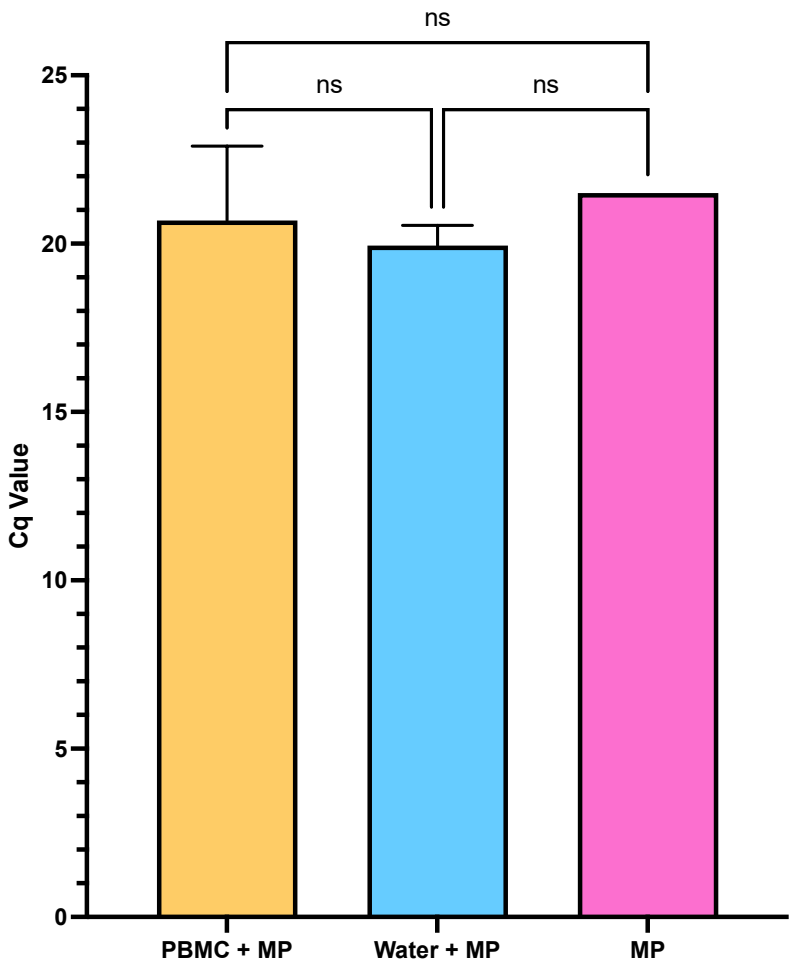
To investigate whether PBMCs had an inhibitory effect on the qPCR detection of Media Plus Actiphage Rapid™ assay negative control, both were combined in a qPCR reaction by using Media Plus samples as the template that had previously been prepared as a negative control for Actiphage Rapid™ assay and used in a qPCR reaction and had given an early Cq value.

PBMCs were extracted from bovine whole blood samples using Ficoll-Paque PLUS (section 2.2.3.2.) and Actiphage Rapid™ assay performed. DNA was purified and screened by qPCR for the presence of any MAP DNA. Any extractions determined to be MAP negative by qPCR were used for experimentation. Briefly, 2.4 µl of DNA from the Media Plus sample was mixed with 2.4 µl of a MAP-negative DNA from the PBMC samples to form the test template. As a control, 2.4 µl Media Plus DNA was added to 2.4 µl sterile water, so the template DNA was diluted to the

same level. All 4.8 µl was used as template for the qPCR reaction using Bio-T Kit® *M. avium paratuberculosis* (Biosellal; France, section 2.5.3.1.).

In the absence of PBMCs, Media Plus Cq value was calculated at Cq 21.51, however in the presence of PBMCs, the detection of Media Plus was earlier (mean Cq 20.69). Despite this, there was no significant difference in the shift of the MP Cq value through the addition of MAP negative template ($p > 0.05$, $p = 0.7554$) (figure 3B.14). This suggests that there were no inhibitors within the extracted and purified DNA from PBMCs eliciting a later Cq value from the MP negative control, nor was there any DNA within the extracted PBMC sample to force a premature detection of Media Plus.

Figure 3B.14 Effect of qPCR detection of Media Plus when spiking into DNA extracted and purified from peripheral blood mononuclear cells



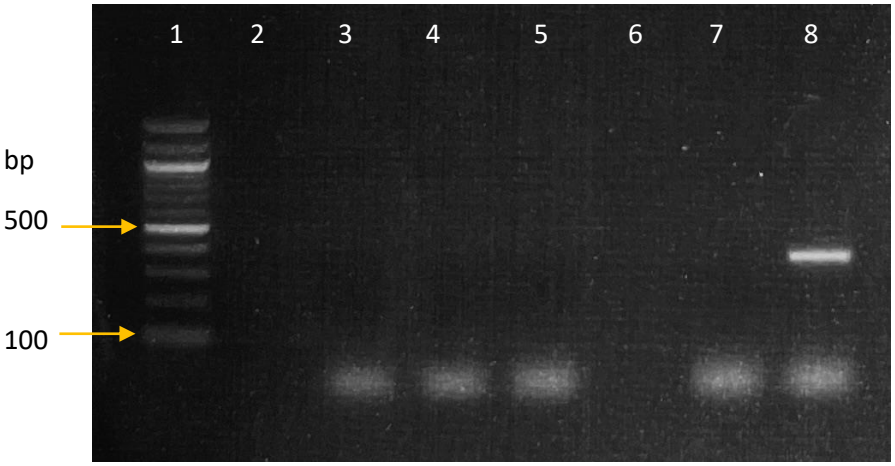
PBMCs were extracted from bovine whole blood using Ficoll-Paque PLUS density gradient and Actiphage Rapid™ assay performed. DNA was purified and screened for the presence of MAP DNA. Any MAP negative samples were mixed 1:1 with Media Plus that had also undergone Actiphage Rapid™ assay (■). As controls, Media Plus was mixed 1:1 with sterile water (■) or Media Plus Actiphage Rapid™ assay negative control was used on its own (■).

4.2.6.2. Endpoint nested PCR of Media Plus negative control

Since mycobacteria-negative PBMCs did not have any effect on the Cq value of Media Plus (section 3B.2.6.1.), it was decided to test whether the positive signal recorded for the Media Plus negative control sample was also detectable by endpoint PCR. As Actiphage® Reagent is propagated using *M. smegmatis* as the host, there will be trace amounts of *M. smegmatis* DNA within the Actiphage® reagent along with the Mycobacteriophage DNA. Therefore, the aim here was to determine whether there is any contaminating DNA contributing the Cq value of the Media Plus in the presence of Actiphage® regent. To do this, the Actiphage Rapid™ assay was performed on Media Plus in the presence and absence of Actiphage® reagent. Nested endpoint PCR was performed on the samples using primers targeting IS*900* from MAP (section 2.5.2.5.). Nested PCR was used as this is more sensitive than standard endpoint PCR and could potentially detect low levels of contamination that might not be detected by standard endpoint PCR.

Analysis of the agarose gel after electrophoresis showed that despite Media Plus giving a positive signal in qPCR reactions (Cq < 30), when performing nested PCR, Media Plus remained MAP DNA negative. There was no detection of a 294 bp MAP band in lanes containing Media Plus without Actiphage® reagent or Media Plus with Actiphage reagent. The positive control of 10^2 MAP cells did show the presence of a 294 bp band as expected (Figure 3B.15).

Figure 3B.15 Nested IS*900* PCR of Media Plus in the presence or absence of Actiphage® reagent



Actiphage Rapid™ assay and DNA purification were performed on Media Plus in the presence of absence of Actiphage® reagent. Nested IS900 PCR was performed on DNA purification eluates to determine if there were any contamination of the control that caused detection by qPCR. The presence of a 294 bp band indicates the detection of MAP IS900 within the sample. L-R: Lane 1: 100 bp molecular marker; Lane 2: Media Plus without Actiphage; Lane 3: Media Plus without Actiphage; Lane 4: Media Plus with Actiphage; Lane 5: Media Plus with Actiphage; Lane 6: gap; Lane 7: Water (NTC); Lane 8: MAP 10^2 cells.

3B.2.7. Applications of Actiphage Rapid™ assay to aid in detection of MAP from farmed deer

Chapter 3 (part A and part B) has shown that Actiphage Rapid™ assay and Bio-T® *M avium paratuberculosis* qPCR kit can be used to detect MAP from the blood of farmed deer whilst optimisation of both in-house qPCR (master mix from Empirical Bioscience; USA) and a commercially available qPCR kit (Biosellal; France) has allowed for detection of MAP from PBMCs of cows using Actiphage Rapid™ assay. Therefore, experiments were performed to compare the in-house qPCR to the commercially available kit to determine if a sensitive detection of MAP could be achieved from the blood of non-bovine ruminants.

3B.2.7.1. Comparison of qPCR to detect mycobacteria from PBMCs extracted from farmed deer

The blood of farmed deer was used to compare the use of Actiphage Rapid™ assay and either an in-house qPCR (section 3B.2.1.) and Bio-T® qPCR kit for the detection of MAP.

Peripheral blood mononuclear cells from 103 individual farmed deer were isolated using ACK methods section 2.2.3.1. and mycobacterial DNA extracted using Actiphage Rapid™ assay (section 2.4.4.) and purified as per section 2.5.1.1. All 103 samples were subject to qPCR using Bio-T® *Mycobacterium avium paratuberculosis* qPCR kit (Biosellal; France). For in-house qPCR, nine samples were chosen based off their result from the Bio-T® *M avium paratuberculosis* qPCR.

There was no evidence of qPCR inhibition when using the Bio-T® qPCR kit as amplification was observed for all samples on the Cy5 acquisition channel which

measures for amplification of the internal positive control. ΔCq analysis of qPCR results was implemented and each deer was categorised into one of three groups: strong MAP positive, weak MAP positive or MAP negative. A positive sample was determined to be any sample that produced a Cq value lower than that of the Media Plus. A sample would be classified as a strong positive if ΔCq > 2. Negative samples were those that had a Cq value higher than the Media Plus negative control.

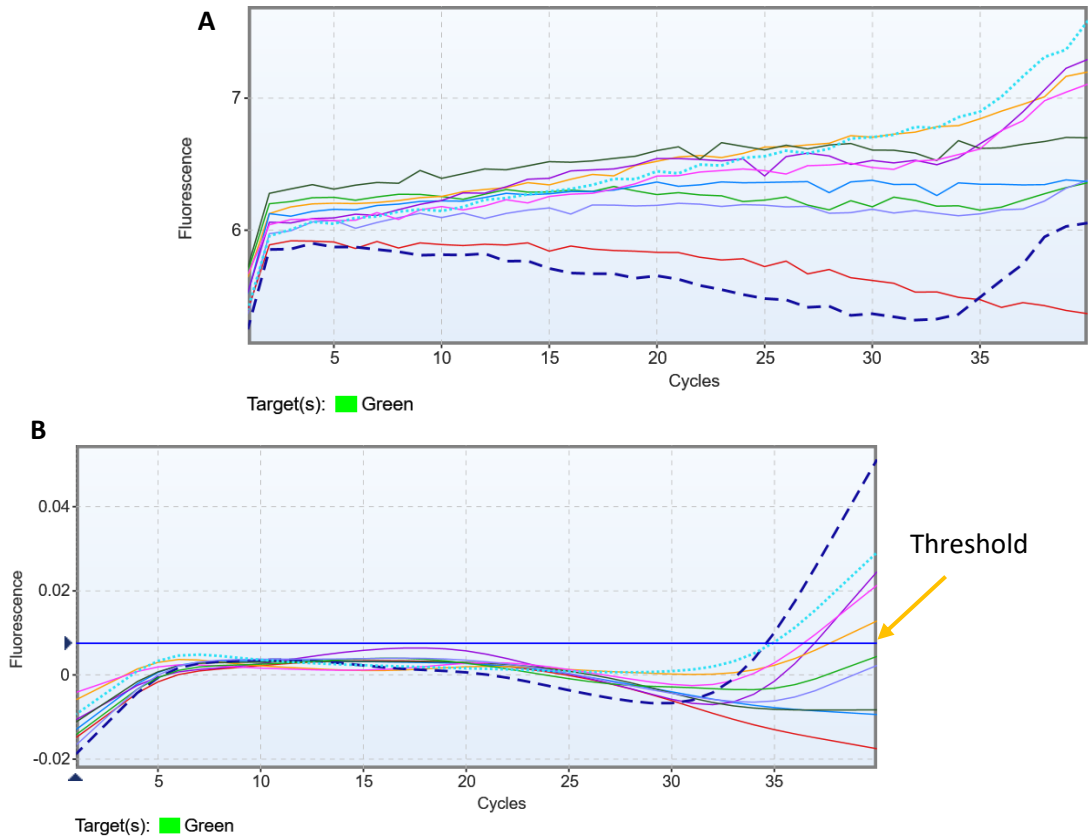
From the 103 deer samples tested, one sample tested as strong positive (ΔCq = 3.83), 34 samples were weak positive (Cq earlier than Media Plus but had a ΔCq < 2) and 68 samples were negative. Nine samples were selected to be used to compare detection of MAP using Empirical qPCR kit. One sample classed as strong MAP positive, four samples classed as weak MAP positive, and four samples categorised as MAP negative were randomly selected.

Amplification of all samples and both the positive and negative control was not well defined when using Empirical qPCR kit through analysis of amplification plots using Q-Rex software. There was very little amplification through analysis of the raw data (figure 3B.16.). As no IAC internal control was added to the Empirical qPCR reaction it cannot be determined if there were any qPCR inhibition through analysis of qPCR results.

Furthermore, all Cq values recorded from the Empirical qPCR were late signals (later than cycle 34 of 40). Sample 102, that was determined to be a strong positive result when using Biosellal qPCR kit (Cq 26.85), was determined to be negative by the Empirical kit (Cq not detected). Samples (ID 42, 56 and 97)

produced Cq values greater than the Cq value of the Media Plus when using Empirical qPCR (table 3B.6.).

Figure 3B.16. qPCR amplification plots of samples using in-house qPCR kit



*Raw (**panel A**) and normalised (**panel B**) data amplification plot of Actiphage Rapid™ assay samples extracted from PBMCs of deer and detected using an in-house qPCR (Empirical Bioscience; USA). Target detection used the FAM fluorophore which is detectable on the green channel using RotorGene-Q. MAP DNA was used as a positive control (---) and Media Plus used as a negative control (...). Poor amplification of samples and low levels of fluorescence can be seen for all samples and controls. MP negative control also gave detectable similar to that of the positive control.*

Table 3B.6. Detection of MAP from farmed deer using either a commercial or in-house qPCR kit

SAMPLE ID	CQ BIOSELLAL (FAM)	CQ EMPIRICAL (FAM)	RESULTS BASED ON BIOSELLAL QPCR
2	ND	ND	Negative
3	ND	ND	Negative
41	30.29	ND	Weak positive
42	29.84	36.96	Weak positive
56	30.38	37.70	Weak positive
57	30.26	ND	Weak positive
97	31.32	36.43	Negative
98	30.71	ND	Negative
102	26.85	ND	Positive
MEDIA PLUS	30.68	34.92	Negative Control
MAP 10^{-2} DILUTION	24.61	34.62	Positive Control
WATER	ND	ND	Non-template Control

103 farmed deer were tested using Actiphage Rapid™ assay and a commercially available qPCR kit (Bio-T Mycobacterium avium paratuberculosis). After analysis of qPCR results it was concluded that there was 1 strong positive animal (ΔCq > 2), 34

weak positive (ΔCq < 2) and 68 negative animals (Cq value higher than MP). Nine animals were selected to compare qPCR results to an in-house qPCR where primer sequences were known to have good homology to MAP strains isolated from non-bovine hosts. Cq values for both qPCR assays are shown in the table above. Samples and controls that did not produce any fluorescent signal that rose above threshold levels remained not-detected (ND).

3B.3. DISCUSSION

The work described in this chapter was aimed to try and optimise qPCR methods to compliment detection of mycobacteria by the Actiphage Rapid™ assay. When developing a diagnostic kit many factors need to be taken into consideration, including probe concentration and target, inclusion of internal amplification controls and Actiphage batch variability. These factors need to be acknowledged, and work done to attempt to reduce the risk of these affecting the diagnostic test. There was variable success when designing and using novel qPCR kits for the detection of MAP and MTBC when these kits were compared to those that are currently available from commercial companies.

3B.3.1. Design and performance of an in-house qPCR kit for detection of Mycobacteria

Whilst attempts were made to create a qPCR kit in-house to aid in sensitive detection of MAP and MTBC (section 3B.2.1. and 3B.2.2.), more work needs to be done before such a kit is capable of rivalling sensitivity of commercial kits already available.

There was success in using the IS900RLP probe developed by Donaghy *et al.* (2011) to detect MAP DNA after probe concentration optimisation. Even though many companies suggest 0.25 µM to be the optimal concentration of probe for use in qPCR (QuantaBio, 2023), the experiments carried out as part of this study found there was little difference in detection of MAP comparing the two concentrations of probe. Despite the IS900RLP probe being able to successfully detect MAP, optimal probe concentration was determined using purified MAP DNA (section 3B.2.1.1.),

however when using the same qPCR assay for detection of MAP cells spiked into PBMCs and detection of MAP from PBMCs extracted from deer samples, overall fluorescence from the qPCR detection was poor (section 3B.2.1.4.). This lack of fluorescence observed could simply be down to repeated freeze-thaw of fluorescent probe causing degradation and reduced performance activity. Alternatively, inhibitors present in the sample could have also contributed to this low signal from blood samples. Heme, lactoferrin, nitrocellulose, ethanol, heparin and immunoglobulin G (IgG) are all known qPCR inhibitors that can either release iron ions that compete with DNA template, precipitate DNA or bind to DNA (Hedman and Rådström, 2013). All these inhibitors appear at some point in the Actiphage Rapid™ assay between blood collection (heparin, heme, lactoferrin), PBMC extraction (IgG) or DNA purification (nitrocellulose, ethanol). Through binding, precipitating or competing with DNA within the qPCR reactions this would leave qPCR probe and/or primers that is unable to bind to target DNA, in turn causing lower fluorescence compared to when purified DNA is used as template. Further experiments can be performed to determine the extent these potential inhibitors are impacting the qPCR reactions, including attempted removal such as using an ion chelating resin to eliminate some potential contaminants.

Another issue that is important to note with the use of IS900RLP probe is that the annealing temperature for this probe does not align with the requisites of a good probe as outlined by Rodriguez *et al.* (2015). An important requisite for probe suitability in a qPCR reaction is that the annealing temperature of the probe needs to be between 8 and 10 °C higher than that of the primers (Wittwer *et al.*, 1997). Designing qPCR assays with dual-labelled probes requires careful coordination of

primer T_m, however, in the case of the IS900RLP probe and the P90/91 primer pair, the probe has an annealing temperature that is 6 °C lower than the primers. During the denaturation step of a qPCR cycle, the temperature is increased to over 90 °C to dissociate any double-stranded DNA, then for primers and probes to anneal to the target template, the temperature is reduced to anywhere between 50 °C and 70 °C until the desired primer annealing temperature is achieved. Amplification and extension of the target DNA starts as soon as primers bind to the sequence, therefore, if the probe binds to the target at the same time, or after the primers bind, the polymerase may begin replication of target that does not contain bound probe. As a result, new DNA will be synthesized without associated probe degradation and, therefore, will not be detected as an increase in fluorescence. As the IS900RLP probe used in the current qPCR experiments did not meet this requirement, more work would need to be performed with other probes, preferentially those with an annealing temperature 8-10 °C higher than that of the primers to determine if overall, this influenced the current experiments.

Despite initial experiments highlighting the potential of using IS900RLP probe in qPCR assays with P90/P91 primer pair, more work needs to be performed to be fully satisfied that this probe provides optimal detection of mycobacterial DNA sequences from biological samples. There are number of published primer and probe combinations for the detection of MAP targeting both the multicopy IS*900* insertion element and single copy gene targets such as *F57* (Donaghy *et al.*, 2011, Imirzalioglu *et al.*, 2011, Acharya *et al.*, 2017). These published primer and probe sets can be explored as potential candidates for the MAP qPCR to see if an improved sensitivity in the detection of MAP can be achieved.

Whilst probe choice may have been a problem with the detection of MAP using the in-house qPCR, the P90/P91 primer pair which was used presented potential issues. Whilst primers can vary in length between 15 and 30 bases, longer primers can potentially provide greater specificity, although this comes with a cost as these longer primers can also cause a decrease in PCR efficiency. This is especially found when using environmental DNA samples, thus leading to a reduction in yield and quality of PCR product (Lim *et al.*, 2011). As both the P90 forward and P91 reverse primer have a length of 27 bp, they are both at the upper end of the desirable primer length, therefore, there could be issues in decreased PCR efficiency.

Furthermore, at the time when this primer pair was designed, the PCR method used was endpoint PCR where PCR products of 413 bp in length were acceptable. However, for qPCR, shorter PCR products with a maximum amplicon length of 150 bp are recommended (Thornton and Basu, 2011). Larger amplicons than 150 bp are more susceptible to the formation of secondary structures (Toouli *et al.*, 2000). Moreover, smaller amplicons are more desirable compared to those over 150 bp in length as they are more likely to denature fully during qPCR denaturation cycles, thus allowing the primers and probe to anneal to their complementary target sequences more effectively. As the P90/91 primer pair creates a 413 bp product, there is a risk that qPCR sensitivity can be lost. Whilst this factor has not been investigated in the current research by comparison of the detection of MAP using a P90/91 primer pair and primer pair specifically designed for qPCR use, evidence has been produced to support the idea that using the P90/91 and IS900RLP combination is less effective at detection of MAP than a

commercially available MAP qPCR kit produced by Biosellal (France) (section 4.2.4.2.).

Although a loss of sensitivity was not tested with MAP based qPCR, two different primer concentrations were tested with the MTBC qPCR assay to determine if sensitivity could be increased. Although the higher concentration of primers (1 µM final concentration) had better detection of samples, this assay was unable to detect the positive control cells. In contrast, the lower concentration of 0.5 µM primers detected the positive control but not the samples. There was consistent amplification of the internal control with all samples having the IAC amplifying within 0.45 cycles of each other when using 0.5 µM primers. When the primer concentration was increased to 1 µM, the sensitivity of target DNA improved however a later signal for the IAC was also produced. As the IAC was added to the assay in a competitive format, the internal control may be outcompeting the target DNA for the primers, resulting in MTBC not being detected when lower primer concentrations were being used.

Although increasing the primer concentration to a final concentration of 1 µM increased the sensitivity of the qPCR for target detection, an opposite effect was observed with the PCR positive control. The PCR positive control was not detected when a final concentration of 1 µM primers was used, however a Cq value of 20.90 for the PCR positive control was observed when a final concentration of 0.5 µM was used. This discrepancy in results, in particular the positive control, that should have a similar Cq value every time, demonstrates the unreliability of this specific result and therefore this result alone cannot be used to draw conclusions as

to whether increasing the primer concentration can improve detection of mycobacteria using the in-house qPCR kit.

Another important feature of a successful qPCR kit is the incorporation of an internal amplification control, which allows the user to monitor for inhibition. This is particularly essential when the qPCR is being used as a diagnostic kit as the bacterial DNA that is being detected has been extracted from a sample that can potentially contain a lot of biological, or chemical, components capable of contributing to PCR inhibition. In the design of the MAP qPCR for the current research, IAC was incorporated in a non-competitive format despite the fact Hoorfar *et al.* (2004) recommend competitive IAC, as this way reduces the risk of unwanted interactions between two different primer pairs and two different molecular probes.

It was found that the qPCR detection of MAP DNA extracted via Actiphage Rapid™ assay performed more efficiently when there was no IAC in the reaction (section 3B.2.1.2.). As the synthetic IAC construct contains primer sites for both IS*6110* from MTBC and IS*900* from MAP, the IAC can used in either a competitive or non-competitive format. For the detection of MAP, a non-competitive format was followed, with the inclusion of the T4/T5 primer pair which detect IS*6110* in the master mix. Despite this, there may have been some competitive binding of the target sequence primers (P90/P91 primer pair) to the IAC construct. As a result, there would have been fewer free primers available in the reaction master mix to anneal to MAP template DNA, thus causing MAP to be detected 2.05 cycles later when IAC was in included within the qPCR. Consequently, this reduced the

sensitivity of the qPCR assay. Another potential cause as to why MAP was detected at a later cycle when IAC was included in the master mix, is because as there were two primer pairs in the reaction, there is a risk of dimerization between the two pairs. Such structures would result in fewer free primers to anneal to their target, therefore reducing sensitivity of the PCR. This demonstrates that further optimisation is required to obtain sensitive detection of the target DNA where a similar limit of detection can be achieved irrespective of whether IAC is included in the reaction.

Despite guideline recommendations to add IAC in a competitive format, issues also arose when a competitive format was used for the detection of MTBC. Efficiency of qPCR reactions was far better in the absence of IAC DNA (101.8 %) compared to in the presence of IAC DNA (282 %), suggesting that there may be competition between target DNA and IAC DNA for primers. IAC DNA was not quantified before use in qPCR assays therefore it cannot be determined if there were a much greater concentration of IAC DNA added to the reactions. If this were to be the case, then it would be expected that IAC DNA would be outcompeting the target DNA.

Hoorfar *et al.* (2004) set out a list of ten recommendations for optimal IAC use within diagnostic PCR (table 3B.7). Of the ten recommendations listed, six were confidently met within the current experiments. The four recommendations that were not met are highlighted in the table in grey. Further optimisation of the qPCR needs to take into consideration those criteria that were not met. For example, quantifying the IAC control and ensuring that a low enough concentration is added

so as to reduce the risk of interfering with the target DNA and primers, however in a great enough concentration that IAC is consistently detectable within all samples.

As IAC is an important component of a diagnostic PCR assay, there needs to be more confidence in the qPCR assay when IAC is included in the master mix, before the in-house qPCR can be used alongside the Actiphage Rapid™ assay to detect MAP from clinical samples.

Despite the fact that IAC is essential in qPCR as an indicator of inhibition, IAC is not sufficient to conclude qPCR failure. Other factors can contribute to the failure of the qPCR in detecting positive clinical samples including detection limit, qPCR assay sensitivity and effective extraction efficiency (Lubeck *et al.*, 2003). This is particularly critical when attempting to detect subclinical cases, where minimal levels of bacteria may be present in the sample. Therefore, even if IAC was working at the optimal levels, there are various other factors that need to be acknowledged to attain efficient and sensitive detection.

Table 3B.7 Considerations when designing an internal amplification control for qPCR

RECOMMENDATION NUMBER	RECOMMENDATION
1	Target DNA and IAC used in competitive format to reduce risk of interactions between two primer pairs
2	IAC amplicon should be easily distinguishable from the target DNA amplicon
3	Amplification efficiencies of target and IAC do not need to be identical
4	IAC should be plasmid DNA or purified PCR product
5	IAC to be detectable by specific hybridization probe
6	Only use highly purified template
7	Concentration of IAC should be predetermined
8	IAC added to a master mix and aliquoted to ensure equal distribution into all tubes
9	Polyallomer tubes used for diluting IAC DNA and stored at high concentrations (>10^3 copies)
10	Amount of IAC used in each qPCR reaction should be as low as possible whilst also consistently eliciting a signal throughout the amplification

Taken from Hoorfar *et al.* (2004). Ten recommendations to creating an optimal qPCR assay that includes an internal amplification control. Recommendations not used in this work are highlighted in grey.

3B.3.2. Evaluation of Empirical and Biosellal qPCR kits

Overall, the qPCR kit designed by Empirical Bioscience still requires further optimisation before the kit can be comparable to the commercially available Biosellal qPCR kits for detection of MAP and MTBC. When investigating reaction efficiency, the Biosellal kits outperformed the Empirical kit, irrespective of whether the target was MAP or MTBC. Despite this, every qPCR assay with diluted MTBC and MAP gDNA had a qPCR efficiency that was above the acceptable range of 90-110 %. Although this high qPCR efficiency for all samples was not investigated, high efficiency is often attributed to pipetting errors, either through pipette calibration or end user errors whilst performing serial dilutions, or contamination (ThermoFisher). Despite this, statistical analysis showed there was no difference in the performance of both qPCR kits in their detection of mycobacterial DNA.

When a comparison of the Empirical qPCR kit was performed against the commercially available kit by Biosellal for detection of mycobacteria from clinical samples, it was shown that the Cq value of samples when using the Biosellal kit were lower in comparison to their in-house kit counterparts. Cq value when using Empirical qPCR kit was 3.95 cycles later than Biosellal on average, the biological significance of which is that Empirical qPCR kit is detecting approximately 10-fold less cells. The Empirical qPCR kit did not detect any of the clinical samples tested, however, the Biosellal kit detected 2 samples, suggesting a greater sensitivity in the detection of MAP from PBMCs. As the samples were from animals of unknown disease status, it was unpredictable how late detection of mycobacteria would be, however, a larger set of samples, including from animals of known disease status,

would allow for a greater understanding of the sensitivity of both qPCR assays and allow for a better comparison to be drawn.

3B.3.3. Actiphage Rapid™ assay components and their effect on qPCR variability

Different components of the Actiphage Rapid™ assay were investigated to determine which, if any, components would contribute to variability in detection of mycobacteria. Although experiments showed that different batches of Actiphage® did not have a significant impact on qPCR detection of mycobacteria, only 10^7 and 10^8 Actiphage particles per assay were tested. Experiments were not performed to further decrease the total number of Actiphage® particles per assay, although it can be assumed that this would have a detrimental effect as with lower number of phage particles a lower probability of these phage attaching to mycobacterial cells would result. Nevertheless, this work shows that although Actiphage® reagent is enumerated by phage titre as per quality control and should contain 10^9 Actiphage® particles per vial as standard, even when only 10^7 Actiphage® particles are added per assay this does not impact performance of the Actiphage Rapid™ assay. This allows for some flexibility in human error when propagating and enumerating Actiphage reagent and gives some confidence that despite simple errors Actiphage Rapid™ assay will still perform correctly with either 10^7 or 10^8 Actiphage® particles per assay.

As also seen in Chapter 3, Media Plus negative Actiphage Rapid™ assay control sporadically gives a signal above the threshold in qPCR assays. The ΔCq method established in Chapter 3 is still required to be used for analysis of samples. Further investigations in section 3B.2.6. showed that PBMCs were not causing any

off-target effects eliciting a false-positive single for the negative control, nor was contamination always an issue, as shown by negative nested PCR assays. The evidence indicates that whilst PBMCs are not contributing to these Cq values, there may be other components of the Actiphage assay causing unspecific binding and unwanted fluorescence from the negative control.

In summary:

1. The newly developed in-house qPCR assays for detection of MAP and MTBC show promise but require further optimisation to match the performance and sensitivity of commercial kits
2. There were complications in incorporating an internal amplification control both in a competitive and non-competitive format
3. Variations in Actiphage Rapid™ components did not significantly affect qPCR performance indicating robustness
4. Occasional false-positive signals in negative controls suggest potential non-specific fluorescence or interference from Actiphage Rapid™ assay components, warranting further investigation

CHAPTER 4

INVESTIGATING IS*900* BASED qPCR METHODS FOR DETECTION OF MAP FROM PBMCS OF CAPRINE CLINICAL BLOOD SAMPLES

4.1 INTRODUCTION

Chapter 3 explored whether the Actiphage Rapid™ assay could be used with qPCR for detection of mycobacteria from clinical samples. Whilst it was found that qPCR was compatible with the Actiphage® assay, there was a need for ΔCq analysis methods to be implemented due to fluorescent signals from the Media Plus negative control. Chapter 3B aimed to analyse and optimise an in-house qPCR kit that could produce similar results to that of the commercially available kit used for the analysis of the deer blood samples. Although there was partial success in creating this in-house qPCR, there is still further optimisation to be done before this can be a part of the diagnostic kit. The majority of optimisation in Chapter 3 lacked the use of clinical samples therefore, it remains under-explored whether this in-house qPCR kit can be as successful in detecting MAP from naturally infected, non-bovine animals such as goat.

Goats are part of the family Bovidae and subfamily Caprinae, and like deer, as used in Chapter 3A, are non-bovine animals susceptible to Johne's disease. Disease is more difficult to spot in goats compared to in cattle with symptoms including weight loss, occasionally lumpy faeces and poor milk yields as the disease advances (USDA, 2024). MAP can be found in the raw milk of goats, causing a potential risk to public health. However, a meta-analysis on prevalence and diagnostic tests adopted for detection of MAP in goats found that overall MAP infection prevalence was determined to be 8.24 % (de Lacerda Roberto *et al.*, 2021), although detection of MAP was by ELISA and PCR based methods which can often lead to underreporting of the disease.

Kubala *et al.* (2021) have previously shown that Actiphage Rapid™ assay can successfully be used to isolate MAP positive breeding groups within herds of farmed deer. This, along with results from Chapter 3, show that Actiphage Rapid™ can be used for detection of MAP from non-bovine species using a commercially available qPCR kit (Biosellal; France), however sensitivity and specificity of the assay with the in-house qPCR kit (Empirical Bioscience; USA) remains underexplored. The aim of the work described in this chapter was to determine whether sensitivity and specificity of qPCR assays used to aid in detection of MAP from the blood of goats, can be improved using primers with known homology to IS*900,* in addition to using analysis methods implemented in Chapter 3 and to determine whether the in-house qPCR kit can be comparable to the commercially available qPCR kit when using clinical samples.

Aims of this chapter are as follows:

1. Determine sensitivity and specificity of qPCR assays for detection of MAP from goat blood
2. Determine whether in-house qPCR assays are comparable to commercially available qPCR kits

4.2 RESULTS

4.2.1. Detection of MAP from caprine PBMCs comparing two different PBMC extraction methods

Whilst detection of MAP from deer using Actiphage Rapid™ assay and qPCR has already been demonstrated (section 3A.2.3.), detection of MAP from goats using the Actiphage Rapid™ assay and qPCR remains unexplored. Experiments were performed to determine whether a sensitive detection of MAP from goats could be achieved using the Bio-T Kit® *M. avium paratuberculosis*. In Chapter 3B, Actiphage Rapid™ assay components were investigated to determine their effect on the detection of mycobacteria from whole blood samples, however the effect of the white blood cell extraction method was not investigated. Therefore, to determine whether PBMC extraction methods also affected qPCR sensitivity for detection of MAP from goats, PBMC lysis using either ACK (section 2.2.3.1.) or Ficoll-Paque PLUS (section 2.2.3.2.) were also investigated.

Blood samples from goats were obtained from an independent third-party veterinary practice. All animals had previously been tested using ELISA and were assigned a laboratory ID from 1 to 41. PBMCs were isolated from blood samples using either a Ficoll-Paque PLUS extraction method (lab ID 1-15 only) (section 2.2.3.2.) or an ACK extraction method (all samples) (section 2.2.3.1.). All samples were subject to qPCR using Bio-T® Kit *Mycobacterium avium paratuberculosis* qPCR kit (Biosellal; France) (section 2.5.3.2.). There were no evident signs of inhibition through analysis of Cy5 internal control acquisition channel for either set of samples.

Although Media Plus negative controls produced a signal above threshold level (Cq = 29.66 ± 0.615), when the qPCR products were resolved on an agarose gel, again there were no signs of contamination due to an absence of band at ~320 bp (data not shown). Therefore, based on the conclusions made in Chapter 3A, ΔCq analysis was implemented to compensate for any Media Plus negative control that creates a signal above the threshold level. Any sample with a ΔCq ≥ 2 cycles before the negative control was determined to be a positive result. From the 15 samples tested with both PBMC extraction methods, 33.3 % of animals tested MAP positive using ACK PBMC extraction method, compared to 80 % of animals using the Ficoll PBMC extraction method. There was a 33.3 % agreement rate between the two different extraction methods for MAP positive animals (Table 4.1).

Paired t test analysis shows there was a significant difference in ΔCq value between detection of MAP from cells using ACK PBMC extraction compared to Ficoll PBMC extraction ($p < 0.05$, $p = 0.0049$). All samples that tested negative with Ficoll also tested negative with ACK, however not all animals that tested MAP positive using Ficoll PBMC extraction were detected when ACK was used as the PBMC extraction method.

Despite the lower efficacy of ACK extraction method to detect MAP from whole caprine blood in comparison to Ficoll based methods with the first 15 samples, when ACK was used as the chosen PBMC extraction method for samples 16-41, only one sample (ID 37) tested negative after applying ΔCq analysis. However, as Ficoll PBMC extraction methods were not performed on samples 16-41 comparisons could not be made with the results from the ACK PBMC extraction.

Table 4.1. qPCR results from the detection of MAP from the blood samples of an ELISA positive herd of goats comparing Ficoll and ACK extraction methods

Lab ID	*ACK Cq value*	*ΔCq*	*Ficoll Cq value*	*ΔCq*	*ΔCq (ACK-Ficoll)*
1	Neg 27.95	1.28	Neg 29.11	0.99	-1.16
2	Neg 30.46	-1.23	Neg 29.63	0.47	0.83
3*	Neg 32.67	-3.44	Pos 26.09	4.01	6.58
4	Neg 30.04	-0.81	Pos 24.03	6.07	6.01
5*	Neg 31.30	-2.07	Pos 26.08	4.02	5.22
6	Pos 26.41	2.82	Pos 25.50	4.60	0.91
7	Pos 26.9	2.33	Pos 27.04	3.06	-0.14
8	Pos 26.02	3.21	Pos 26.26	3.84	-0.24
9	Neg 28.68	0.55	Neg 32.59	-2.49	-3.91
10	Pos 26.67	2.56	Pos 25.53	4.57	1.14
11*	Neg 30.79	-1.56	Pos 26.31	3.79	4.48
12	neg 27.88	1.35	Pos 27.37	2.73	0.51
13	Neg 27.34	1.89	Pos 26.23	3.87	1.11
14	Pos 26.49	2.74	Pos 26.45	3.65	0.04
15	Neg 29.86	-0.63	Pos 27.00	3.10	2.86
MP	29.23		30.10		
MAP 10^2 cells	20.02		15.18		

qPCR results comparing two different PBMC extraction methods (ACK and Ficoll) using Biosellal qPCR kit. ΔCq analysis was performed on all samples. Samples with a ΔCq ≥2 were determined to be a positive result. Samples with an '' tested inconclusive with ELISA testing, all others tested positive.*

4.2.2. Spiking samples with qPCR kit positive control

Due to the significant difference of results between Ficoll-Paque PLUS and ACK PBMC extraction methods, experiments were performed to determine if there were any PCR inhibitors present within samples extracted from PBMCs using ACK extraction method. Two samples were spiked with the Bio-T kit® *Mycobacterium avium paratuberculosis* qPCR kit (Biosellal; France) positive control (EPC). Samples were chosen based on their qPCR results from section 4.2.1. Sample ID 4 had a Cq value of 30.04 meaning that this sample was scored as a MAP-negative sample, whereas sample ID 8 had a Cq value of 26.02 meaning this sample was scored as MAP-positive. These two samples were chosen as the experimental matrix and 1 µl EPC was spiked into 3.8 µl of each sample DNA to from the sample template and qPCR performed as per section 2.5.3.2.

A shift in Cq value was observed with both samples, with both samples giving an earlier detectable signal when spiked with EPC (ID 4 had a ΔCq of -6.79, ID 8 had a ΔCq of -3.66 with an average ΔCq of -5.23 cycles between samples spiked with EPC and samples not spiked (Table 4.2). When the results for these two samples were compared with that for the EPC that was not spiked into any PBMC extractions, this had a Cq value of 19.90, a signal was detected 3.35 cycles later for ID 4 and 2.46 cycles later for ID 8.

Table 4.2 Effect of DNA extracted from goat PBMCs on Cq values of qPCR positive control (EPC) DNA.

SAMPLE	CQ VALUE OF SAMPLE BEFORE SPIKING	CQ VALUE OF SAMPLE AFTER SPIKING
ID4	30.04	23.25
ID8	26.02	22.36
MEDIA PLUS (NEGATIVE CONTROL)	29.23	27.79
EPC ALONE (POSITIVE CONTROL)	18.58	19.9

qPCR Kit positive control (EPC) was spiked into two DNA samples extracted from goat PBMCs; ID 4 classed as MAP-negative and ID 8 classed as MAP-positive. Samples were spiked with 1 µl EPC. As a control, 1 µl EPC was used as a template alone.

4.2.3. Comparison of ELISA results and Actiphage Rapid™ assay results for detection of MAP from blood of goats

Prior to blood sample collection, ELISA tests were performed for all 41 animals by an independent third-party. ELISA test results were then compared to Actiphage Rapid™ assay results to determine whether Actiphage Rapid™ could prove to be a more sensitive diagnostic test than ELISA.

Based on the comparison of the PBMC extraction methods for goat blood samples, the ACK extraction method (section 2.2.3.1.) was then performed on all the remaining samples ready for use with Actiphage Rapid™ assay (2.4.4). The presence of any MAP DNA in all samples was detected Bio-T® *Mycobacterium avium paratuberculosis* qPCR kit (Biosellal; France) as per section 2.5.3.2.

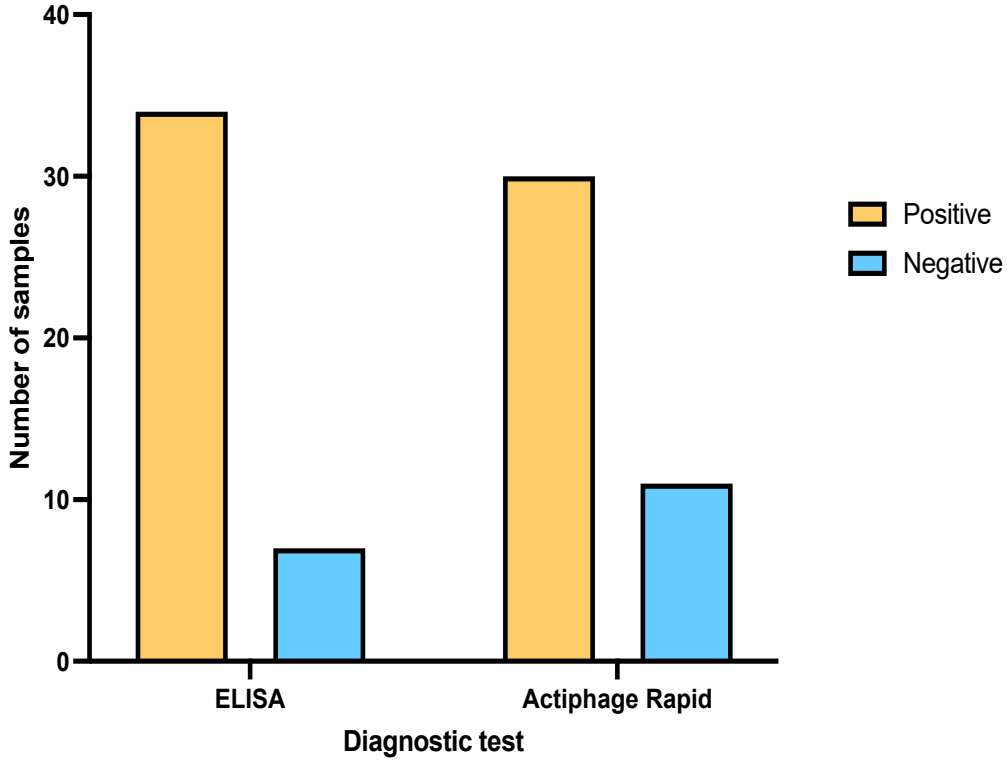
From the 41 blood samples, seven gave an inconclusive test result with ELISA testing (ID 3, 5, 11, 19, 22, 28 and 40). Using data obtained from samples where PBMCs were extracted using the ACK method only, 30 samples gave a MAP-positive test result and 11 a MAP-negative ($\Delta Cq \leq 2$) (Figure 4.1.). Of those were MAP-negative, three samples also tested inconclusive with ELISA testing. The remaining 8 Actiphage Rapid™ MAP-negative samples all gave a positive ELISA test result.

Considering the qPCR data when Ficoll was used as the PBMC extraction method, all animals that tested ELISA-inconclusive would be classed as MAP-positive using Actiphage Rapid™ assay. Samples with lab IDs 3, 5 and 11, which were all scored as inconclusive using the ELISA test and were scored as MAP- negative

when using PBMCs extracted using the ACK method, all gave a MAP-positive result when using Ficoll as the PBMC extraction method.

Clearly this experiment revealed that using the Ficoll and ACK PBMC extraction methods can influence the pattern of results gained and this requires more understanding, but these experiments were performed just prior to the COVID closure of the University and because of this, there was not an opportunity to continue the study using more animal samples. However, the results showed there was a general agreement between ELISA and Actiphage Rapid™ testing results, the difference being that the Actiphage® test results detect on-going infection, whereas the ELISA test only indicates exposure to the organism that has resulted in an antibody response.

Figure 4.1. Comparison of ELISA and Actiphage Rapid™ assay for detection of MAP from the blood of goats



41 goats were tested for MAP using both and ELISA and Actiphage Rapid™ assay. When tested with ELISA, 34 animals tested positive, and 7 animals tested inconclusive. In comparison, 30 animals tested positive with Actiphage Rapid™ and 11 tested MAP negative.

4.2.4. Comparison of in-house qPCR kit and commercial qPCR kit for detection of MAP from PBMCs of goats

Given the investment in developing their own qPCR kit, the company wished to determine if this performed any better with different sample types. Therefore, the Bio-T Kit® *Mycobacterium avium paratuberculosis* qPCR kit was compared to a qPCR assay designed in-house (see section 3B.2.1.), using the caprine samples. Results from Chapter 3B show that although the Empirical qPCR assay to detect MAP using P90/P91 primer pair and IS900RLP qPCR probe was possible, the qPCR assay was still not fully optimised. Therefore, the company was interested in using the Empirical master mix with a different combination of primers and probe that were being developed in the lab for other PhD research (Handley-Hartill, 2023). Primers and probes used for these experiments are listed in table 4.3. To do this comparison between qPCR kits, DNA from goat samples with Lab ID 1-15 that were from PBMCs extracted using ACK method were used.

It was found that 83.3 % of samples that gave a MAP-positive test result the using Bio-T® *Mycobacterium avium paratuberculosis* qPCR kit also gave a positive result using the in-house qPCR assay. One sample, Lab ID 12, gave a positive result using the in-house qPCR however but a negative test result using the Bio-T® qPCR kit. When using the Bio-T® qPCR kit, this sample was scored as negative due to having a ΔCq compared to the Media Plus negative control of < 2 cycles (ΔCq = 1.35). Therefore, it is possible that it was a weak positive that contained low numbers of cells. In contrast the MAP-positive control which contained ~10^2 MAP

cells grown in culture showed better amplification with a strong detectable signal rising above the threshold levels at 23.31 cycles.

When low numbers of mycobacterial cells are present in a clinical sample, some stochastic effects are seen, and it was shown using human blood samples that increasing the number of samples tested increased the likelihood of gaining a positive result from IGRA-positive, asymptomatic TB contacts (PBD Biotech, unpublished data).

Despite the in-house qPCR detecting positive results for samples through Cq analysis, analysis of the normalised qPCR data plots shows there was very little amplification of samples. There was a lack of good amplification (Figure 4.2) despite the internal control channel showing no signs of PCR inhibition (data not shown). To overcome the lack of amplification seen from the samples, primer concentrations for this in-house qPCR assay were increased 4-fold to a final concentration of 2 µM per qPCR reaction.

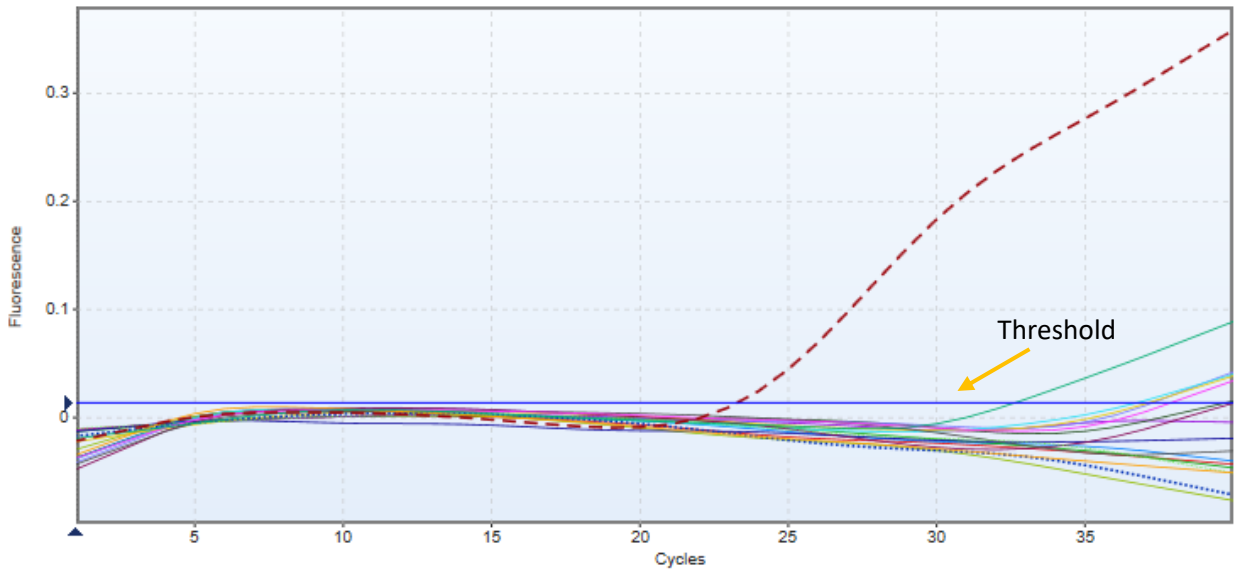
When final primer concentration was increased, an increase in positive samples was observed. All positive samples when using the Bio-T® qPCR kit and the previous in-house qPCR were also positive when primer concentrations were increased. A further 5 samples were also determined to be positive by Cq analysis after an increase in primer concentration in comparison to qPCR performed using Bio-T® qPCR kit (Table 4.4). Despite the increase in positive results, there was still a lack of amplification curves observed from the samples and late Cq values. Only one sample had a Cq value of less than 30.

Table 4.3 Primer and probe sequences used for in-house qPCR detection of MAP

TARGET	PRIMER/PROBE NAME	SEQUENCE (5'-3')
IS900	MAP F Realtime	AATGACGGTTACGGAGGTGGTT
IS900	MAP R Realtime	AGCGATGAGCAAGGCGATCA
IS900	MAP RT Probe	FAM-TGCGCGTCGTCGTTAATAACC-BHQ1

Primer and probe sequences used for optimisation of an in-house qPCR assay for detection of MAP from caprine PBMCs. Primers and probes were developed by Handley-Hartill (2023).

Figure 4.2. Normalised qPCR data plot of DNA extracted from caprine PBMCs and detected using an in-house qPCR



Normalised qPCR data plot generated by Q-Rex software showing qPCR amplification of MAP DNA extracted from caprine PBMCs using ACK extraction method and detected using an in-house qPCR assay. Sample ID 1-15 (solid lines of various colours) all showed very little amplification with many samples only just rising above threshold levels. The MAP-positive control (---) contained ~10^2 MAP cells grown in culture.

Table 4.4. Comparison of Cq values from qPCR assays to detect MAP DNA from caprine PBMCs using Bio-T Kit *M. avium paratuberculosis* or an in-house qPCR using two different primer concentrations

	BIO-T® KIT *M. AVIUM PARATUBERCULOSIS*		IN-HOUSE QPCR		IN-HOUSE QPCR (4X CONCENTRATION OF PRIMERS)	
SAMPLE LAB ID	**TEST RESULT (Positive or Negative)**	**Cq value**	**TEST RESULT (Positive or Negative)**	**Cq value**	**TEST RESULT (Positive or Negative)**	**Cq value**
1	-ve	27.95	-ve	ND	-ve	ND
2	-ve	30.46	-ve	ND	-ve	ND
3*	-ve	32.67	-ve	ND	+ve	31.13
4	-ve	30.04	-ve	ND	+ve	27.95
5*	-ve	31.3	-ve	ND	+ve	34.92
6	+ve	26.41	+ve	39.75	+ve	35.62
7	+ve	26.9	+ve	37.9	+ve	37.06
8	+ve	26.02	+ve	37.29	+ve	36.16
9	-ve	28.68	-ve	ND	-ve	ND
10	+ve	26.67	+ve	36.76	+ve	34.53
11*	-ve	30.79	-ve	ND	+ve	30.66
12	-ve	27.88	+ve	37.15	+ve	33.37
13	+ve	27.34	+ve	32.65	+ve	36.31
14	+ve	26.49	-ve	ND	+ve	36.2

15	-ve	29.86	-ve	ND	+ve	36.03
MP		29.23		ND		37.18
MAP 10^2		20.02		23.31		22.92

Caprine DNA samples with lab ID 1-15 were used. Samples marked with '' produced an inconclusive ELISA test result. The other samples gave a MAP-positive ELISA test result. Samples that were determined to be MAP-positive by qPCR are highlighted in orange. Samples where there was no fluorescent signal above threshold levels were scored as not detected (ND).*

4.2.4.1 BLAST analysis of MAP specific primers

Due to overall poor amplification of IS*900* sequence using MAP F Real-Time primer (section 4.2.4.), a basic local alignment search tool (BLAST) analysis (National Institute of Health; USA) was performed to determine whether these primers lacked specificity to MAP strains isolated from non-bovine ruminants.

BLAST analysis results showed that 58.3 % of MAP strains isolated from caprine animals had a query cover < 100 % compared to 28 % of strains isolated from *Ovies aries,* 8.2 % of strains isolated from *Bos taurus,* and 0 % for Buffalo and *Homo sapiens*. Based on these BLAST results and poor qPCR data produced using this primer, it was decided to not proceed with using the primer for any more qPCR experiments.

4.3. DISCUSSION

Detection of MAP from the blood of farmed goats by Actiphage Rapid™ assay and qPCR using the Bio-T® qPCR kit by Biosellal (France) is more sensitive than detection using in-house qPCR kits and is also more sensitive than ELISA based assays.

Even though the in-house qPCR assays appeared to be able to detect MAP as there was an 83.3 % agreement rate between the in-house qPCR and the qCR kit by Biosellal, the assays robustness can still be questioned. When detecting MAP from goats, fluorescence and amplification of target sequences remained weak and in low levels. Cq values produced for samples using in-house qPCR often had a Cq over 30. This is further supported by the lack of amplification, low fluorescence and rough amplification curves observed from normalised data plots. By cycle 30 of a qPCR, probe degradation, auto-fluorescence and low levels of bacterial target DNA can all contribute to background noise and a late Cq value. It is therefore questioned whether these positive results using the in-house qPCR assay are true positives, or a by-product of minimal detection of target DNA, non-specific binding of eukaryotic DNA and probe degradation. In addition, the gain setting on the RotorGene-Q (Qiagen; Germany) could have contributed to fluorescence not being in the optimal range. Adjustment of raw baseline fluorescence to keep within the optimal range is often deemed as essential when optimising a qPCR. Without these adjustments, baseline fluorescence can influence PCR performance causing less accurate measurements of samples (Reynisson *et al.*, 2006). Many qPCR instruments such as ABI 7700 (Applied Biosystems; USA) allow for gain adjustment

and optimization, unlike the RotorGene-Q which relies on an automatic gain calibration function to provide a predetermined baseline fluorescence for samples. This inability to adjust the gain measurement on the instrument may have contributed to the low background fluorescence and lack of sensitivity shown in section 4.2.4 for the detection of MAP from goats using an in-house qPCR assay.

In addition to inability to adjust gain, inappropriate storage of probes and choice of fluorophore could have also contributed to an increase in background and a lower signal to noise ratio. Fluorogenic probes that have undergone prolonged exposure to light are more susceptible to degradation, which means they can release free dye into the reaction mixture contributing to background noise. Therefore, probes that have undergone several freeze-thaw cycles may have experienced more probe degradation than probes being used for the first time.

Furthermore, the primers used in the in-house qPCR for detection of MAP from goat blood were primers that had a similar issue to that of the P90 primer which lacked specificity to non-bovine ruminant target DNA sequences, in particular goats. This could also explain the poor results. Lack of primer specificity can result in underestimation of target DNA within samples. To overcome this problem and to assess whether poor primer design contributed to poor amplification of target DNA, the P90short primer, which was designed to have increased specificity to MAP strains isolated from non-bovine ruminants, needs to be used for these qPCR assays as a comparison.

The choice of PBMC extraction method can have an impact in detection of MAP. If only ACK instead of Ficoll was used for PBMC extraction from whole goat

blood, then many samples would have remained undetected leading to false-negative results. The current data suggests that the Ficoll PBMC extraction method performed better as 80 % of samples were recorded as positive samples compared to only 33.3 % being positive when using ACK PBMC extraction method. There was also a significant difference in ΔCq between the ACK and Ficoll PBMC extraction methods. Despite this difference, it was shown that although there were inhibitors diluting the detection of kit positive control (EPC) when this was spiked into samples, there was only a maximum of 3.35 cycle shift, suggesting that overall, ACK extractions weren't having a large detrimental effect causing almost total inhibition of qPCR detection of MAP. Nevertheless, spiking EPC into ACK PBMC extractions did shift the Cq over 3 cycles later, so any animals that may have been in a sub-clinical stage of infection, therefore having a lower number of MAP cells within their body, could be going undetected as there is still a reduction in sensitivity. Whilst both ACK and Ficoll PBMC extraction methods separate erythrocytes from other components of blood, ACK lysis does not separate out granulocytes or platelets. In contrast, Ficoll, due to working on a density gradient, mostly isolates lymphocytes and monocytes. As a result, extractions performed using ACK can carry forward more contaminants that can reduce qPCR sensitivity compared to when Ficoll is used. Other contaminants that are at higher risk of not being removed properly by ACK PBMC extraction are haemoglobin and lactoferrin, found in the ACK buffer, both of which can interact with *Taq* polymerase, reducing qPCR efficiency (Al-Soud and Rådström, 2001, Sidstedt *et al.*, 2018). Kermekchiev *et al.* (2009) developed a mutant *Taq* polymerase through point mutations and an N-terminal deletion, which confers greater resistance to PCR inhibitors, including haemoglobin and IgG.

Currently it is not known if the *Taq* polymerase used in this research is the wild type, or a modified version. Development of a qPCR assay with the mutant enzyme described by Kermekchiev *et al.* (2009) with samples extracted from whole blood using ACK extraction method and comparing this to a known wild type *Taq* polymerase and the Biosellal qPCR kit, would provide further insight into qPCR inhibitors originating from the original blood sample and determine whether these barriers could be overcome.

Differences between ACK and Ficoll PBMC extraction methods from caprine blood can be extended to comparison of ELISA results. Differences between ELISA testing and Actiphage Rapid™ assay were only seen when using ACK as the chosen white blood cell extraction method. Samples that tested positive for ELISA remained undetected when using Actiphage Rapid™ assay and ACK white blood cell lysis methods. When using Ficoll as the extraction method, similar results were achieved between Ficoll-Actiphage Rapid™ testing and ELISA testing. This difference supports the conclusion that Ficoll is a better extraction method than ACK by achieving a higher purity of target PBMCs and minimising blood-based qPCR inhibitors. Furthermore, some samples that were MAP-positive using Ficoll extraction were reportedly inconclusive with ELISA testing. ELISA testing can produce inconclusive results for numerous reasons, including borderline signals from OD readings falling close to the cut-off range, high background signal-to-noise ratio caused by non-specific binding of antibodies or conflicting replicates. Additionally, as ELISA is an antibody response, animals with sub-clinical infection may not have elicited a strong immune response and therefore their infection remained undetectable. Actiphage Rapid™ assay is designed to overcome these issues through increased sensitivity,

less ambiguity because of minimal background signal-to-noise ratios and lack of unspecific binding of primers and probes.

This chapter has demonstrated that Actiphage Rapid™ assay may have greater sensitivity than ELISA assays, as eight out of eleven animals that tested inconclusive with ELISA testing were deemed to be Actiphage Rapid™ positive. Despite this success, when using in-house qPCR background noise and unspecific binding of primers and probes due to Media Plus negative controls were shown, producing a detectable signal above threshold levels despite not showing any signs of contamination. Therefore, without further optimisation, the Actiphage Rapid™ assay does not overcome all issues that arise from using ELISA testing for detection of MAP from goats.

In summary:

1. Actiphage Rapid™ assay had greater sensitivity than ELISA assays
2. The in-house qPCR kit shows signs of background noise and unspecific binding therefore do not perform as well as commercial qPCR kits

CHAPTER 5

ELUCIDATING APPLICATIONS OF ACTIPHAGE RAPID™ ASSAY AS A LYSING AGENT AND FOR RFLP ANALYSIS

5.1. INTRODUCTION

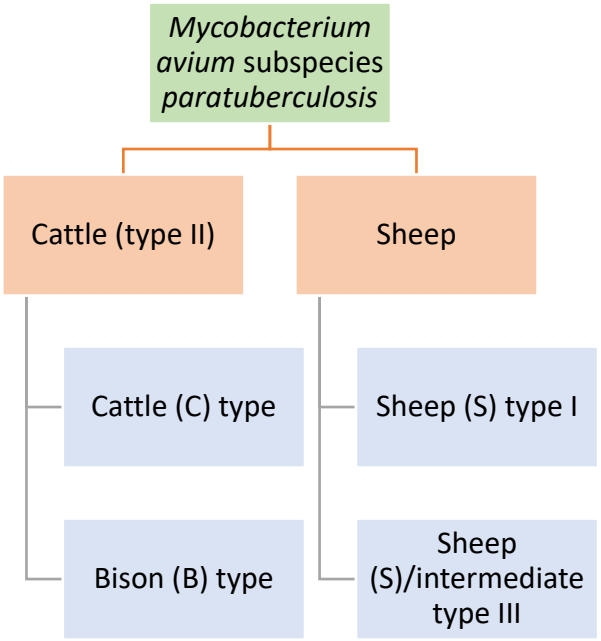
Previous Chapters explored the applications and limitations of the Actiphage Rapid™ assay when used with qPCR to detect mycobacteria and provided a clearer understanding of how qPCR variability can affect detection. Moreover, it has been demonstrated that Actiphage Rapid™ assay used with a commercial qPCR kit can be used to detect mycobacterial infection from naturally infected ruminants including cattle, goat, and deer. However, this was just extension work building on original studies using end-point PCR (Swift *et al.*, 2020), but there was potential that other information can be obtained using Actiphage Rapid™ assay and associated analysis methods through further development of qPCR assays. This is particularly of interest when there is a desire to not only detect the presence or absence of mycobacteria from samples, but also to differentiate between sub-types within a *Mycobacterium* complex. Furthermore, as animal hosts are also susceptible to mixed strain infections, meaning they can become concurrently infected with multiple strains of a single pathogenic species (Byrne *et al.*, 2020), using Actiphage Rapid™ assay and qPCR to identify mixed strain infections would be advantageous. Mixed strain infections can affect both humans and animals suffering from mycobacterial infections. From an epidemiological perspective, the ability to differentiate both strains and sub-types within a *Mycobacterium* complex is advantageous as it can create a better understanding of spread of disease through populations allowing for more effective control programs, herd management and disease eradication.

This Chapter explores one of the two pathologically important complexes of mycobacteria, namely *Mycobacterium avium* complex (MAC). Members of this complex include *Mycobacterium avium* subspecies *avium* (MAA), *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Mycobacterium avium* subspecies *hominissuis* (MAH) and *Mycobacterium avium* subspecies *silvaticum* (MAS), however, a focus will be placed on MAP and MAA.

MAP, the causative agent of Johne's disease in ruminants, can be separated into three different subtypes: Sheep-type (S-type), Cattle-type (C-type) and Bison-type (B-type) (figure 5.1). Classification into subtypes was traditionally performed by techniques based on single nucleotide polymorphisms (SNPs) within insertion sequences, Originally, characterisation into these groups was performed using techniques such as restriction enzyme analysis to detect local polymorphisms in specific DNA sequences (Whittington *et al.*, 1998). More recently, the validity of classifying of strains into these groups has since been confirmed through whole genome sequencing approaches and using molecular analysis tools.

Unlike IS*900*, which is an insertion sequence exclusive to MAP, IS*1311* is found in all members of the *M. avium* complex and is used as a method to detect the DNA from these bacteria using a single primer pair. IS*1311* is a multi-copy sequence present in 7-10 copies per genome, making the insertion sequence a good PCR target as it increases the likelihood of detection when low cell numbers are present, compared to targeting single copy signature sequences.

Figure 5.1 Lineages and sub-types of *Mycobacterium avium* subspecies *paratuberculosis*



M. avium subspecies paratuberculosis (green) can be divided into two major lineage (orange) and four sub-lineages (blue). C-type strains are predominantly isolated from cattle, B-type strains are a derivative of C-type strains usually isolated from bison and S-type strains primarily isolated from sheep. Intermediate S-type strains share a close genetic relationship with type I.

In addition to the multi-copy nature of the insertion sequence, polymorphic variations within the sequence mean that a more in-depth analysis of the genetic sequence allows differentiation of members within MAC. For instance, IS*1311* insertion sequence contains five, highly conserve point mutations that differentiate MAA from MAP (see Table 5.1 for details). These point mutations can also dictate the presence of absence of *Hinf*I and *Mse*I restriction sites (figure 5.2.). Analysis of 351 isolates using polymerase chain reaction restriction enzyme analysis (PCR-REA) by Hodgeman *et al.* (2021) demonstrated the C-type lineage to be highly monomorphic as there were only 20 SNP differences separating all C-type isolates investigated, highlighting the usefulness and accuracy of exploiting molecular typing techniques to discriminate between subtypes. Typically, subtypes are isolated from their respective host, for example S-type MAP strains are usually isolated from sheep, whereas C-type strains are less likely to be found in sheep and more likely to be isolated from cattle. However, subtypes can be known to spread to other animals with reports of S-type MAP strains can spreading to cattle (Stevenson *et al.,* 2009). Understanding MAC subtype can tell us a lot about epidemiology, where the disease may have originated and how it has spread.

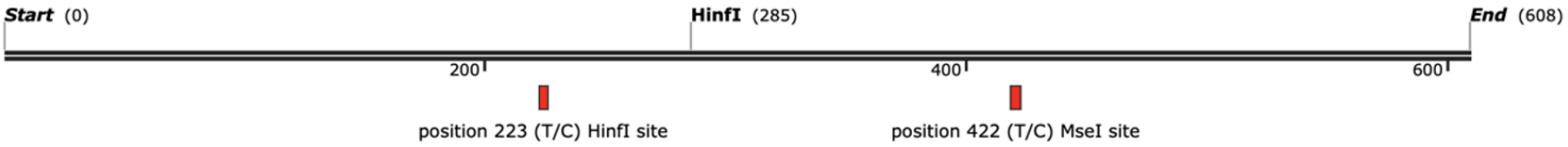
Traditionally, techniques used to differentiate subtypes of MAC included mycobacterial interspersed repetitive unit and variable-number tandem repeat (MIRU-VNTR), whole genome sequencing (WGS), pulse-field gel electrophoresis (PFGE) and PCR-REA. The method of PFGE uses restriction enzymes to digest genomic DNA, which is then separated through a gel matrix using electrical currents that periodically change direction. PFGE provides a profile of the entire genome in comparison to PCR-REA.

Table 5.1 Single nucleotide polymorphisms present in IS*1311* of *Mycobacterium avium* complex

SPECIES/TYPE	POSITION IN IS*1311*					
	68	**223**	**236**	**422**	**527**	**628**
MAA	T	C	T	T	G	T
MAP S-TYPE	C	C	C	C	A	C
MAP C-TYPE	C	C/T	C	C	A	C
MAP B-TYPE	C	T	C	C	A	C

Single nucleotide polymorphisms present in the IS1311 insertion sequence of members of the M. avium complex which can be used to differentiate subtypes. At position 223 in IS1311 C-type MAP strains can possess copies of the insertion sequence containing a C nucleotide as well as copies of the insertion sequence containing a T nucleotide.

Figure 5.2 IS*1311* amplicon depicting location of *Hinf*I and *Mse*I restriction sites



The 608 bp PCR product of IS1311 PCR using M56F and M119R. HinfI restriction site at position 285 is present in all members of the M. avium complex. The presence of a HinfI restriction site at position 223 is dictated by the presence of a T nucleotide. MAC lineages with a C nucleotide at this position will not have a HinfI restriction site. The presence of an MseI restriction site at position 422 is determined by a T nucleotide at this position. MAC lineages with a C nucleotide at this position will not have an MseI restriction site.

Although both methods use restriction enzymes to fragment DNA, PCR-REA only provides an overview of a select region of the bacterial genome (Sharma-Kuinkel *et al*., 2016, Conde *et al.,* 2022).

Before genome sequencing became more possible, PFGE was often considered to be the gold standard in molecular typing methods, but the method is time-consuming and, with the need for specialised lab equipment, PFGE can also be expensive (Neoh *et al.*, 2019). Similarly, WGS and the respective analysis, which allows for high resolution clarity of strain diversity (Wibberg *et al.,* 2020), requires time and money, both of which are restricted in diagnostic assays. Whilst MIRU-VNTR attempts to overcome these limitations by only requiring thermal cyclers and agarose gel visualisation systems, both of which are widely available in most laboratories, this method has been reported to both over- and underestimate the relationship between strains as some repetitive elements can be unstable, or due to suboptimal typing technique (Ahlstrom *et al.*, 2015, Imperiale *et al.*, 2017).

An alternative approach to discriminating between SNPs within IS*1311* is using PCR-REA assay. PCR-REA for the differentiation of MAC strains first described by Whittington *et al.* (1998), exploits the restriction pattern of specific enzymes to digest and fragment IS*1311* DNA sequences.

PCR-REA can be broken down into two parts. The first part of the test involves amplification of the IS*1311* insertion sequence from purified DNA, using specific primers that amplify a large, specific, section of the insertion sequence (608 bp of the 1213 bp insertion sequence is amplified). The section of insertion sequence targeted includes a single nucleotide polymorphism (SNP) at location 224

bp that dictates the presence or absence of a *Hinf*I restriction site and an SNP at 423 bp that dictates the presence or absence of an *Mse*I restriction site. After amplification of the insertion sequence, restriction enzymes are used to target and digest the amplified sequence. Digested DNA products are visualised through gel electrophoresis where different strains can be discriminated based on differences in fragmented band patterns.

Another insertion sequence important and present in MAA is IS*901*. This 1472 bp insertion sequence shares 60 % homology to IS*900* found in MAP, but unlike MAP is not present in every strain of MAA (Kunze *et al.*, 1991). Reports suggest that the presence of IS*901* contributes to the virulence of the Mycobacterial strain where the insertion sequence resides (Pavlik *et al.*, 2000). Furthermore, multi-drug-resistant *M. avium* strains are increasingly becoming a threat, with resistance being harboured on IS*901* elements amongst a few other genes (Algammal *et al.*, 2021). Therefore, detection of the IS*901* insertion sequence may be important in controlling virulence and determination of antibacterial resistance profile of MAA strains.

This Chapter describes experiments that aimed to explore whether Actiphage Rapid™ assay can be used as a lysing agent to aid in extraction of mycobacterial DNA and allow for detection and sub-typing of mycobacterial infection. In addition, experiments were performed to determine whether PCR-REA could be developed into a real-time PCR format using high resolution melting analysis to supplement the qPCR assays optimised in previous chapters to allow for additional epidemiological information to be obtained.

The aims of Chapter 5 are as follows:

1. Evaluate the use of Actiphage Rapid™ assay with IS*1311* specific PCR-REA
2. Evaluate the usefulness of Actiphage Rapid™ assay as a lysing agent for sequencing
3. Use Actiphage Rapid™ assay to determine phage-host range of uncharacterised mycobacteriophage
4. Develop IS*1311* PCR-REA into a real-time format for detection and sub-typing of *Mycobacterium*

5.2. RESULTS

5.2.1. Standard methods of detecting and differentiating *Mycobacterium avium* complex

To initiate this study, ten MAP strains were obtained from Moredun Institute (Scotland; UK) (section 2.1.4.). All strains were known to be independent isolates of MAP from various sources and animal hosts, but were of unknown subtype. Primary experiments were performed on these MAP strains to confirm that all strains could be identified as MAP using standard methods of Actiphage Rapid™ assay and endpoint PCR. Once strains were confirmed as MAP, subtyping was performed using PCR-REA (section 2.5.2.4.) to determine lineage of strains.

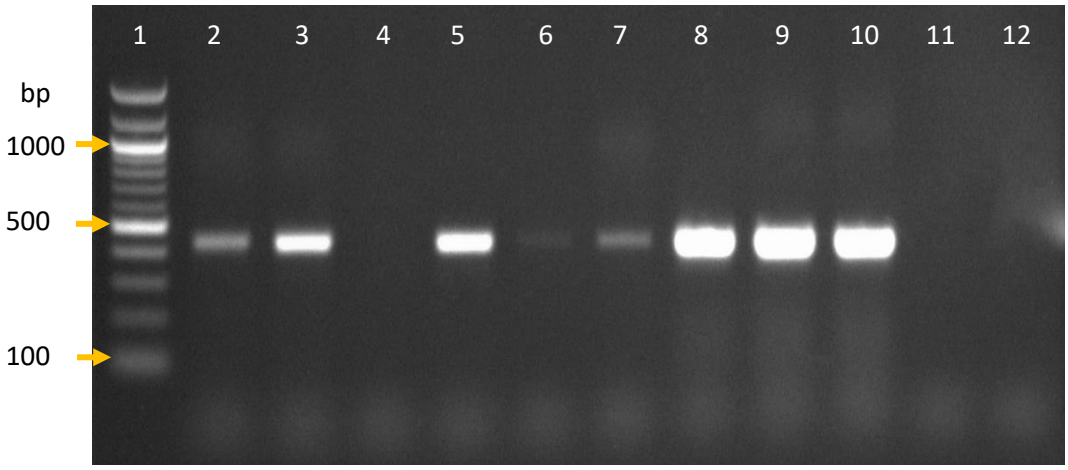
5.2.1.1. Confirmation of Mycobacterium avium subspecies paratuberculosis strains using IS900 and F57 specific PCR

Upon receipt of the ten MAP strains (information in section 2.1.4.), all isolates were sub-cultured as per section 2.1.2.1. for 8 weeks. Actiphage Rapid™ assay was used to extract DNA from the cultures (section 2.4.4.). Endpoint PCR was performed to confirm the identity of isolates to ensure they were MAP before any further analysis could take place. Two MAP specific endpoint PCR assays were performed targeting both the multi-copy insertion sequence IS*900* (section 2.5.2.1.) and the single copy gene F*57* (section 2.5.2.2.). IS*900* PCR has high sensitivity due to the multi-copy nature of the insertion sequence, whilst the highly conserved F*57* sequence has high specificity. IS*900* PCR used the P90short primer, a primer modified from the published primer P90 (Millar *et al.*, 1995), due to its increased

specificity to MAP strains isolated from non-bovine hosts as previously described in Chapter 3.

All ten MAP isolates were confirmed to be MAP by detection of a 413 bp PCR product using the IS*900*-specific primers (data not shown). Eight out of ten MAP isolates were also confirmed by the detection of a 439 bp F*57* PCR product. However, for two isolates (M71/03 and M266/04) no F*57* PCR product was generated and isolate B4 only produced a very faint band after visualisation of the agarose gel (Figure 5.3.) despite DNA from the equivalent of 10^3 cells of each isolate added per assay. These results suggested there was some genomic variation in this set of MAP strains.

Figure 5.3 Visualisation of PCR products from F*57*-specific PCR of ten MAP isolates



DNA was extracted from MAP strains using Actiphage Rapid™ assay and PCR performed targeting the single copy F57 gene. PCR products from F57-specific primers were then visualised on a 1.5 % (w/v) agarose gel stained with ethidium bromide and viewed under UV light. Positive results were confirmed by the presence of a 439 bp band.

Samples loaded on the gel were: Lane 1: 100 bp Molecular size marker. Lanes 2-11, PCR products produced from DNA extracted from different MAP isolates: Lane 2, strain 235G; Lane 3, strain 213G; Lane 4, strain M71/03; Lane 5, strain M113/05; Lane 6, strain B4; Lane 7, strain M110/05; Lane 8, strain ATCC 19851; Lane 9, strain M112/05; Lane 10, strain 218; Lane 11, strain M266/04. Lane 12; RNase-free water was used as a no-template control.

5.2.1.2. Sub-typing Mycobacterium avium complex strains of unknown lineage using restriction enzyme analysis

Once all strains of were confirmed to be MAP, IS1*311* PCR-REA (section 2.5.2.4.) was performed on these ten isolates as well, as three other isolates from the *Mycobacterium avium* complex (MAC) to determine lineage of all these strains (information on strains in section 2.1.4.). Of the thirteen isolates, there was one strain previously confirmed to be an *M. avium* strain and used as a reference type-strain (MAA type strain), one known cattle type (C-type) MAP strain (ATCC 19851), two suspected but unconfirmed *M. avium* strains (#7210 and #7212) isolated from a person presenting at hospital with NTM infection and nine MAP strains of unknown lineage.

PCR amplification of a 608 bp product of the IS*1311* insertion sequence using the M56/M119 primer pair (Marsh *et al.*, 1999) was successful for all strains tested, confirming them to be members of the *M. avium* complex. Restriction enzyme analysis of PCR amplicons (PCR-REA) using restriction enzymes *Mse*I and *Hinf*I (section 2.5.2.4) was used to identify the species and lineage of isolates.

PCR-REA confirmed that the two suspected *M. avium* strains received from a clinical infection (#7210 and #7212) were *M. avium* subspecies. *avium* according to the band pattern produced (Table 5.2). Of the nine MAP strains of unknown lineage, 4 were identified as C-type MAP (218, M113/05, M110/05 and M112/05), 3 were identified as S-type MAP (B4, M266/04 and M71/03) and one MAP strain was identified as belonging to the Bison lineage (213G). Poor resolution of PCR products on the agarose gel meant that there were difficulties in confidently identifying

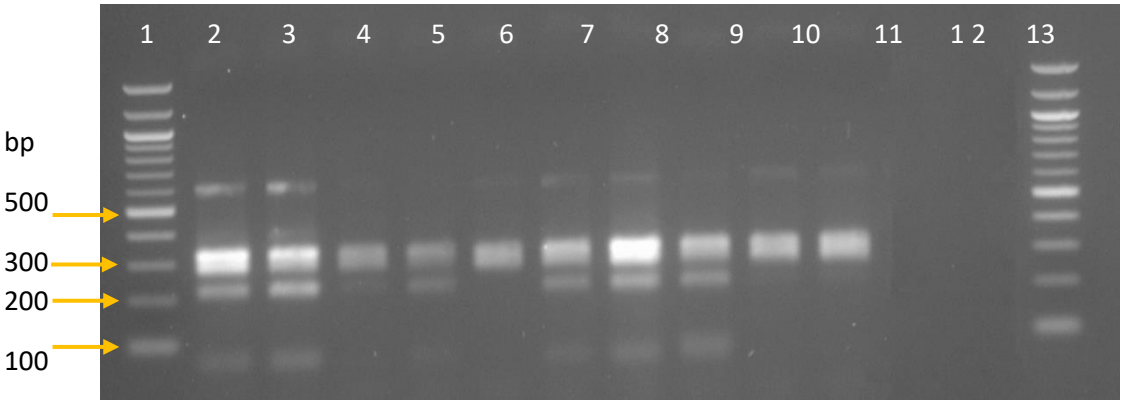
lineage of strain 235G and it remained unclear whether this strain was B-type or C-type (Figure 5.4). Any bands observed at 600 bp were undigested PCR product due to lack of efficiency of *Hinf*I and *Mse*I.

Table 5.2. Predicted band size patterns of members of MAC after PCR-REA

***M. AVIUM* COMPLEX TYPE**	**RESTRICTION DIGEST BAND PATTERN (BP)**
MAP BISON (B) TYPE	67, 218, 323
MAP CATTLE (C) TYPE	67, 218, 285, 323
MAP SHEEP (S) TYPE	285, 323
M. AVIUM* SUBSPECIES *AVIUM	134, 189, 285

Predicted band size patterns of members of the Mycobacterium avium complex after amplification of a 608 bp section of IS1311 insertion sequence found in all members of MAC. Amplified PCR product is digested with restriction enzymes MseI and HinfI. Restricted fragments were separated on an agarose gel and visualised under UV light. Band sizes were determined by comparison with a 100 bp molecular marker.

Figure 5.4 Visualisation by gel electrophoresis of restriction digest products from ten MAP strains



DNA was extracted from 10 MAP strains using the Actiphage Rapid™ assay. The IS1311 MAA signature sequence was targeted by PCR then PCR products digested with restriction enzyme MseI and HinfI. Products of restriction digest were resolved on a 1.5 % (w/v) agarose gel. Lineage of each MAP strain was determined by analysis of band patterns (see Table 6.2.).

Samples loaded on the gel were: Lane 1: 100 bp Molecular size marker. Lanes 2-11, PCR-REA restriction fragments produced from DNA extracted from different MAP isolates: Lane 2, strain ATCC 19851; Lane 3, strain 218; Lane 4, strain 235G; Lane 5, strain 213G; Lane 6, strain B4; Lane 7, strain M113/05; Lane 8, strain M110/05; Lane 9, strain M112/05; Lane 10, strain M266/04; Lane 11, strain M71/03. In Lane 12 the sample used for the Actiphage assay contained Media Plus only (negative control). Lane 13; RNase-free water was used as a no-template control. Lane 14: 100 bp molecular size marker.

5.2.2. Effect of strain type on detection of MAP by qPCR analysis

Chapter 3A and 3B have both shown that Actiphage Rapid™ can be used to extract MAP DNA from viable cells and in turn, this DNA can be detected by qPCR using the Bio-T® *Mycobacterium avium paratuberculosis* qPCR kit (Biosellal; France). It has also been highlighted that MAP strains isolated from non-bovine host animals may lack sensitivity to PCR detection due to lower sequence homology to commonly used MAP primers (section 4.2.4.1.). To this point, when using qPCR to identify MAP, strains used for experimental matrices in Chapter 3B were known C-type strains, and MAP isolated from bovine, caprine and Cervidae hosts (Chapter 3A, 3B and 4) were not characterised by sub-type. By using known MAP strains from all three different lineages (C-type, S-type and B-type), the question remains whether it is possible to see any qPCR variability or a lack of sensitivity especially from S-type and B-type strains.

All MAP strains previously identified by lineage in section 5.2.1.2. were enumerated using the Actiphage® Core assay prior to use in the qPCR assay (section 2.4.6.). Briefly, MAP cells were incubated with Actiphage® Reagent for 1 h at 37 ˚C. After 1 h, virucide was added to reaction vessels and incubated under constant rotation for 5 min to ensure virucide was covering all internal surfaces of the reaction vessel. Then, 10-fold serial dilutions were performed on samples before pouring on soft agar plates containing Media Plus, *M. smegmatis* sensor cells and 7H10 molten agar. Plates were left to set and incubated at 37 ˚C for 18 h and plaques counted, and MAP enumerated. For each strain, 10^3 cells were used per Actiphage Rapid™ assay and 5 µl of purified DNA template was used per qPCR

reaction (section 2.5.3.2.). In addition, a series of 10-fold diluted DNA samples from strain ATCC 19851 were included in the qPCR assay as a reference standard and to allow a standard curve to to be created to determine qPCR efficiency.

Analysis of the results indicated that there were no signs of qPCR inhibition through observation of amplification of internal positive control on the Cy5 acquisition channel, but despite this the qPCR efficiency was calculated to be 116 %,

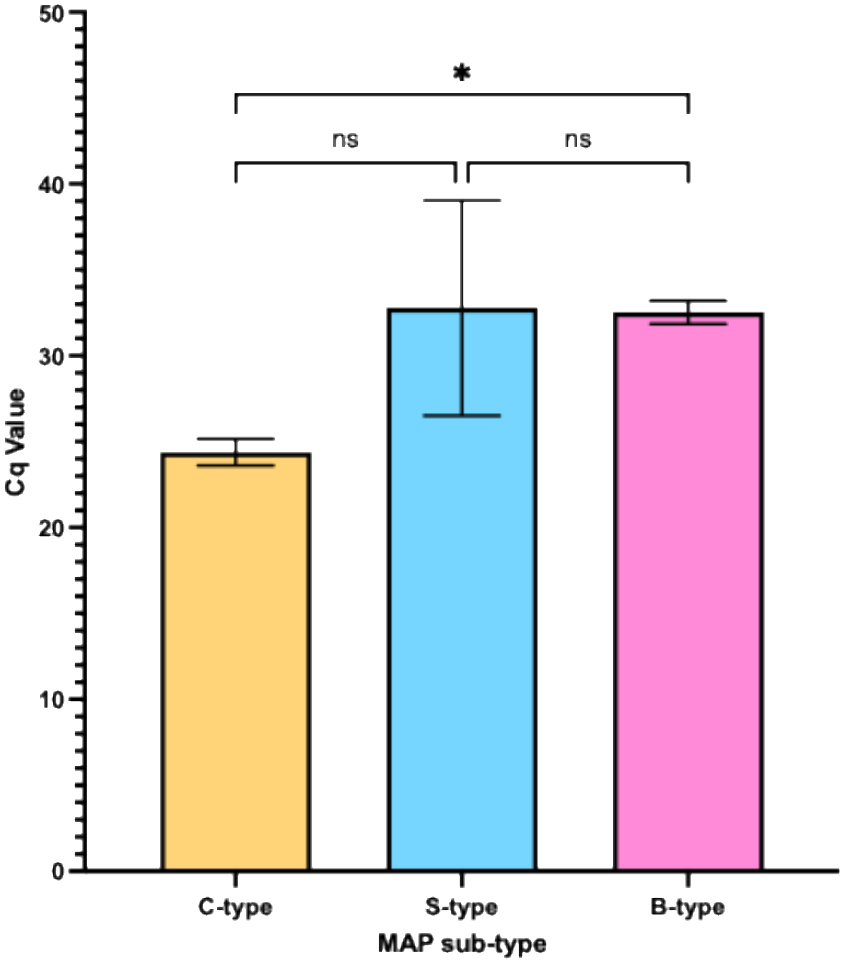
The sub-type of each MAP strain had been determined by IS*1311* PCR-REA (section 5.2.1.2.) except for strain 235 for which the sub-type identification was unclear. So, for this strain, the qPCR results were compared to those of the S-type, B-type and C-type strains to determine whether the qPCR Cq value for strain 235 followed a similar pattern to any of those of known type.

From the qPCR assay results, five strains identified as C-type strains through PCR-REA (ATCC 19851, 218, M113/05, M110/05 and M112/05) on average had an earlier Cq value than those strains typed as B-type or S-type. C-type strains had a mean Cq value of 24.38 ± 0.7764 cycles, compared to Cq 32.78 ± 6.270 cycles for S-type strains and Cq 32.52 ± 0.6788 cycles for B-type strains. Welch ANOVA analysis shows there is a statistical significance between the three subtypes of MAP (p = 0.0298). Dunnett's T3 multiple comparisons test showed there was no significant difference between C-type and S-type (p = 0.4987) and S-type and B-type (p = 0.2567), however there was a significant difference between Cq values between C-type and B-type MAP strains (p = 0.0166) (Figure 5.5).

This suggests that when using Actiphage Rapid™ assay and qPCR for detection of MAP from clinical samples, there is reduced sensitivity of MAP

detection when detecting S-type and B-type strains which could lead to false-negative results.

Figure 5.5 Mean Cq value of detection of three different MAP sub-types by qPCR



MAP strains of three presumptive sub-type based on results from IS1311 PCR-REA were enumerated, and 10^3 cells were added per Actiphage Rapid™ assay. DNA extracted from Actiphage Rapid™ was then detected through qPCR using Bio-T Mycobacterium avium paratuberculosis qPCR kit (Biosellal; France). Chart shows results for C-type MAP (■), S-type MAP (■) and B-type MAP (■).

5.2.3. The use of Actiphage® reagent as a lysing agent to extract DNA for whole genome sequencing

The Actiphage Rapid™ assay has proven to be a successful lysing agent to aid in subtyping of MAC strains (section 5.2.1. and section 5.2.2.). To investigate additional applications and limitations of Actiphage Rapid™ assay, focusing on Actiphage Rapid™ as a lysing agent, experiments were performed to determine whether the assay could be used as a DNA extraction tool to allow for sequencing on mycobacteria.

To carry out this investigation, cultures of both *M. bovis* BCG and *M. smegmatis* were enumerated using the Actiphage Core assay (section 2.4.6.) prior to use in Actiphage Rapid™ assay (section 2.4.4.). For this experiment 10^5 cells of *M. bovis* BCG cells and 10^6 cells of *M. smegmatis* were used in Actiphage Rapid™ assays. As a negative control, an assay was performed using Media Plus containing 10^7 D29 phage particles. DNA was purified and concentrated as per section 2.5.1.1. and DNA sequenced by whole genome sequencing by MicrobesNG (Birmingham; UK) using Illumina short reads. Bioinformatics of raw reads was performed by Dr Conor Meehan (Nottingham Trent University, Nottingham; UK) and data was provided containing information including taxID, number of reads, number of unique reads and abundance.

The sequencing profile of the DNA recovered from the experiment containing 10^5 *M. bovis* BCG cells showed that Mycobacteriophage including D29, Chy4 and Chy5 were in high abundance in the sample. Similar results were found with *M. smegmatis* sequencing profile. The high abundance of D29 was as expected

as this was used as the lysing agent and DNA was co-purified with *M. bovis* BCG DNA. Low-level detection of Mycobacteriophage Chy4 and Chy5 likely reflects either cross contamination, or sequence homology within phage clusters. As Mycobacteriophage Chy4 had 0 unique reads, this reading was likely a false positive. Mycobacteriophage Chy5 had 9 unique reads therefore this reading was likely due to minor contamination events as other, Mycobacteriophage were also being used in the same laboratory at the time. Minor reads assigned to *M. bovis* BCG represent conserved genomic regions, likely shared with *M. bovis* causing ambiguous alignments or misassignment during sequencing. Minor reads from non-mycobacterial species can be attributed to low levels of contamination, or again misassignment during sequencing due to sequence homology.

Results also showed that 435 different species were detected in the *M. smegmatis* DNA sample, in comparison to 229 different species in the *M. bovis* BCG sample and 40 different species for bacteriophage in Media Plus sample. As previously mentioned, abundance, number of reads and number of unique reads all need to be taken into careful consideration with these results. Although there were 435 different species and 229 different species detected for *M. smegmatis* and *M. bovis* BCG respectively, a lot of these species' assignments had very low numbers of unique and total reads ($\leq$ 10 reads for both total and unique reads). For the sample containing *M. bovis* BCG DNA, 92 % of species recorded had $\leq$ 10 total reads, whilst 97 % of species recorded had $\leq$ 10 unique reads. For *M. smegmatis* DNA, 85 % of species recorded had $\leq$ 10 total reads and 95 % of species recorded $\leq$ 10 unique reads. Therefore, the assignment of these species was likely due to background

noise, or low-level contamination. These findings highlight the need for careful interpretation of low read hits in metagenomic datasets.

In the samples containing bacteria, the target organism was never recorded to be of the highest abundance for either the *M. smegmatis* or *M. bovis* BCG samples. However, this may be expected as bacteriophage was used as the lysing agent, during the viral replication cycles it would be expected that viral DNA would be in higher abundance than that of the target DNA. Although *M. bovis* BCG was used, a higher number of reads was obtained for *M. bovis* and *M. tuberculosis* (Table 5.3) with 3109 reads and 3520 reads, respectively. There were only 8 reads in total and 0 unique reads for *M. bovis* BCG despite this being the template DNA used for sequencing. This may have been due to misassignment of *M. bovis* BCG reads as *M. bovis* due to similarity between genomes.

When *M. smegmatis* was used as template DNA, although mycobacteriophage was recorded as being in the highest abundance, *M. smegmatis* was the bacterium in the highest abundance within the sample.

Table 5.3 Whole genome sequencing results profile of *M. bovis* BCG DNA template extracted using Actiphage Rapid™ assay

NAME	TAXID	NUMBER OF READS	NUMBER OF UNIQUE READS	ABUNDANCE
MYCOBACTERIUM PHAGE D29	28369	7005	6068	0.984195
MYCOBACTERIUM PHAGE CHY4	1327947	1216	0	0.00651833
MYCOBACTERIUM BOVIS	1765	3109	385	0.00561622
MYCOBACTERIUM PHAGE CHY5	1327948	1230	9	0.00353288
MYCOBACTERIUM TUBERCULOSIS	1773	3520	66	5.64E-05
PROPIONIBACTERIUM ACNES	1747	10	1	2.57E-05
SULFOLOBUS SOLFATARICUS	2287	4	4	2.28E-05
MYCOBACTERIUM BOVIS BCG STR. PASTEUR 1173P2	410289	8	0	1.48E-05

MYCOBACTERIUM SMEGMATIS	1772	50	16	1.00E-05

Table shows first nine sequencing result reads from M. bovis BCG DNA extracted using Mycobacteriophage D29 in Acitphage Rapid™ assay as the lysis method for extraction of DNA for Illumina short read sequencing. TaxID is the taxonomy identifier number from the NCBI database.

5.2.4. Analysis and detection of single nucleotide polymorphisms from Sanger sequencing

Whole genome sequencing had limited success in detection of *Mycobacterium* when using Actiphage Rapid™ as a lysing agent due to lack of specificity to the target organism. As Sanger sequencing can be used with purified PCR products directed at a specific region within the target genome, thus providing specificity, experiments were performed to determine whether this approach could be used to see if Actiphage Rapid™ can be used as a lysing agent to identify polymorphic variations within IS*1311* of MAC species.

5.2.4.1. Detection and profiling of single nucleotide polymorphisms from Sanger sequencing data

DNA for each of the thirteen MAC strains was extracted from cells using Actiphage Rapid™ assay (section 2.4.4.). Endpoint PCR was used to amplify IS*1311* insertion sequences from each MAC strain (section 2.5.2.4.). Instead of restriction digest analysis, the presence of a 600 bp product was confirmed by gel electrophoresis and the PCR product was purified (section 2.5.1.1.). DNA concentration and integrity for each sample was determined by Nanodrop, and DNA samples with a A260/280 ratio between 1.8 and 2.0 were considered pure enough for sequencing. DNA was diluted to achieve a final concentration of 5 ng μl^{-1}, when necessary, to prepare the samples for sequencing. DNA underwent TubeSeq Sanger Sequencing (Eurofins; Germany) and data analysis performed to determine restriction fragment length polymorphisms (RFLPs) in MAC sequences to see if

these were consistent with results gained from PCR-REA of the insertion sequences (section 5.2.1.2).

For data analysis, a reference sequence was used as a benchmark for comparison against the subject sequences. The reference sequence was a *Mycobacterium avium* IS*1311* transposase gene (GenBank accession number U16276.1) taken from the NCBI database (National Institute of Health; USA). Sequenced MAC isolates were aligned to the reference strain using the DNADynamo software (Blue Tractor; UK). This analysis software was also used to perform virtual restriction digests of the DNA sequences using the same enzymes (*Mse*I and *Hinf*I) which were used for IS*1311* PCR-REA.

Two strains (M266/04 and 235G), despite being detectable on IS*1311* PCR as well as having appropriate DNA concentrations and A260/280 ratios when measured by Nanodrop, failed sequencing quality checks by Eurofins and therefore no DNA sequence data was obtained for these samples. These strains were therefore omitted from the analysis. Analysis of the remaining DNA sequences was performed using SnapGene Viewer (Dotmatics; USA) and DNADynamo software (Blue Tractor; UK).

A single nucleotide polymorphism within IS*1311* allows for discrimination between MAA strains and MAP strains by restriction enzyme analysis. At position 423/428, MAA strains have a T nucleotide whereas MAP strains have a C nucleotide. By having a T nucleotide at this position, a restriction site is created, recognised by restriction enzyme *Mse*I.

Examination of chromatogram data from sequencing results of the eleven MAC isolates showed that three strains (#7210, #7212 and *M. avium* type strain) had a single homozygous peak resolved as a T base at position 423/428. All other strains had a single homozygous peak resolved as a C base at the same position except for strain 213G which displayed a heterozygous double peak for both a T and a C base at this position (fig 5.6.). Strains #7210 and #7212 were confirmed to be *M. avium* subspecies *avium* whilst the remaining strains with a homozygous peak with a C nucleotide at this position were confirmed to all be strains of MAP.

For those strains with a homozygous peak for a C nucleotide at 423/428, SNP variation at position 222/224 was analysed. Typically, a polymorphism at position 222/224 can be used to allow for differentiation between members of the *avium* complex and subtype MAP strains into S-type, B-type and C-type. At this position, if the nucleotide is a T, a *Hinf*I restriction site is present, however if the nucleotide at this position were a C base this alters the ability for *Hinf*I to recognise this sequence as a restriction site and therefore the sequence would not be digested.

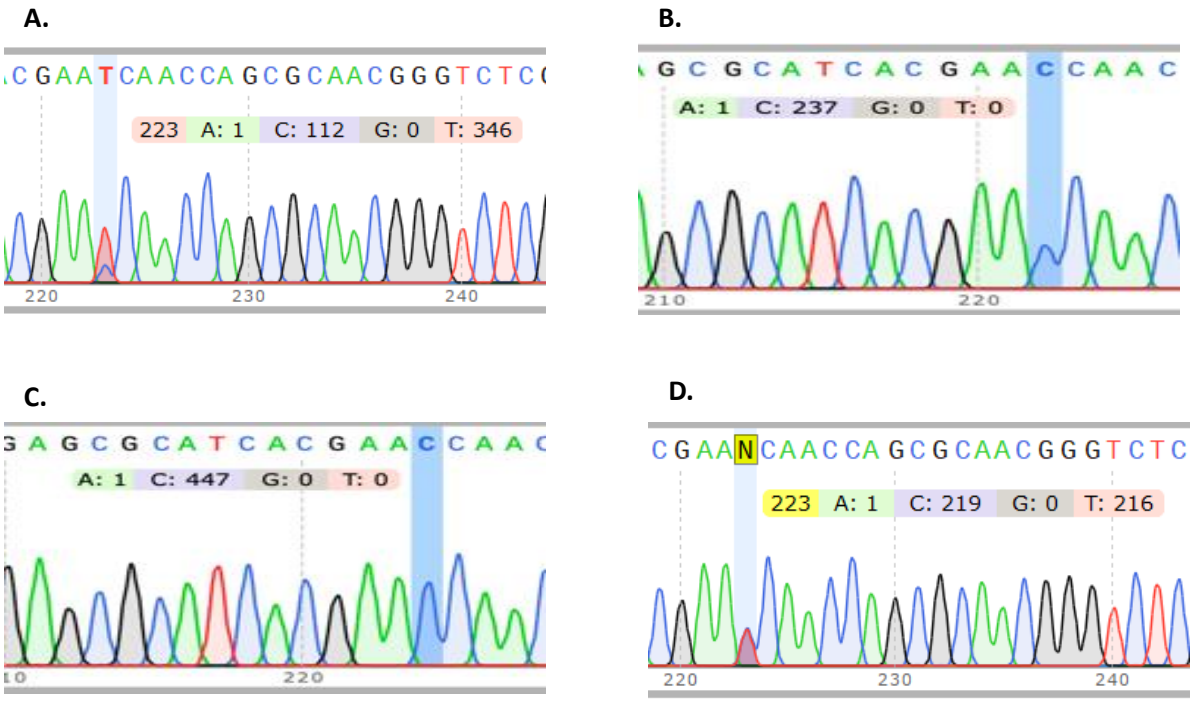
Strain 218 had a single homozygous peak resolved as a T nucleotide at position 222/224. Further analysis of strain 218 showed the presence of another *Hinf*I restriction site at location 288. The presence of these two restriction sites means that the DNA is restricted into three fragments: 67 base pair fragment, 218 base pair fragment and a 323 base pair fragment. This band pattern was observed through both a virtual restriction digest (DNADynamo; UK) and standard PCR-REA enabling this strain to be identified as a B-type strain.

S-type strains of MAP (B4 and M71/03) as well as *M. avium* subspecies *avium* (*M. avium* lab strain, #7210 and #7212) had a single homozygous peak resolved as a C nucleotide. C-type MAP strains had an 'N' at position 222/224 due to a heterozygous double peak (ATCC 19851, M112/05, M71/03, 213G, M110/05 and M113/05) (Figure 5.7).

Figure 5.6 Alignment of MAC sequences highlighting SNP at position 423/428

IS1311 insertion sequences of eleven MAC strains were aligned to show an SNP at 423/428. Presence or absence of a T nucleotide at this location determines whether there an MseI restriction site within the insertion sequence. All MAP strains have a C at position 423/428 apart from strain 213G which had an N, whereas three M. avium strains possess a T at this position (variation in sequence highlighted on image in red).

Figure 5.7 Chromatogram analysis for the detection of SNPs at 222/224 in MAC strains



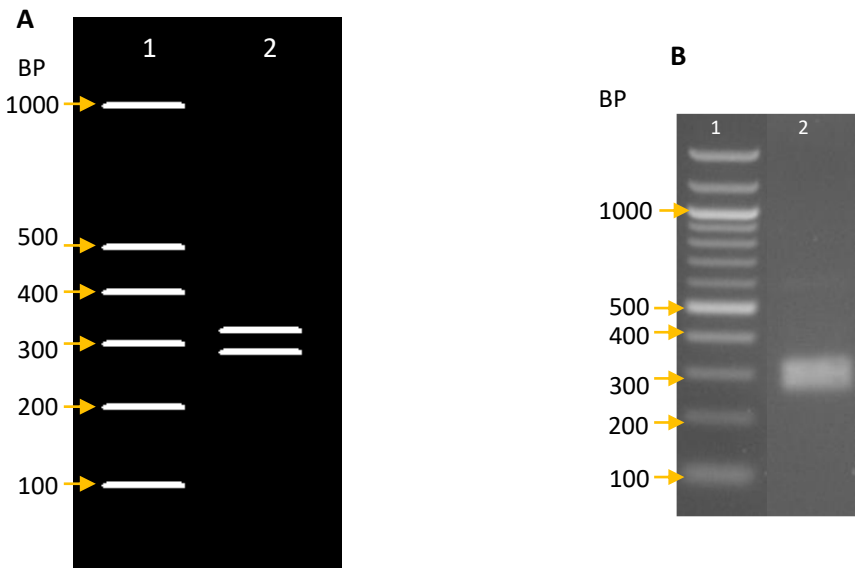
M. avium complex DNA was isolated using Actiphage Rapid™ assay and the IS1311 sequence amplified by IS1311 PCR. Amplicons were sent for Sanger sequencing and sequences analysed using SnapGene Viewer. Analysis of chromatogram data showed that ***(A)*** *strains labelled as B-type MAP by PCR-REA had a single homozygous peak resolved as a T nucleotide.* ***(B)*** *strains found to be S-type MAP by PCR-REA had a single homozygous peak resolved as a C nucleotide.* ***(C)*** *strains found to be M. avium subspecies avium also had a single homozygous peak resolved as a C nucleotide.* ***(D)*** *strains that were C-type by PCR-REA had a heterozygous double peak at position 222/224.*

5.2.4.2. Comparison of virtual and traditional restriction digests to identify sheep-type strains

Strain B4 was primarily identified as an S-type strain through PCR-REA (section 5.2.1.2.) and was shown to have a single homozygous peak for a C nucleotide at position 222/224 and a single homozygous peak for a C nucleotide at position 423/428 (section 5.2.4.1.). Further analysis of strain B4 DNA sequence shows the presence of a single recognition site for the restriction enzyme *Hinf*I at position 286. The presence of this singular restriction site means DNA is restricted into two fragments: a 285 base pair fragment and a 323 base pair fragment. This band pattern was observed through both a virtual restriction digest (DNADynamo; UK) and standard PCR-REA (figure 5.8).

The second MAP strain (M71/03) identified as S-type through a traditional PCR-REA had a peak fluorescent intensity score of 225 for a C nucleotide at position 222/224 however, also had a peak fluorescent intensity score of 68 for a T nucleotide at this position. Despite generating peak values for two nucleotides, the base at this position was called as a C nucleotide. Quality value (QV) score for this nucleotide was >40 as such there is a low probability (0.01 % chance) of the position being incorrectly labelled.

Figure 5.8. Virtual and traditional PCR-REA from an S-type MAP strain



*Virtual restriction digest **(Panel A)** and traditional restriction digest **(Panel B)** of IS1311 from MAP strain B4 using restriction enzyme HinfI.*

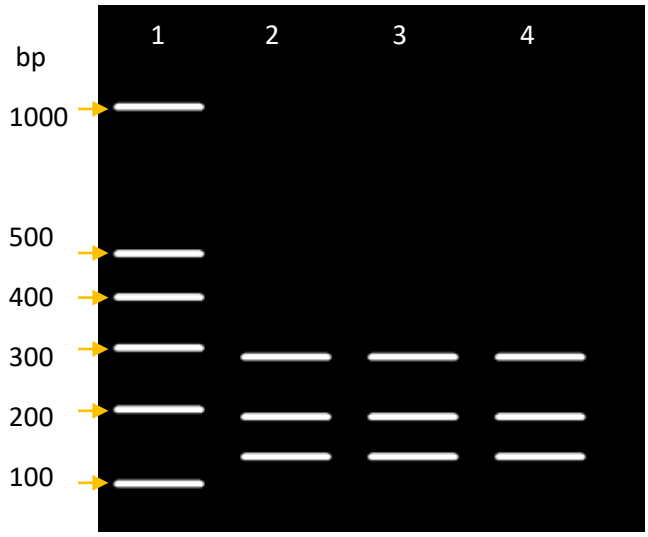
***Panel A**: DNA amplified by endpoint PCR targeting IS1311 in MAP strain B4 was sent for Sanger sequencing. Using the sequence data, a simulation agarose gel and restriction was run using DNADynamo software. L-R: Lane 1: Virtual molecular marker in 100 bp increments; Lane 2: Simulation restriction digest of IS1311 sequence from strain B4 using HinfI showing the presence of a 285 bp and a 323 bp product. **Panel B**: DNA amplified by endpoint PCR targeting IS1311 in MAP strain B4 and digested using HinfI restriction enzyme then visualised on a 1.5 % (w/v) agarose gel. L-R: Lane 1: 100 bp molecular marker; Lane 2: fragmented DNA products of strain B4 showing the presence of a 285 bp and a 323 bp product.*

5.2.4.3. Virtual and traditional restriction analysis to identify M. avium subspecies avium strains

From sequence analysis, strains with a single homozygous peak for a C base at position 222/224 in addition to a single homozygous peak for a T base at position 423/428 were preliminarily identified as *M. avium* subspecies. *avium* strains (section 5.2.4.1.). Out of the sequence data from eleven strains, three were identified as *M. avium* subspecies *avium* (#7210, 7#212, *M. avium* lab strain). The presence of a T base at position 222/224 creates a *Hinf*I recognition site within this sequence. In addition to this restriction site, an *Mse*I restriction site is also present when there is a T nucleotide at position 423/428.

In the presence of both *Mse*I and *Hinf*I, the DNA sequence of strains #7210, #7212 and *M. avium* lab strain can be digested into three fragments: a 134 bp fragment, a 189 bp fragment and a 285 bp fragment. Virtual restriction digest and standard PCR-REA results agreed with sequence analysis, showing that IS*1311* insertion sequence of all three strains could be fragmented in accordance with this band pattern (figure 5.9).

Figure 5.9 Virtual restriction digest of MAC strains presumptively identified as *M. avium* subspecies *avium* through sequence analysis



DNA extracted from MAC strains using Actiphage Rapid™ assay were amplified using IS1311 specific primers. Amplified PCR products were subjected to Sanger sequencing and virtual restriction digests simulated. Both virtual and standard restriction digests used restriction enzymes HinfI and MseI to digest the 608 bp PCR product. All three strains (M. avium lab strain, #7210 and #7212) were successfully identified as M. avium subspecies. avium through simulation of a virtual restriction digest due to fragmenting into a 134 bp fragment, a 189 bp fragment and a 285 bp fragment. L-R: Lane 1: virtual molecular marker in 100 bp increments; Lane 2: Simulation restriction digest of IS1311 sequence from strain #7210; Lane 3: Simulation restriction digest of IS1311 sequence from strain #7212; Lane 4: Simulation restriction digest from M. avium lab strain.

5.2.4.4. Sequencing chromatogram analysis of Cattle-type strains

After successful identification of two S-type strains (B4 and M71/03) and three MAA strains (#7210, #7212, *M. avium* lab strain) through sequence analysis, virtual restriction digest and standard PCR-REA (section 5.2.4.2. and section 5.2.4.3.) the remaining six MAC strains were analysed to see if they too could be categorised. These strains all resolved as C-type MAP strains through PCR-REA analysis (section 5.2.1.2.). For analysis of these samples, quality value (QV) scores and peak fluorescent intensity score information were both taken into consideration during analysis.

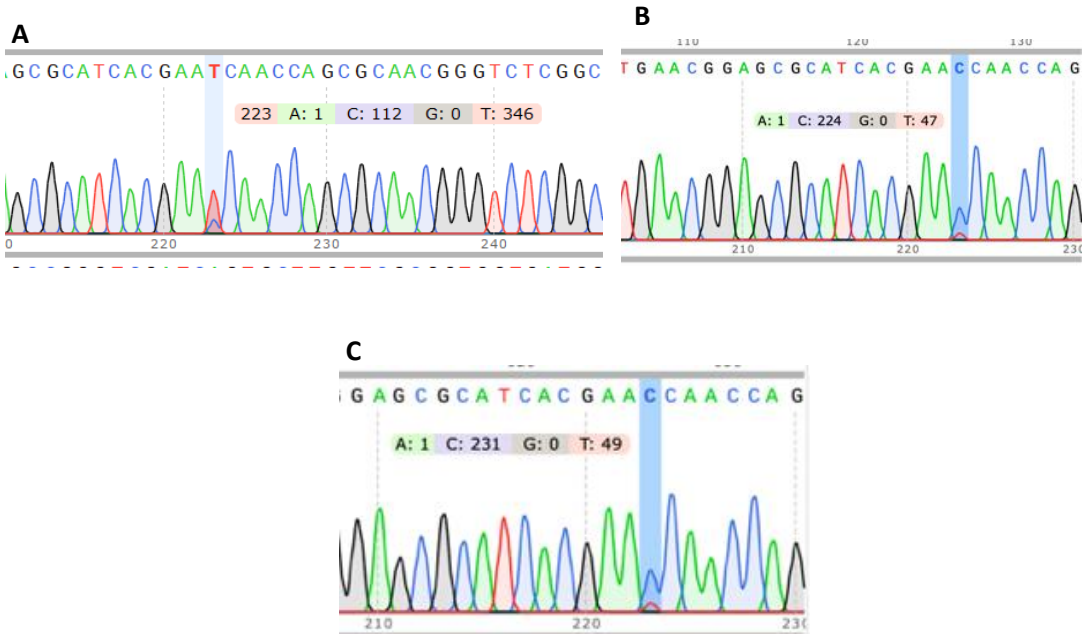
Cattle type MAP can possess both C and T nucleotides at position 222/224 within the multiple copies of IS*1311*. Two strains, ATCC 19851 and M112/05 were given an N nucleotide at this position, therefore chromatogram information was analysed to determine whether the N nucleotide was due to poor read quality or there was true a heterozygous double peak.

Analysis of these two strains showed that the N nucleotide at this position was because of a heterozygous double peak for both C and T bases. Both strains had a high QV score (QV > 40) (Figure 5.10 A) at this position suggesting there is a low probability (0.01 % chance) this position was called incorrectly. This suggests strains ATCC 19851 and M112/05 possess multiple copies of both C and T allelic variants at position 222/224 within IS*1311*. Copies that have a T nucleotide at position 222/224 have a *Hinf*I restriction site, whereas the presence of a C nucleotide at position 222/224 removes the recognition site.

The remaining three strains which were all C-type MAP through PCR-REA but did not have an N nucleotide at position 222/224 were analysed. Strain 218 had a peak fluorescent intensity score of 112 for a C nucleotide and a peak fluorescent intensity score of 346 for a T nucleotide (QV = 34) (Figure 5.10 B). Through virtual restriction digest, this makes strain 218 a B-type strain as position 222/224 was called as a T nucleotide which creates a *Hinf*I recognition site.

Strains M110/05 and M113/05 both had peak fluorescent intensity scores >230 for a C nucleotide and <50 for a T nucleotide (figure 5.10 C). Quality value score for both strains was 31 therefore there is approximately a 0.1 % chance of these bases being called incorrectly. Both strains had a C nucleotide called at position 222/224 meaning there is no *Hinf*I recognition site, making these two strains S-type strains by way of virtual restriction digest.

Figure 5.10 Chromatogram analysis showing allelic variation at 222/224 for three MAP strains



Chromatogram analysis showing allelic variation at position 222/224 of the IS1311 insertion sequence for three different MAP strains which were all shown to be C-type strains through PCR-REA. ***A.*** *Strain 218 has an asymmetrical double peak with the nucleotide called as a T. Chromatogram analysis shows this base has an intensity value of 112 for a C nucleotide and an intensity value of 346 for the T nucleotide.* ***B.*** *Strain M113/05 has a C nucleotide resolved at position 222/224. The C nucleotide has an intensity value of 224 compared to an intensity value of 47 for T nucleotide.* ***C.*** *Strain M110/05 also has a C nucleotide at position 222/224 with an intensity value of 231, compared to T nucleotide with an intensity value of 49 at this position.*

5.2.5. Use of qPCR dissociation curves to detect restriction fragment length polymorphisms in *Mycobacterium avium* complex

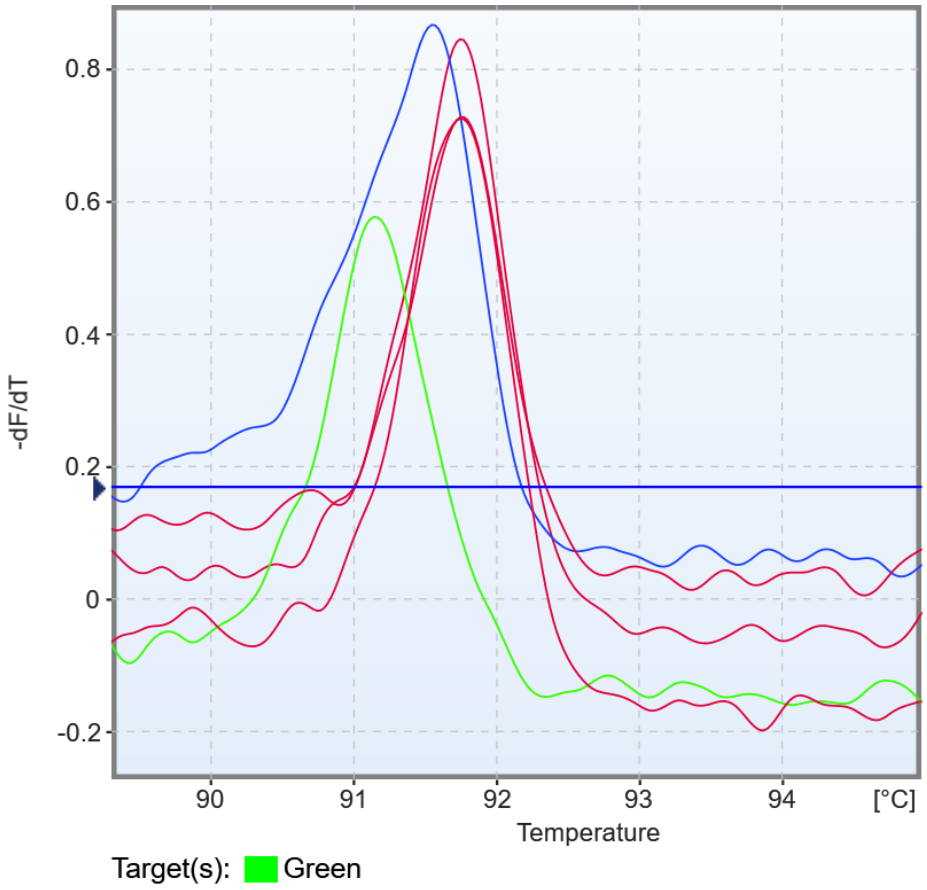
Although sequence analysis and virtual restriction digests were shown to successfully identify S-type, B-type and *M*. *avium* subspecies. *avium* subtypes, due to the complexity of SNP variation, Sanger sequencing and virtual restriction digest methods were unable to identify C-type strains (section 5.2.4.). However, using SNP information, primers were designed to detect a section of IS*1311* which included these SNPs that differentiate the MAC strains and subtypes to determine whether qPCR dissociation curves could identify polymorphisms within the IS*1311* sequence of different subtypes of MAC. Both melt curve analysis and high-resolution melting (HRM) methods were used. Both methods involve raising the temperature of the reaction mixture containing amplified PCR products in small increments. For melt curve analysis, after the final step in qPCR the temperature was adjusted to 65 ˚C and held for 1 min 30 s before the temperature being raised by 0.5 ˚C every 5 s until temperature reached 95 ˚C. Fluorescent measurements were acquired on FAM acquisition channel continuously. For high resolution melting, after final qPCR step, the temperature was adjusted to 65 ˚C and held for 1 min 30 s before being raised in 0.1 ˚C increments every 1 s until a final temperature of 95 ˚C was reached. Fluorescent measurements were acquired on FAM acquisition channel continuously. Both melt curve and high-resolution melting analyses were performed on the Q-Rex software (Qiagen; Germany) using the 'melt curve' and 'high resolution melting' plug-ins.

A portion of the IS*1311* sequence which contains restriction sites for both *Mse*I and *Hinf*I was identified to be used for primer design. The sequence was entered into Primer Quest (Integrated DNA Technologies; USA) choosing the option 'two primers and intercalating DYE, qPCR' to identify candidate primers for the assay. Four primer pairs were identified by the software, all of which were then subject to a BLAST search analysis (NCBI; USA) to determine homology of the primer pair to MAC.

Out of the four primer pairs, the primer pair that had the best homology to MAC was chosen. This primer pair had a forward primer with 100 % homology with MAC, including strains of MAP isolated from both bovine and ovine hosts. The remaining three primer pairs had 100 % homologous matches to organisms other than MAC including *Pseudonocardia broussonetiae* and *Burkholderia pseudomallei* which are soil dwelling organisms known to infect cattle. The sequence information for primer pair chosen (IS*1311*F and IS*1311*R) to be used to aid in development of a melt curve analysis is listed in Table 2.1.

Results show that when the temperature was raised by 0.1 °C every 1 s using high resolution melting, a difference in peaks could be observed between C-type MAP strains, B-type MAP and MAA (Figure 5.11.). C-type MAP had a melt peak of 91.8 ˚C, B-type MAP had a melt peak of 91.6 ˚C And MAA had a melt peak of 91.1 ˚C. This suggests that the use of high-resolution melting analysis may be able to differentiate between subtypes of MAP based on their single nucleotide polymorphisms.

Figure 5.11 High-resolution melt analysis comparing dissociation of IS*1311* PCR product of C-type and B-type MAP and *M. avium* subspecies. *avium*



C-type MAP strains (M112/05, 218 and ATCC 19851) (__), B-type MAP strain (213G) (__) and M. avium subspecies avium (type strain) (__) were subject to Actiphage Rapid™ assay. Extracted mycobacterial DNA was then purified and SYBR Green qPCR with additional high resolution melting analysis performed to determine whether a difference in peaks could be observed between different MAC subtypes. Three different peaks were observed for the three different MAC strains. C-type MAP had a melt peak of 91.8 ˚C, B-type MAP had a melt peak of 91.6 ˚C And MAA had a melt peak of 91.1 ˚C.

5.2.6. Elucidating host range of Mycobacteriophage with *Mycobacterium avium* subspecies *avium* strains

The Actiphage Rapid™ assay currently utilises a broad host range Mycobacteriophage D29. When this assay is combined with IS*1311* targeted PCR it has shown to be successful in several applications including identification of members of the *M. avium* complex (section 5.2.1.) and to also subtype members of the complex to further differentiate strains (section 5.2.1. and section 5.2.4.). Therefore, experiments were performed to determine whether Actiphage Rapid™ assay could be suitable to investigate the host range of different Mycobacteriophage available in the laboratory collection which had not previously been characterised as being able to infect MAA strains.

Two *M. avium* subspecies *avium* (MAA) strains (#7210 and #7212), isolated from a patient presenting with symptoms of NTM infection were primarily identified using IS*1311* PCR-REA (section 5.2.1.2.). These strains were subject to Actiphage Rapid™ assay using seven different Mycobacteriophage (section 2.1.5.) as the lysing agent and detection performed by IS*901* specific PCR. IS*901* insertion sequence is exclusive to MAA and no other members of the *avium* complex.

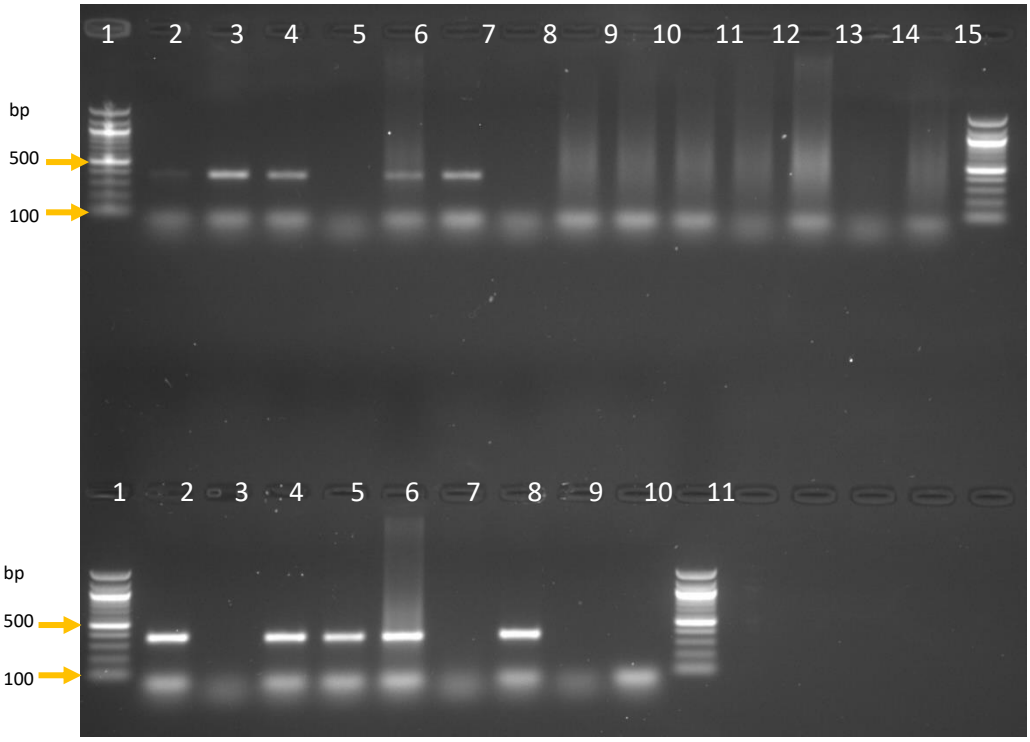
Despite Mycobacteriophage D29 being successfully used to detect strain #7212 with *IS1311* PCR, strain #7212 was not detected by IS*901* PCR by any of the seven Mycobacteriophage. Mycobacteriophage D29, bacteriophage U2 and bacteriophage U4 were able to be used for detection of both isolate #7210 and the *M. avium* reference strain. Bacteriophage U1 and U5 were only able to be used for

detection of isolate #7210 whilst bacteriophage U3 and TM4 were only able to detect *M. avium* lab strain (Figure 5.12).

To determine whether detection of the IS*901* insertion sequence from isolate #7210 and *M. avium* lab strain was due to high concentrations of extracellular DNA (eDNA) within the original stock cultures, Actiphage Rapid™ assays were repeated in both the presence and absence of bacteriophage.

For both strains with positive IS*901* results, (*M. avium* reference strain and #7210), only bacteriophage which presented a positive result from the previous PCR were used to determine effects of eDNA. For isolate #7210 Mycobacteriophage D29, U1, U2, U4 and U5 were used in the assays. For the *M. avium* strain bacteriophage D29, U2, U3, U4 and TM4 were used in the assays. A difference in band intensity could be seen for all samples with a brighter band observed for samples in the presence of the relevant bacteriophage compared to samples in the absence of bacteriophage (data not shown). This indicates that although some eDNA was present in cultures of these bacteria grown in the laboratory, the phage was able to infect these strains as more DNA was detected after phage treatment.

Figure 5.12 visualisation of IS*901* PCR products from *M. avium* isolates using seven different Mycobacteriophage for cell lysis



Type strain analysis of IS901 PCR products using DNA extracted using the Actiphage Rapid™ assay format and seven different bacteriophages. ***Top gel*** *Lane 1: 100 bp molecular size marker, Lanes 2-8; isolate 7210 infected with different bacteriophage: Lane 2, D29; Lane 3, U1; Lane 4, U2; Lane 5, U3; Lane 6, U4; Lane 7, U5 and Lane 8, TM4. Lanes 9-15; isolate 7212 infected with different bacteriophage: Lane 9, D29; Lane 10, U1; Lane 11, U2; Lane 12, U3; Lane 13, U4; Lane 14, U5 and Lane 15, TM4. Lane 16: 100 bp molecular size marker.* ***Bottom gel L-R****; Lane 1: 100 bp molecular size marker. Lanes 2-8 isolate M. avium infected with different bacteriophage: Lane 2, D29; Lane 3, U1; Lane 4, U2; Lane 5, U3; Lane 6, U4; Lane 7, U5 and Lane 8, TM4. Lane 9: Actiphage negative control (Media Plus), Lane 10: RNAase-free water (no-template PCR control), Lane 11: 100 bp molecular size marker*

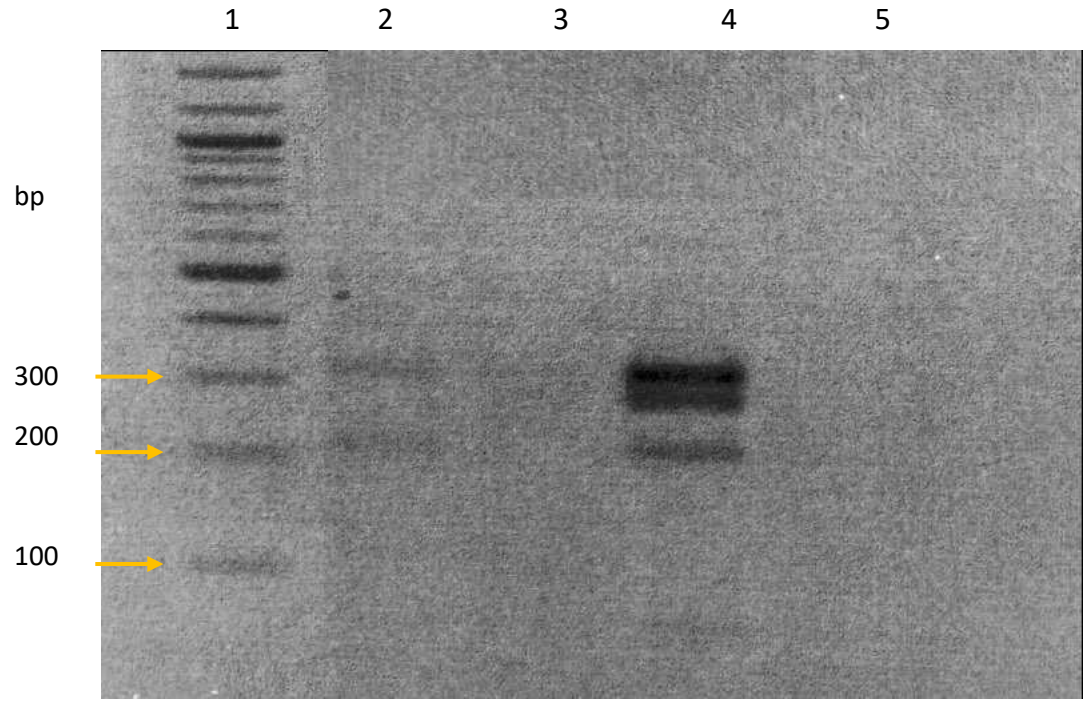
5.2.7. Real-life applications of Actiphage Rapid™ and restriction enzyme analysis to detect *Mycobacterium avium* complex infection in naturally infected animals

Actiphage Rapid™ assay has been used to successfully identify mycobacterial infection from the blood of numerous animals including cattle and deer (Swift *et al.*, 2020, Kubala *et al.*, 2021). The aim of this experiment was to demonstrate that in addition to being able to identify MAC infection from the whole blood of animals, REA can be used on the DNA recovered to identify strain sub-type.

PBMCs from cattle and parakeets of unknown MAC status were isolated as per section 2.2.3.2. and subject to Actiphage Rapid™ assay. PCR amplification of IS*1311* was performed on all samples (section 2.5.2.4.). A total of 5 µl of PCR product was analysed by agarose gel electrophoresis. A 600 bp band was observed for all samples confirming the presence of MAC infection within the animals. The remainder of amplified PCR product was subjected to REA using *Hinf*I and *Mse*I (section 2.5.2.4) and analysis of band patterns performed (information in table 5.2).

Based on restriction digest band pattern results, B-type MAP was detected from the whole blood of cattle after Actiphage Rapid™ and IS*1311* PCR-REA. Due to the nature of the sample being a clinical sample therefore expected low numbers of MAP cells present, bands on agarose gel were very faint. As a control, 10^2 MAP cells of known C-type lineage (strain ATCC 19851) were used as the Actiphage Rapid™ positive control (figure 5.13). *M. avium* subspecies *avium* has also successfully detected from whole blood of parakeets by Actiphage Rapid™ assay and IS*1311* PCR-REA due to the presence of a 285 bp, 189 bp and 134 bp band when restriction digest products were visualised after gel electrophoresis (figure 5.14).

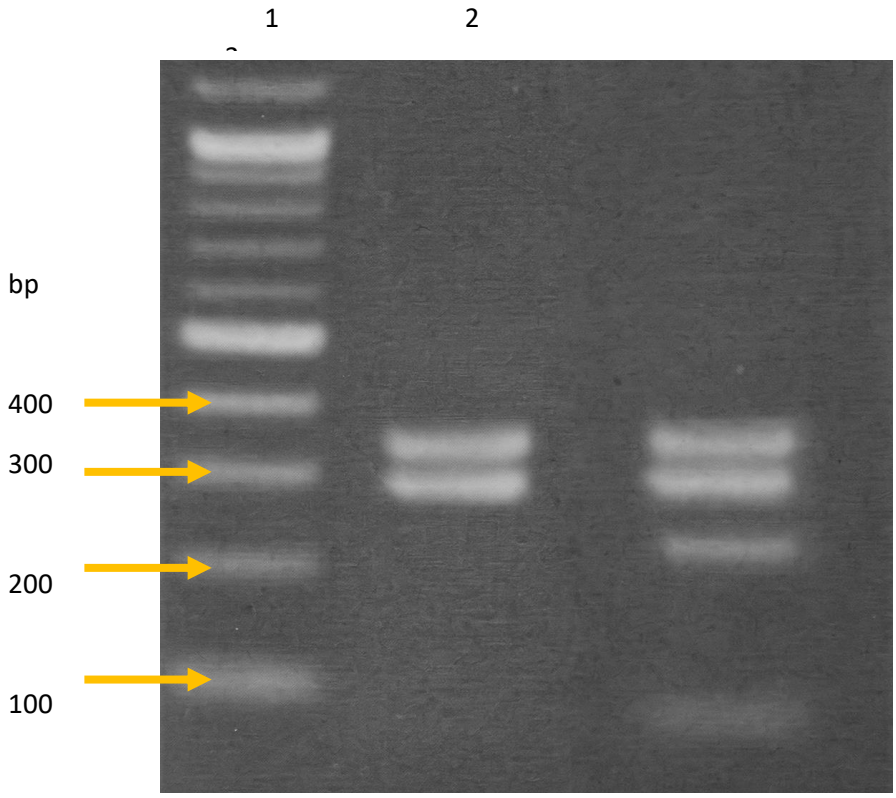
Figure 5.13 Visualisation of IS*1311* PCR-REA products from DNA extracted from PBMCs of cattle



Mycobacterial DNA was extracted from bovine PBMCs and signature IS1311 sequence amplified before enzyme digest by HinfI and MseI. Restriction fragments of PCR products were then visualised on a 1.5 % (w/v) agarose gel following electrophoresis and band patterns analysed to determine MAP lineage.

Samples loaded on the gel were: Lane 1, 100 bp molecular marker, Lane 2, clinical sample 1 from cattle whole blood, Lane 3, clinical sample 2 from cattle whole blood, Lane 4, 10^2 MAP ATCC 19851 positive Actiphage Rapid™ assay control (C-type), Lane 5, RNase-free water used as a non-template negative control

Figure 5.14 Visualisation of IS*1311* PCR-REA products from PBMCs extracted from parakeets



Mycobacterial DNA was extracted from the PBMCs of parakeets and IS1311 signature sequences detected by PCR before restriction digest using HinfI and MseI restriction enzymes. Restriction fragments were visualised by gel electrophoresis on a 1.5 % (w/v) agarose gel and band patterns analysed.

Samples loaded on the gel were: Lane 1, 100 bp molecular marker, Lane 2, clinical sample 1 from parakeet whole blood, Lane 3, clinical sample 2 from parakeet whole blood

5.3. DISCUSSION

5.3.1. Use of Actiphage Rapid™ assay and sequencing for detection of mycobacteria

The work described in this Chapter explored additional applications of Actiphage Rapid™ assay including subtyping members of *M. avium* complex through sequencing, melt curve analysis and PCR-REA. Although sequencing and virtual restriction digests proved partially successful, they were unable to identify C-type strains due to the complexity of SNP variation within their IS*1311* and a lack of clear heterozygous double peak present in potential C-type strains of MAP (section 5.2.4.4.). Discrepancies in peaks could be due to the nature of the presence of variations in IS*1311* in C-type MAP strains. IS*1311* is present in multiple copies within the genome and in C-type strains within these multiple copies, C-type MAP possess copies of IS*1311* which have a T nucleotide at position 222/224 but also, they have copies with a C nucleotide at this position too (Bryant *et al.*, 2016). It remains unclear how many copies of the C-T allelic variation of IS*1311* C-type MAP contain, although the present results provide evidence to suggest there is variation of the distribution of C and T nucleotide variation between different strains, based on base calling scores from the different C-type MAP strains from Sanger Sequencing (section 5.2.4.4.). Strains of MAP that gave an ambiguous result through sequence analysis and virtual restriction digest could still be C-type strains but may have copies of both C-T allelic variations of the insertion sequence at an uneven distribution, which gave the smaller peaks. This would also explain why the quality value score for these strains were lower compared to other strains which were

easily identifiable. If some C-type strains only possessed one copy of an SNP compared to up to nine of the other variation, for example, then after exponential PCR amplification, there will be significantly fewer copies of the lower copy number allelic variant of the insertion sequence.

The complexity of the IS*1311* composition in C-type strains means they are more challenging to subtype by the Sanger sequencing methods used in this chapter, in comparison to B-type and S-type MAP strains. The results demonstrate that Sanger sequencing is not a reliable option for an accurate differentiation of C-type MAP subtypes when using IS*1311* as the target. At present, it is not clear whether the discrepancies between PCR-REA and Sanger sequencing results is due to the polymorphic nature of IS*1311*, or because of the potential unreliability of IS*1311* PCR-REA. Bryant *et al.* (2016) have previously highlighted through WGS of 141 global MAP isolates that some strains identified as C-type strains had an S-type *IS1311* PCR-REA profile, concluding that PCR-REA using IS*1311* as the target sequence may not always be the most reliable for differentiation of MAP subtypes.

To answer this question, WGS analysis of the MAC strains would enable further insight into this variation and distribution amongst different MAP strain subtypes. Additionally, other PCR targets can be utilised and compared against IS*1311* PCR-REA to determine if there is a better way to distinguish C-type and S-type MAP strains. Large sequence polymorphisms (LSPs) are alternative polymorphic sequences to SNPs, that show variation between S-type, C-type and *M. avium* strains (Bannantine *et al.*, 2012). The presence of $LSP^{A}20$ is unique to the C-type MAP genome, presence of $LSP^{A}4$-II is unique to S-type MAP and the absence of

LSPA17 is a polymorphism unique to *M. avium* subspecies *avium*. Semret *et al.* (2006) have previously shown that by targeting these specific regions of the genome using a multiplex PCR approach of one forward and two reverse primers, it is possible to differentiate between MAC subtypes.

Despite discrepancies with the technique of using amplified IS*1311* PCR products for Sanger sequencing to determine subtype of MAC strains, the results demonstrate that Actiphage™ Rapid assay can be used as the lysing agent process to allow for the detection of mycobacteria for this purpose.

Although previously there have been successful attempts at using WGS for detection of mycobacterial infection and the detection of SNPs within sequences when DNA extraction was performed using mechanical shearing methods (Witney *et al.*, 2017), when using bacteriophage as the lysing agent, the results of this experiment using WGS was partially unsuccessful. As bacteriophage are propagated using *M. smegmatis*, trace amounts of *M. smegmatis* DNA can be found in the bacteriophage stocks. WGS results found that DNA concentrations of off-target DNA sequences were too high, pulling up multiple hits including D29. Although this shows that bacteriophage can be used as a lysing agent for WGS, using mycobacteriophage and WGS as a diagnostic aid is unsuitable when the infection cause is unknown due to the high number of different species recorded at varying levels of abundance. Although this was only tried once, this could be repeated several times using varying concentrations of phage and cells to see if contaminating DNA could be diluted out of the sample.

5.3.2. high-resolution melt analysis for subtyping mycobacteria

High-resolution melt analysis approaches to subtyping MAC strains have shown some preliminary success, however, the qPCR assay is yet to be fully optimised. For there to be more confidence in this assay, a much larger sample size needs to be used. If a melt curve analysis approach can be fully optimised to successfully detect SNP variations within MAP sequences consistently and accurately, there is potential for these fundamentals to be applied to MTBC to extrapolate additional data, such as drug resistance of MTBC isolates. Most antibiotic resistance mutations within the MTBC genome are present in only one or two genes within 12 bp of the start codon of the resistance gene (Walker *et al.*, 2022). With this information and through analysis of the WHO catalogue of *M. tuberculosis* complex mutations associated with drug resistance, signature sequences could be identified as a target for qPCR melt curve analysis to enable differentiation of drug resistant and drug sensitive MTBC isolates. This would significantly enhance the diagnostic process of MTBC infection allowing for a more targeted approach to treatment as well as reducing costs and time associated with WGS which is often required to obtain this data.

6.3.3. Isolation and identification of mycobacterial strains causing infection from humans

Using Actiphage™ Rapid assay with five uncharacterised Mycobacteriophage has demonstrated the ability of identification of multiple strain infections using phage typing. At present there is very little research into strain typing using phage,

with papers reporting on this in the 1970's and using more traditional plaque assay techniques similar to the Actiphage Core assay (Mankiewicz and Liivak, 1975, Bates *et al.,* 1976). Whilst these early reports of identifying multiple strain infections were performed on the identification of MTBC, the current research has demonstrated that the use of different phage with Actiphage Rapid™ assay can also be useful in identifying multiple strain infections from MAC. This research has also shown how Actiphage Rapid™ assay has been used to identify avirulent strains of MAC co-infecting a human patient.

In summary:

1. Actiphage Rapid™ assay was successfully used to aid in sub-typing MAC strains
2. Sanger sequencing of IS*1311* was unreliable for differentiating C-type MAP due to complex allelic variation and mixed SNP copies
3. Using mycobacteriophage as a lysing agent for WGS resulted in high contamination from *M. smegmatis* DNA, reducing accuracy from a diagnostic perspective
4. HRM qPCR showed promise for rapid sub-typing of MAC

CHAPTER 6

USE OF ACTIPHAGE RAPID™ ASSAY FOR DETECTION OF MYCOBACTERIA FROM MILK

6.1. INTRODUCTION

Previous chapters have demonstrated an analysis of the Actiphage Rapid™ assay from whole blood samples. However, many of the early studies utilising the Actiphage® Core assay and endpoint PC focussed on detection of Mycobacteria in milk samples (Stanley *et al.*, 2007, Botsaris *et al.*, 2010, Gerrard *et al.,* 2018). At the time of this study, using Actiphage Rapid™ assay with qPCR to detect mycobacteria from milk samples remained under explored.

Milk somatic cells are a collection of cells found within the milk of animals made up of milk producing epithelial cells from glands and leukocytes from blood. Whilst somatic cells are omnipresent in the milk of animals, levels can fluctuate throughout the animal's lifetime and can often be used as an indication of mammary health and milk quality of animals (Alhussien and Dang, 2018). For example, an elevated level of epithelial cells is because of early and late lactation, whereas higher levels of leukocytes are caused by infections such as mastitis or Johne's disease. Furthermore, milk and dairy products have been identified as potential sources of *M. bovis* infection in humans, particularly in regions where bovine tuberculosis is endemic (Tigre *et al.,* 2011, Silva *et al.,* 2018). Therefore, the development of sensitive and specific methods for the detection of *Mycobacterium* species from milk is essential for ensuring food safety and protecting public health. As Mycobacteria are able to persist within leukocytes after phagocytosis (Hosseini *et al.*, 2016), isolated somatic cells make a good target for the Actiphage Rapid™ assay. Despite successes of using Actiphage® core assay for detection of MAP from milk somatic cells (Botsaris *et al.*, 2013, Gerrard *et al.*, 2018), there are many known

components of milk that have shown to affect bacteriophage infection of other bacteria. For instance, *Listeria* phage A511, has been shown to be less effective at infection and lysis of *Listeria* spp. in the presence of fat, whey and casein fractions of milk (García-Anaya *et al*., 2020). Similarly, both O'Flaherty *et al.* (2005) and Gill *et al.* (2006) found interactions of bovine whey proteins and fat globules with *S. aureus* cell surface inhibited bacteriophage K interactions with the bacterium. Milk proteins can interact with bacterial cellular membranes to prevent attachment of bacteriophage, in turn reducing sensitivity of phage-based PCR detection assays. Therefore, for optimisation and development of Actiphage Rapid™ assay for detection of Mycobacteria from milk somatic cells, these factors need to be taken into consideration, as first described by Stanley *et al.* (2007).

Culture-based methods have been traditionally used for the detection of *Mycobacterium* species from milk. These methods involve the growth of bacteria on specific media, such as Herrold's Egg Yolk Medium (HEYM), Lowenstein-Jensen or Middlebrook agar, followed by identification based on biochemical tests and/or PCR. However, these methods are time-consuming, taking several weeks to obtain results, require antimicrobials to suppress growth of other organisms and may lack sensitivity due to the low concentration of mycobacteria in milk samples (Spahr and Schafroth, 2001). Commercial kits are currently available that are specifically designed to overcome the issues of traditional culture methods being time consuming and allow for a rapid isolation and detection of Mycobacteria from milk. Three of these kits are described in Table 6.1. The issue with these kits is there is still a lack of sensitivity, with one of the kits specifying a sensitivity from milk of 84

% (*Myocbacterium avium* subspecies *paratuberculosis* antibody test kit, Veterinary Medical Research and Development; USA), whilst Selim *et al.* (2013) found that the limit of detection using Adiapure® PARATB milk kit (Adiagene; France) was between 100 and 1000 MAP cells per ml, dependant on target primers used despite Adiagene listing the sensitivity as between 6 and 60 Map cells per ml. Furthermore, these diagnostic kits use ELISA testing or total DNA extraction from milk, neither of which can discriminate between active or past infection.

More recently, next-generation sequencing (NGS) technologies have been applied for the detection of *Mycobacterium* species from milk. NGS-based methods can detect and identify multiple *Mycobacterium* species in a single sample, providing a more comprehensive assessment of the microbial community present in milk. NGS-based methods have been used to detect *M. bovis* and other *Mycobacterium* species from milk samples, showing high sensitivity and specificity (Carneiro *et al.*, 2022). However, these methods may be expensive and require specialized equipment and expertise.

The work in this Chapter aimed to explore whether the use of Actiphage Rapid™ assay could provide a method of detection which can be both rapid and sensitive to allow for the detection of *Mycobacterium* in milk samples from the dairy industry. Experiments were also performed to determine whether the effect of inhibitory milk components on the Actiphage® assay could be minimised to allow for optimised Actiphage Rapid™-PCR detection of *Mycobacterium* species.

The aims of this chapter are as follows:

1. Determine whether Actiphage Rapid™ assay could be used for detection of *Mycobacterium* from milk samples
2. Investigate whether milk components inhibitory to Actiphage Rapid™ assay could be minimised during somatic cell extraction

6.1. Commercially available kits designed for detection of MAP from milk samples

NAME	DETECTION METHOD	SAMPLE TYPE	SENSITIVITY	SPECIFICITY	COMPANY
***MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* ANTIBODY TEST KIT**	ELISA	Bovine sera and milk, caprine sera	93.11 % bovine serum 84.8 % bovine milk 100 % caprine serum	90 % bovine serum 82.1 % bovine milk 95.5 % caprine serum	Veterinary Medical Research and Development
CATTLETYPE® MAP AB	ELISA	Cattle, sheep and goat serum, plasma and milk	Not specified	Not specified	Qiagen
ADIAPURE PARATB MILK	PCR	Bovine milk	6-60 bacteria ml^{-1}	Not specified	Adiagene

Details of three different commercially available kits for the detection of Mycobacteria from milk. Two kits (Mycobacterium avium subspecies paratuberculosis antibody test kit and CattleType® MAP Ab) are ELISA immunological tests whilst Adiapure ParaTB milk is a PCR-based detection method.

6.2. RESULTS

6.2.1 Adaptations of current cream fraction removal methods from milk to limit Actiphage® inhibition

The method for isolation of somatic cells from milk in preparation for Actiphage® assays (section 2.2.3.3.) involves a centrifugation step to pellet somatic cells and separate the cream layer from the milk. The cream layer is then removed using a sterile spatula. This often leaves residual cream within the conical sampling tubes which poses a risk of contributing to Actiphage® inhibition as fat globules amongst other milk components are known inhibitors of phage. To determine whether there is a more effective method of removing this cream layer and minimising risks of Actiphage® inhibition experiments were performed to investigate different filters and their ability to aid in cream removal.

6.2.1.1. Use of plastic and mesh inserts for conical tubes to aid in cream removal

Different methods of cream removal were compared to the standard method currently used (using a sterile spoon to remove cream fraction as per section 2.2.3.3.). For samples where mesh inserts and strainers were used, these were placed in 50 ml conical tubes prior to addition of milk and centrifugation. These cell strainers and mesh inserts were adapted to enable them to sit inside the 50 ml conical based tubes used for testing. Whole, pasteurised milk was used, purchased from a local supermarket, and each sample experimentally inoculated with 10^3 *Mycobacterium avium* subspecies *paratuberculosis*. Six different variations of mesh inserts or strainers were tested and compared against the standard method of cream removal,

and as a control, milk not experimentally inoculated with MAP was also tested. After somatic cell extraction, Actiphage Rapid™ assay was performed on all samples (section 2.4.4.) and IS*900* qPCR performed to detect presence of MAP from samples.

The efficacy for each method in removing the cream layer is described in Table 6.2. Starlab cell strainers (Starlab; UK) overall performed better in removing the cream layer in comparison to Costar™ Netwell™ inserts (Corning; USA). When using Starlab cell strainers, less additional cream removal was required. All samples where Starlab cell strainers were used, irrespective of cell strainer size, only required a quick wipe of the conical tubes after cell strainer removal to discard any residual cream. In contrast, Costar™ Netwell™ samples required further handling with the need for a sterile spoon to remove the large excess of cream which was not trapped on the Costar™ Netwell™ inserts. However, this difference in results is most likely not due to different brands of strainers but rather the Costar™ Netwell™ inserts were a larger size (400 µm mesh size) than all Starlab cell strainers used (100 µm to 40 µm mesh size).

For detection of MAP from these samples, qPCR was performed as per section 2.5.3.5. No internal amplification controls were used in these experiments, therefore signs of qPCR inhibition could only be monitored by analysis of the standard curve which was performed on the same qPCR run as the samples. qPCR efficiency was calculated to be 148 % suggesting an over-efficiency of the qPCR reaction, indicating that inhibition was occurring.

Despite the use of cell strainers improving the efficacy of cream layer removal from centrifuged milk, there was no difference in qPCR detection of these samples

compared to those using adapted Costar™ Netwell™ mesh inserts. All samples had a Cq in the range of 28.29 and 34.82 irrespective of the cream removal process implemented (Table 6.3.). Mean Cq value for samples using strainers and inserts was Cq 31.82 (SD = 2.14). One sample; Starlab 100 µm nylon cell strainer, did not generate a signal that rose above the threshold and remained undetected. The positive control sample (10^3 MAP cells in Media Plus) had a Cq value of 24.61. There was a ΔCq of 7.21 cycles between mean Cq for samples using strainers and inserts and this positive control sample. This suggests that a component within milk is causing less sensitive detection of MAP from milk when using Actiphage Rapid™ assay.

Resolution of qPCR products on a 1.5 % (w/v) agarose gel confirmed qPCR results as there was no difference in band intensity for the samples that gave a Cq value between 28.59 and 34.82. The sample that remained undetected by qPCR also had no visible bands after electrophoresis, suggesting a lack of amplification from the inoculated sample with no Cq value was because of inhibition.

Table 6.2. Observations from using different mesh inserts and strainers to improve detection of cream fraction removal from whole milk

SAMPLE ID	INSERT	TESTING CONDITION	COMMENTS AFTER CENTRIFUGATION (2500 X G FOR 15 MIN)
1	Corning Costar Netwell Insert	24 mm plastic hoop insert, no polyester mesh filter	Cream only partially removed; sterile spoon had to be used to aid in cream removal
2		24 mm plastic hoop insert with 440 µm polyester mesh filter	Small amount of cream stuck to mesh filter; sterile spoon was needed to aid in cream removal
3		440 µm polyester mesh filter, no plastic hoop inserts	Most cream was stuck to mesh filter
4	n/a	Control sample using spoon to remove cream layer	Control sample where all cream was removed with a sterile spoon
5	Starlab cell strainer	100 µm nylon cell strainer	Most of the cream was successfully caught by the cell strainer and removed. Conical tubes only required a wipe to remove excess cream

6		70 µm nylon cell strainer	Cream successfully removed by cell strainer. Conical tubes only required a wipe to remove excess cream
7		40 µm nylon cell strainer	Cream successfully removed by cell strainer. Conical tubes only required a wipe to remove excess cream
8	n/a	Control sample with no spiked MAP cells	Control sample here all cream was removed with a sterile spoon

Different methods aiding in the removal of the cream fraction of whole milk, which is known to be inhibitory to phage were tested. Eight different conditions were tested including adapting Costar Netwell mesh inserts (Corning; USA) and Starlab cell strainers (Starlabs; UK). Unless specified, 10^3 MAP cells were spiked into milk prior to cream separation. After milk separation, Actiphage Rapid™ and MAP specific qPCR were performed.

Table 6.3. qPCR data for detection of MAP from milk using different methods to remove cream fraction

SAMPLE ID	CQ VALUE (FAM)
1	29.82
2	31.57
3	31.35
4 (CONTROL, SPOON REMOVAL)	34.14
5	ND
6	34.29
7	28.59
8 (CONTROL, SPOON REMOVAL, NO MAP)	33.00
MEDIA PLUS NEGATIVE ASSAY CONTROL	ND
10^3 MAP CELLS POSITIVE ASSAY CONTROL	24.61
WATER NON-TEMPLATE QPCR CONTROL	ND

Table shows qPCR results gained when 10^3 MAP cells were inoculated into 50 ml pasteurised whole milk. Different cell strainers and inserts were used to aid in removal of cream fraction during centrifugation (details of each method is listed in Table 7.2.). Sample ID correlates to sample ID from Table 6.2. MAP specific qPCR was performed and fluorescence measured in the FAM acquisition channel. No internal control was used therefore sample inhibition could not be monitored. ND = not detected. 10^3 MAP cells in Media Plus were used as a positive control.

6.2.1.2. Use of different volumes of milk to improve cream fraction removal

Results from section 6.2.1.1. showed that although using mesh inserts with a mesh size ranging from 100 µm to 40 µm improved the efficiency of cream fraction removal from whole milk, overall, there was no difference in qPCR detection of MAP suggesting that none of these methods improved or hindered Actiphage Rapid™ and MAP detection. Therefore, experiments were performed to determine whether altering the sample size of milk from the standard 50 ml to 20 ml would improve cream fraction removal without inhibiting detection of MTBC.

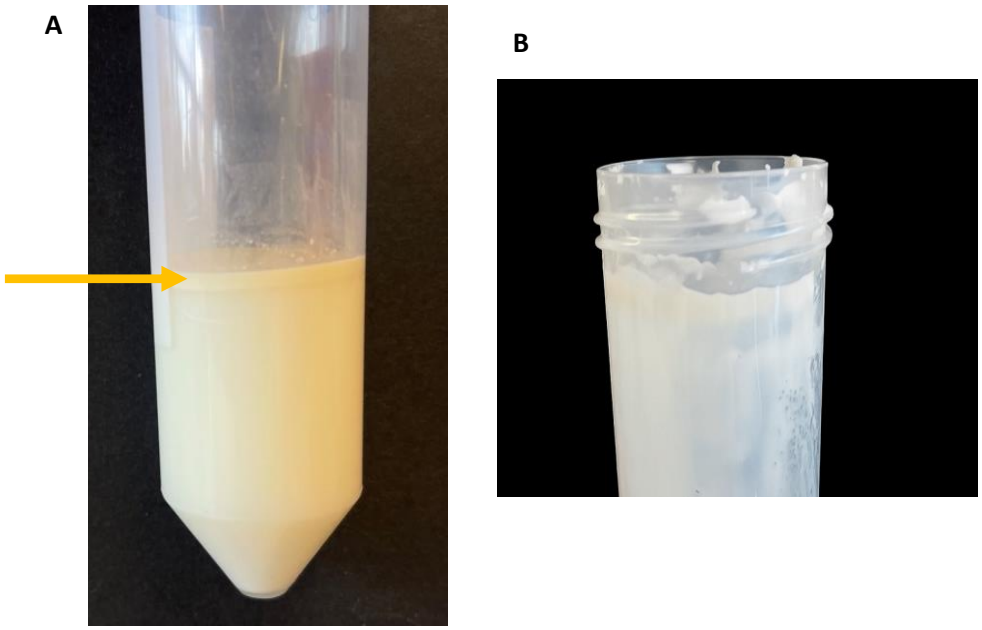
At the time these experiments took place, PBD Biotech Ltd. had been contacted by a commercial farm to test their raw bovine milk for MTBC. Therefore, the following experiments use this raw milk from both bulk milk tanks and individual cattle from a suspect TB herd, instead of supermarket pasteurised whole milk as used in section 6.2.1.1.

A sample volume of either 50 ml or 20 ml of naturally infected raw bovine milk was processed as per section 2.2.3.3. to isolate somatic cells. After somatic cell isolation, samples were subject to Actiphage Rapid™ assay (section 2.4.4.) and *IS6110* endpoint PCR (section 2.5.2.6.).

Using a smaller volume of milk made disposing of the cream layer easier and left less residual cream on the sides of the sampling tubes (Figure 6.1). As expected, the pellet left over after centrifugation, which contains the somatic cells, was smaller however this did make resuspension in Actiphage® reagent easier, reducing the risk of not fully resuspending the sample in Actiphage® reagent. It was also found that

there was less cellular debris to block filter columns, which are used during centrifugation of Actiphage Rapid™ assays after the 3.5 h incubation, again reducing the risk of mycobacterial DNA getting trapped within this debris on top of the filter.

Figure 6.1. Separation of 20 ml and 50 ml raw bovine milk after centrifugation



*Naturally infected raw bovine milk was centrifuged in either 20 ml (**A**)or 50 ml (**B**) volumes to separate cream fraction and pellet somatic cells. Cream fraction (→) when using 20 ml of milk was a lot smaller and easier to remove from the conical tube due to less adherence on the side of the tubes. When using 50 ml of milk, a lot more residual cream was left over and adhering to the sides of the conical tubes after removal of supernatant and cream fraction with sterile spoon.*

6.2.1.3. Determining sensitivity of MTBC detection using smaller sample volume of milk

To determine whether a smaller sample volume of milk would affect the sensitivity of the Actiphage® assay, experiments were performed with bovine milk samples from cows from a herd that was under TB restriction.

Three samples (FR83, FR182 and FR186) were from individual cows that had reacted positively to the single intradermal comparative cervical tuberculin test (SICCT) test, whilst one sample (Jersey) was a bulk milk sample collected from a herd of cattle that had not been subject to SICCT. Somatic cell extraction was performed on all samples as per section 2.2.3.3. using the standard 50 ml of milk, and again as per section 2.2.3.3. with the modification of using a sample volume of 20 ml milk instead. Using a smaller volume of milk made removal of cream fraction layer quicker and easier by decanting supernatant and cream fraction leaving no residual cream in sampling tubes, in comparison to a larger sample volume that required removal by sterile spatula and further wiping down of tubes to remove any residual cream. After somatic cell isolation, all samples were subject to Actiphage Rapid™ assay as per section 2.4.4. and detection of MTBC performed using IS*6110* specific PCR (section 2.5.2.6.). As a comparison, all samples were also subject to Actiphage Core assay (section 2.4.6.) and any presumptive positive samples identified by the formation of plaques on bacterial lawns. DNA was then extracted from these plaques (section 2.5.1.2.) and tested using the same IS*6110*-specific PCR that was used for the Actiphage Rapid™ samples.

Using Actiphage Core assay, the Jersey bulk milk samples produced 5 plaques, FR182 had 2 plaques, FR83 had 5 plaques, and FR186 had 2 plaques. After DNA extraction, all plaque DNA samples produced a faint band of 123 bp in size. FR182 and FR186 milk samples using 20 ml milk in Actiphage Rapid™ assay also had a faint 123 bp band. Jersey bulk milk sample using Actiphage Rapid™ assay also produced a positive IS6110 PCR result, but the band intensity for this sample was a lot brighter than all purified plaque DNA samples and all other Actiphage Rapid™ assay samples (Figure 6.2.). One sample (FR83) produced a negative PCR result using 20 ml milk and Actiphage Rapid™ assay. These results suggest that using 20 ml of milk with Actiphage Rapid™ assay has a similar sensitivity to detection of MTBC from Actiphage® Core assay.

Figure 6.2 Detection of MTBC from raw milk samples using Actiphage® Core assay or Actiphage Rapid™ assay with 20 ml sample volume

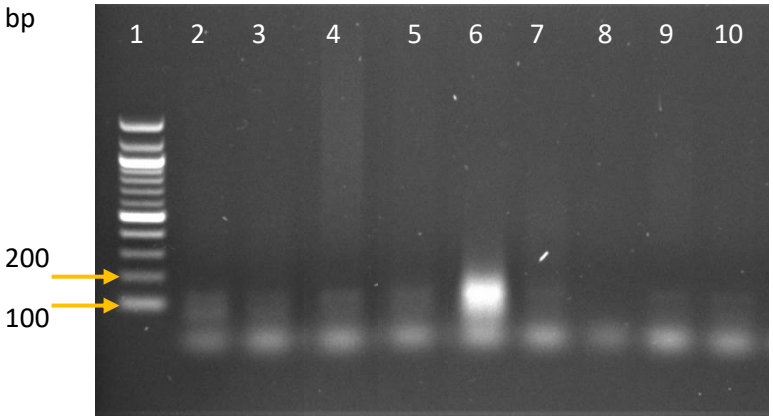


Figure shows results of experiment to detect MTBC DNA extracted from plaques by IS6110 targeted PCR. Expected size of PCR product was 123 bp. Samples on gel are Lane 1: 100 bp molecular size marker; Lane 2, FR182 Core assay; Lane 3, FR186 Core assay; Lane 4, Jersey Core assay; Lane 5, FR83 Core assay; Lane 6, Jersey Rapid assay; Lane 7, FR182 Rapid assay; Lane 8, FR83 Rapid assay; Lane 9, FR186 Rapid assay; Lane 10, M. bovis BCG positive control

6.2.1.4. Comparison of Actiphage Rapid™ and Core assay for detection of MTBC from bovine raw milk using qPCR assay

To determine whether qPCR could be used with DNA recovered from milk samples, mycobacteria were recovered from somatic cells of raw bovine milk for three individual cows (FR83, FR182 and FR186) and one bulk milk tank (Jersey). A volume of 50 ml of milk was used and compared against using a volume of 20 ml of milk. DNA was extracted using Actiphage® Core assay and Actiphage Rapid™ assay. DNA was confirmed as MTBC by Bio-T Kit MTBC (Biosellal; France) as per section 2.5.3.3. Due to volumes of milk received from sampling, samples FR83 and FR182 were only subject to standard Actiphage Core assay and 10 ml Actiphage Rapid™ assay.

For Jersey bulk milk there was no difference in the three methods; the standard Actiphage® Core assay, standard Actiphage Rapid™ assay and 20 ml Actiphage Rapid™ assay all produced a Cq value of 29. For sample FR186, all three methods gave a Cq value within 2.3 cycles of each other. Samples FR83 and FR182 had late Cq values when using standard Actiphage Core assay (Cq > 32) however both remained undetected when 20 ml milk and Actiphage Rapid™ were performed. FR83 also showed signs of total inhibition due to lack of amplification of the internal positive control, therefore a lack of Cq value on the target channel for this sample can be attributed to qPCR inhibition. In contrast, FR182 showed no qPCR inhibition (IPC Cq = 24.10), suggesting this sample is a true negative sample (Table 6.4).

Table 6.4. qPCR detection of MTBC from bovine raw milk using different sample volumes of milk

SAMPLE	CQ VALUE (FAM)	CQ VALUE (CY5)
JERSEY (10 ML RAPID ASSAY)	29.54	24.99
JERSEY (CORE ASSAY)	29.21	25.31
JERSEY (50 ML RAPID ASSAY)	29.05	23.54
FR83 (CORE ASSAY)	34.88	23.89
FR83 (10 ML RAPID ASSAY)	ND	ND
FR182 (10 ML RAPID ASSAY)	ND	24.10
FR182 (CORE ASSAY)	32.27	24.43
FR186 (10 ML RAPID ASSAY)	31.73	23.59
FR186 (50 ML RAPID ASSAY)	30.00	25.35
FR186 (CORE ASSAY)	32.30	23.84

MEDIA PLUS	29.92	24.61
BCG 10^2 CELLS	27.43	21.91
EPC KIT POSITIVE CONTROL	30.14	25.61
WATER NON-TEMPLATE CONTROL	ND	23.50

Somatic cells were extracted from 50 ml raw bovine milk and used in both Actiphage Core and Actiphage Rapid™ assay. As a comparison, somatic cells were extracted from 10 ml raw bovine milk and subject to Actiphage Rapid™ assay. Detection of MTBC was performed using Bio-T Kit MTBC (Biosellal; France). Target DNA fluorescence was measured on the FAM data acquisition channel whilst qPCR inhibition was monitored through fluorescence on Cy5 acquisition channel. Any samples that did not give a signal above threshold levels remained undetected (ND).

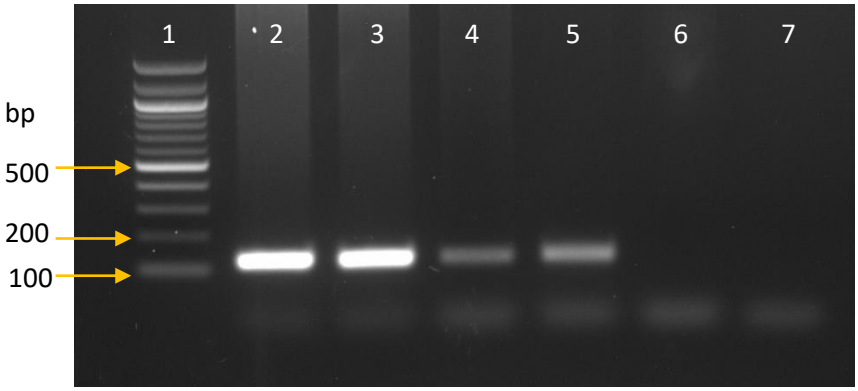
6.2.2. Using Actiphage® Core assay and endpoint PCR to detect the IS*6110* insertion sequence from bulk milk and individual reactor cattle

Results from section 6.2.1. suggest that current cream fraction removal processes were sufficient in removing potential Actiphage® inhibitors therefore no further modifications to somatic cell extraction methods were required at this stage. Experiments were performed to determine whether Actiphage Rapid™ assay could be used to detect MTBC from raw bulk milk samples. As Actiphage® Core assay and endpoint PCR has previously had success in detecting mycobacteria from milk (Botsaris *et al.*, 2013), Actiphage® Core assay was first performed to be used as a baseline comparison for any results obtained from Actiphage Rapid™ assays.

Samples of milk from an unpasteurised bovine bulk milk tank and a cow known to be a TB reactor through the SICCT were tested using the Actiphage® Core assay (section 2.4.6.). Plaques produced from the assay are presumptively identified as detection of *Mycobacterium* species. To confirm the identity of plaques, plaques were picked and purified (section 2.5.1.2.) prior to endpoint PCR to detect the IS*6110* insertion sequence (section 2.5.2.6.). As a positive control for the Actiphage® Core assay, *M. bovis* BCG was used with results from the Actiphage Core assay enumerating the *M. bovis* BCG culture to be 3×10^3 pfu ml^{-1}.

Five plaques were counted on each of the petri dishes from the bulk milk sample and from the individual TB reactor cow. Purified plaques from both samples were confirmed to contain MTBC DNA by the detection of a 123 bp product using IS6110 PCR (Figure 6.3.).

Figure 6.3. Visualisation of IS*6110* PCR products detecting MTBC in bulk milk and an individual cow



Bulk milk sample and a milk sample from an individual TB reactor cow were subjected to Actiphage™ Core assay and presence of MTBC within plaques arising from these milk samples detected by IS6110 PCR. PCR products were visualised on a 1.5 % (w/v) agarose gel, stained with 2.5 % (v/v) ethidium bromide. The presence of a 123 bp PCR product confirms the presence of MTBC within the milk samples. The samples loaded on the gel were: Lane 1: 100 bp molecular size marker, Lane 2; bulk milk sample, Lane 3; Individual TB reactor cow, Lane 4; DNA purified from M. bovis BCG plaques, Lane 5; M. bovis BCG DNA extracted from 10^3 cells using Actiphage Rapid™ assay, Lane 6; Media Plus negative control from Actiphage Rapid™ assay, Lane 7: RNase-free water (no-template PCR control).

6.2.3. Applications of Core- and Rapid™ Actiphage assays in herd management of MTBC

Next, the Actiphage Rapid™ assay and qPCR were used to test these milk samples to determine whether this could provide a quicker alternative to use of the two-day Actiphage® Core method. Investigations were also performed to determine whether Actiphage Rapid™ could provide a useful tool in herd management of MTBC infection.

After the TB breakdown, the herd of Friesen cows separated into two groups based on their SICCT results. Any animal that gave a strong reaction to the bovine PPD but still was scored as negative based on the comparative reaction to the avian PPD, was placed in a separate group. On this basis the herd of Friesen cows were split with 50 cows in the bovine PPD positive group (straw-bed) and the remaining 110 cows assigned to the bovine PPD negative group (cubicle). Once assigned to groups, the two groups remained physically separated from each other and had no opportunities to interact. Unpasteurised bulk milk samples were taken from these two groups, and in addition to bulk milk samples from a herd of Jersey cows from the same farm (Southern England) were provided. All these samples were tested using the Actiphage Rapid™ assay. It was also reported that the Jersey cows had previously tested positive for MAP, however their SICCT results were negative.

6.2.3.1. *Altering Actiphage® reagent particle number per Rapid assay for detection of MTBC from raw milk samples*

It had previously been observed that when performing Actiphage™ Rapid assay, the large, viscous nature of the somatic cell pellet made it difficult to fully resuspend in Actiphage® reagent, thus threatening the sensitivity of the test. To overcome this issue, two different volumes of Actiphage® reagent were used to resuspend the pellet to determine the optimal volume and number of Actiphage particles to use per Actiphage Rapid™ assay and enable detection of MTBC from somatic cells of milk.

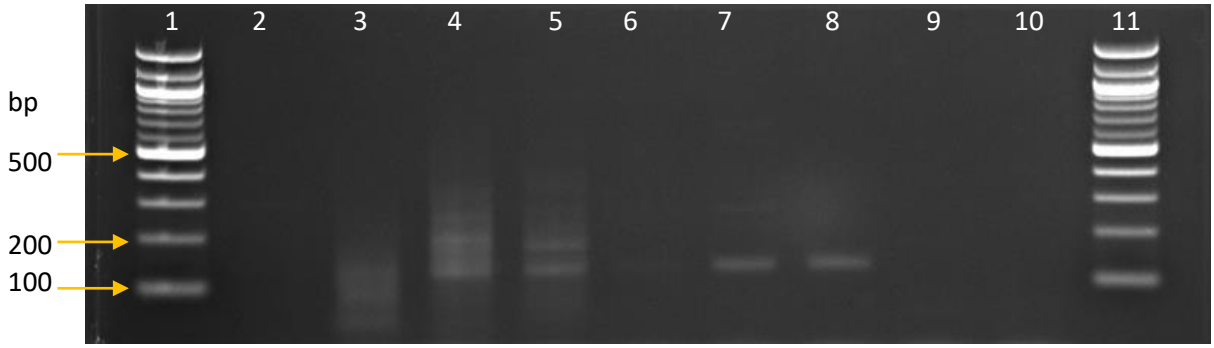
The first volume of Actiphage® reagent was 200 µl as per the standard protocol (section 2.4.4.). This volume of Actiphage® reagent was compared to the use of 1000 µl of Actiphage® reagent. When a total volume of 200 µl Actiphage® reagent was used, approximately 2 x 10^8 phage particles were added per Actiphage assay. In comparison 1000 µl of Actiphage® reagent meant that approximately 1 x 10^9 phage particles were added per assay. Once cells were resuspended, Actiphage Rapid™ was performed as per the standard protocol (section 2.4.4.) and the presence of MTBC within the samples confirmed by IS*6110* PCR (section 2.5.2.6.)

The results showed that the bulk milk samples derived from the group of cattle assigned to the 'straw-bed' group as well as bulk milk from the herd of Jersey cows gave a positive MTBC test result using the Actiphage™ Rapid assay when somatic cell pellets were resuspended in 1000 µl Actiphage reagent. Jersey bulk milk samples also gave a positive MTBC test result when 200 µl Actiphage reagent was used to resuspend the somatic cell pellet. In contrast only a very faint 123 bp band

was detected for the straw-bed group when 200 µl of Actiphage® reagent was used, suggesting that using 10^9 Actiphage particles per assay increased the sensitivity of the test. Milk samples from cattle within the 'cubicle' group gave a negative MTBC test result when both 1000 µl and 200 µl of Actiphage® reagent was used with no visible band through gel electrophoresis (Figure 6.4.).

Results obtained from this testing were used as preliminary results to begin to determine whether individual cows from herds could be identified as MTBC-positive using SICCT interpretation and Actiphage Rapid™ testing of milk samples to improve herd disease management.

Figure 6.4. Visualisation of IS*6110* PCR products from DNA extracted from somatic cells of bulk milk using different numbers of Actiphage® particles per Rapid assay



Somatic cells extracted from bulk milk samples were tested using the Actiphage Rapid™ assay. Somatic cells were extracted in duplicate and tested for MTBC using either 200 µl or 1000 µl of Actiphage® reagent. Mycobacterial DNA was detected using MTBC specific IS6110 PCR and PCR products visualised on a 1.5 % (w/v) agarose gel stained with 2.5 % ethidium bromide.

Samples loaded on the gel were: Lane 1; 100 bp molecular size marker, Lane 2; 'Cubicle' herd sample using 200 µl Actiphage® reagent, Lane 3; 'Cubicle' herd sample using 1000 µl Actiphage® reagent, Lane 4; 'Jersey' herd sample using 200 µl Actiphage® reagent, Lane 5; 'Jersey' herd using 1000 µl Actiphage® reagent, Lane 6; 'Straw-bed' herd using 200 µl Actiphage® reagent, Lane 7; 'Straw-bed' herd using 1000 µl Actiphage® reagent, Lane 8: M. bovis BCG 10^2 cells positive assay control, Lane 9: Media Plus negative assay control, Lane 10: RNase-free water (no-template PCR control), Lane 11: 100 bp molecular size marker.

6.2.3.2. Identification and isolation of MTBC-positive cattle in a herd

After the next compulsory SICCT test of this herd, individual cows that had a positive reaction or tests that were recorded as 'inconclusive' to SICCT testing were identified and isolated. The remainder of the herd were kept in their previous group's 'cubicle', 'straw-bed' or 'Jersey'. One cow from the 'Jersey' group and four cows from the 'straw-bed' group produced a positive reaction to the SICCT test, therefore individual milk samples for these cows were taken (cattle ID FR182, FR186, FR83, J629, FR244, J708). In addition, a further milk sample was taken from an individual cow from the 'Jersey' group as the results for this cow were reported as inconclusive. Bulk milk samples were taken for the remaining cattle within their respective groups.

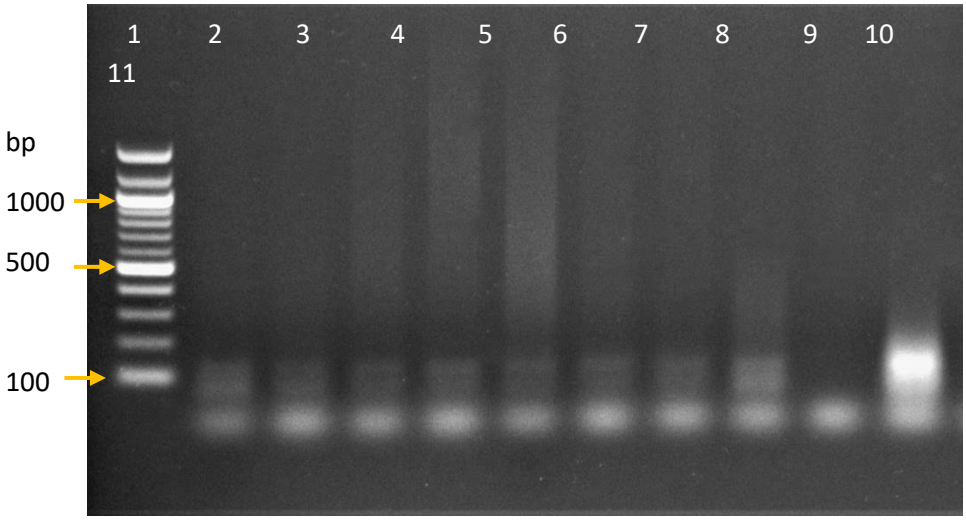
Both Rapid- and Core Actiphage™ assays were performed on the raw milk samples. Despite results from section 6.2.3.1. showing that 1000 µl of Actiphage® reagent improved detection of MTBC from milk samples, for this round of testing, 200 µl of Actiphage® reagent was used for each sample as per Actiphage Rapid™ IFU (section 2.4.4.) as the company wanted further work to validate this result before it was adopted as a change to the IFU.

From the ten milks sampled, 8 out of 10 produced plaques with the Actiphage Core assay. Despite J708 and FR244 having positive SICCT reactor tests, there were no observable plaques by Actiphage Core assay for these two milk samples. All plaques were picked, and DNA extracted from plaques using an extraction and purification kit (section 2.5.1.2.). Both purified plaque DNA and purified DNA from Actiphage Rapid™ assay (section 2.4.4.) were subjected to IS*6110* endpoint PCR (section 2.5.2.6.) to confirm the presence of MTBC.

Endpoint PCR confirmed the plaques extracted from the Actiphage Core assay for all samples to be MTBC through visualisation of a 123 bp product amplified from IS*6110* insertion sequence (Figure 6.5). Similarly, the presence of a 123 bp amplicon for all milk samples was also observed by analysis of gel electrophoresis from IS*6110* PCR using DNA extracted from the Actiphage Rapid™ assay.

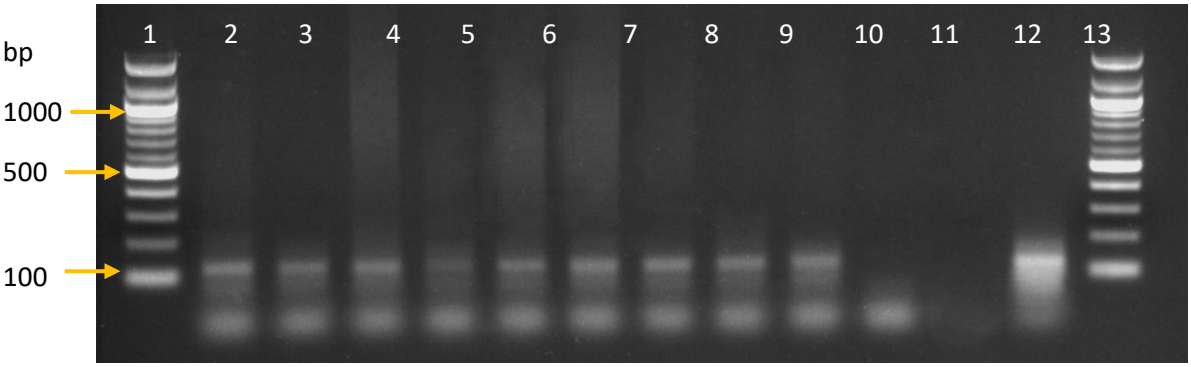
Two samples (J708 and FR244) which had no observable plaques by Actiphage Core assay but showed the presence of a 123 bp amplicon with DNA extracted using the Actiphage Rapid™ assay (Figure 6.6).

Figure 6.5 IS*6110* PCR results for detection of MTBC DNA extracted from raw milk samples using Actiphage® Core assay



Raw milk samples were tested using the Actiphage Core assay. DNA was purified from all plaques and any MTBC DNA present detected by IS6110 endpoint PCR. The presence of a 123 bp product confirmed the presence of MTBC. The samples loaded on the gel were: Lane 1: 100 bp molecular size marker. Lanes 2-9 contain the results of the PCR assay for the following milk samples; Lane 2; FR182, Lane 3; FR186, Lane 4; Cubicle, Lane 5; Jersey A, Lane 6; Straw-bed, Lane 7; Jersey B, Lane 8; FR83, Lane 9; J629. Lane 10 contained the Media Plus sample (Actiphage assay negative control) and Lane 11 contained DNA extracted from 10^2 M. bovis BCG ^{cells} (positive control). Cattle with an 'FR' identification were Fresian cows and those with a 'J' identification were Jersey cows. Cubicle sample was a bulk milk tank sample taken from a herd of cows of negative SICCT status. Straw-bed sample was a bulk milk tank sample taken from a herd of cows previously of reactor SICCT status.

Figure 6.6. IS*6110* PCR results for detection of MTBC DNA extracted from raw milk samples using Actiphage Rapid™ assay



Visualisation of 123 bp PCR products of DNA from IS6110 insertion sequence amplified from raw milk samples using IS6110 endpoint PCR following the Actiphage Rapid assay. The samples loaded on the gel were: Lane 1: 100 bp molecular size marker. Lanes 2-10 contain the results of the PCR assay for the following milk samples; Lane 2: J629, Lane 3: J708, Lane 4: FR 244, Lane 5: FR83, Lane 6: FR182, Lane 7: FR186, Lane 8: Cubicle, Lane 9: Jersey A, Lane 10: Straw-bed. Lane 11 contained the Media Plus sample (Actiphage assay negative control) and Lane 12 was the RNase-free water sample (no-template PCR control). Lane 13 contained DNA extracted from 10^2 M. bovis BCG cells (PCR positive control).

6.2.3.3. Detection of MTBC from bovine milk using Actiphage Rapid™ assay and qPCR

Results from section 6.2.3.2. show that despite two milk samples being MTBC-negative when using Actiphage Core assay, all samples tested positive using Actiphage Rapid™ assay and IS*6110* endpoint PCR. To compare the effectiveness of real-time and endpoint PCR detection, DNA from the nine milk samples was also used as template in qPCR using the Bio-T kit® MTBC (Biosellal; France).

M. bovis BCG cells enumerated to 10^2 cells was used as the Actiphage Rapid™ positive control whilst Media Plus and Actiphage® reagent used as the negative assay control. A volume of 5 µl of each of these controls were run on the qPCR. The positive qPCR control was a 1 µl sample of the qPCR kit exogenous positive control (EPC) and 5 µl of RNase-free water was used as as the no-template qPCR negative control. Also included in the qPCR reaction was an internal positive control (IPC) to detect any qPCR inhibition on the Cy5 acquisition channel.

All four controls showed no signs of inhibition, with all samples producing a detectable signal (Cq value) between cycle 18.96 and 20.89. Two samples (FR83 and FR186) showed internal control inhibition. For FR83 there was no amplification of IPC and for FR186 IPC Cq value was determined to be 37.59. All other samples had a Cq value for IPC detection between 20.03 and 21.93.

As highlighted in previous chapters, when using Actiphage Rapid™ assay and qPCR kits by Biosellal (France), Media Plus negative assay control can often yield a detectable signal. In this instance, Media Plus Cq value was 28.73 and because this

was at least 0.18 cycles earlier than any of the test samples, the ΔCq method could not be applied. Both the Actiphage Rapid™ assay positive control and qPCR kit EPC had earlier detectable signals at Cq 26.69 and Cq 28.05, respectively. In addition, there was no observable correlation between number of plaques produced by Actiphage Core assay, Actiphage Rapid™ assay with endpoint IS*6110* PCR and Actiphage Rapid™ assay with qPCR (Table 6.4). Hence no useful data could be obtained from the qPCR results, again showing that this DNA detection method is more sensitive to sample composition than end-point PCR.

Table 6.5 Comparison of Actiphage Rapid™ qPCR results with Actiphage Rapid™ endpoint PCR and Actiphage Core assay for detection of MTBC from bovine milk

SAMPLE NAME	CQ VALUE FAM	CQ VALUE CY5	ACTIPHAGE RAPID™ ENDPOINT PCR	NUMBER OF PLAQUES FROM CORE ASSAY
J629	29.62	21.17	Positive	2
J708	29.25	21.63	Positive	0
FR83	ND	ND	Positive	5
FR182	30.18	21.32	Positive	2
FR186	ND	37.59	Positive	2
CUBICLE	29.33	21.93	Positive	2
JERSEY	28.91	20.65	Positive	5
STRAWBED	29.62	20.49	Positive	3
FR244	30.73	20.03	Positive	0
BCG 10^2 CELLS	26.69	20.26	n/a	n/a
MP	28.73	20.70	n/a	n/a

KIT CONTROL EPC	28.05	18.96	n/a	n/a
WATER	ND	20.89	n/a	n/a

DNA was extracted from six individual bovine milk samples (from animals J629, J708, FR83, FR182, FR186, FR244) and from three bovine bulk milk samples (Cubicle, Jersey, Straw bed). Both the Actiphage Core assay and Actiphage Rapid™ assay was used with MTBC DNA was detected by endpoint IS6110 PCR (Actiphage Core assay) or by qPCR (Actiphage Rapid™ assay). For qPCR, samples that did not generate a signal above threshold levels for either FAM or Cy5 acquisition channels were determined to be not detected (ND). For qPCR, no samples had a detectable Cq value earlier than that of the Media Plus negative control. All samples were MTBC positive using Actiphage Rapid™ and endpoint PCR and samples J708 (individual cow) and FR244 (individual cow) had no plaques by Actiphage Core assay. All other samples from Actiphage Core assay had plaque DNA confirmed to be MTBC by IS6110 endpoint PCR.

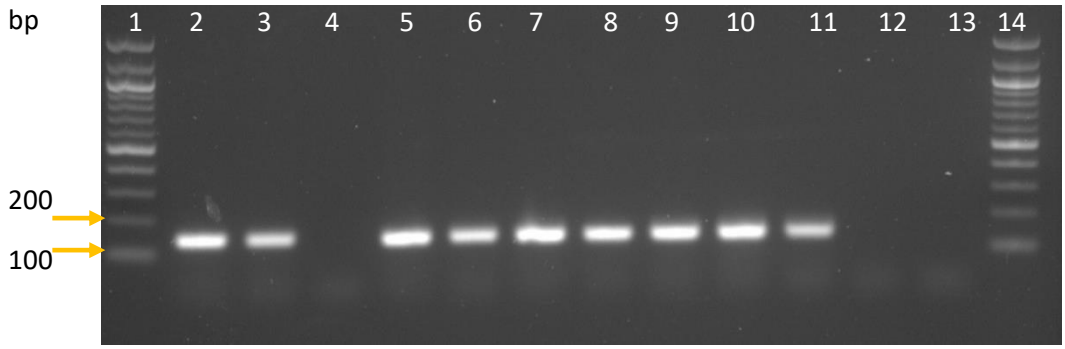
6.2.3.4. MTBC speciation PCR for confirmation of mycobacterium present in milk

The T4/T5 primer pair used for the endpoint PCR assay targets IS*6110* which is present in all members of *M. tuberculosis* complex. Since the results of these tests were going to be used to inform disease management decisions by the farm, a second PCR assay was performed (section 2.5.2.7.) using primers to detect either the presence or absence of Rv1510 locus in RD4 (Taylor *et al.*, 2007). A deletion in this region is specific to *M. bovis* strains and the PCR assay is designed so that a PCR band of 142 bp confirms the deletion of RD4 and therefore categorises the organism as *M. bovis.* A PCR band of 176 bp confirms the presence of RD4 sequences and categorise the *Mycobacterium* as either *M. tuberculosis* or *M. caprae.* No amplification indicates that MTBC strains are not detected. Since the RD4 is present in a single copy in the genome, then this PCR assay requires more template DNA to generate a positive signal compared to the *IS*6110 PCR assays because of the multi copy nature of this element.

DNA from six individual bovine milk samples (FR182, FR186, FR83, J629, FR244, J708) and three bulk milk tank samples (cubicle, straw bed and Jersey) previously extracted by Actiphage Rapid™ assay were used as DNA template for the RD4 PCR assay. As previously mentioned, all samples from individual cattle were from animal classed as reactors based on the SICCT test. All the other animal in the Straw bed bulk milk group showed a strong reaction to bovine PPD but were classed as SICCT negative. All cattle in the cubicle group were SICCT-negative and did not show a strong response to bovine PPD and cows in the Jersey group were also non-reactors.

Out of the 10 samples tested, 9 were confirmed to contain *M. bovis* due to the presence of a 142 bp band (Figure 6.6). The only sample from an individual animal that had a negative PCR result was FR83. Although this sample was positive for IS*6110* PCR, FR83 also showed total inhibition when using qPCR. When centrifuging this sample for somatic cell isolation, it was noted that the milk was very discoloured with a yellow/green hue and almost translucent. The pellet was a lot larger than that of all other samples and difficult to resuspend in Media Plus because it was very viscous (Figure 6.7.). This suggests that this sample was from a cow suffering from mastitis or another infection that was influencing the milk quality and large somatic cell pellet for this sample which contributed to difficulties with PCR amplification of DNA extracted using the Actiphage Rapid™ assay.

Figure 6.6 RD4 flanking PCR to confirm and speciate MTBC infection from bovine milk samples



DNA was extracted from six individual bovine milk samples (FR182, FR186, FR83, J629, FR244 and J708) and three bulk milk samples (cubicle, straw bed and Jersey) using the Actiphage Rapid™ assay. PCR was performing using RD4 flanking PCR. A 176 bp band indicates the detection of either M. tuberculosis or M. caprae. A 142 bp band indicates the detection of M. bovis. The samples loaded on the gel were: Lane 1: 100 bp molecular size marker. Lanes 2-10 contain the results of the PCR assay for the following milk samples: Lane 2; FR182, Lane 3; FR186, Lane 4; FR83, Lane 5; J629, Lane 6; FR244, Lane 7; J708, Lane 8; cubicle, Lane 9; straw bed, and Lane 10; Jersey. Lane 11 contained DNA extracted from 10^2 M. bovis BCG cells (positive control) and Lane 12 contained DNA extracted from the Media Plus negative control; Lane 13 shows the results for the no-template PCR negative control. Lane 14 contains the 100 bp molecular size marker.

Figure 6.7. Comparison of two milk samples after centrifugation to extract somatic cells

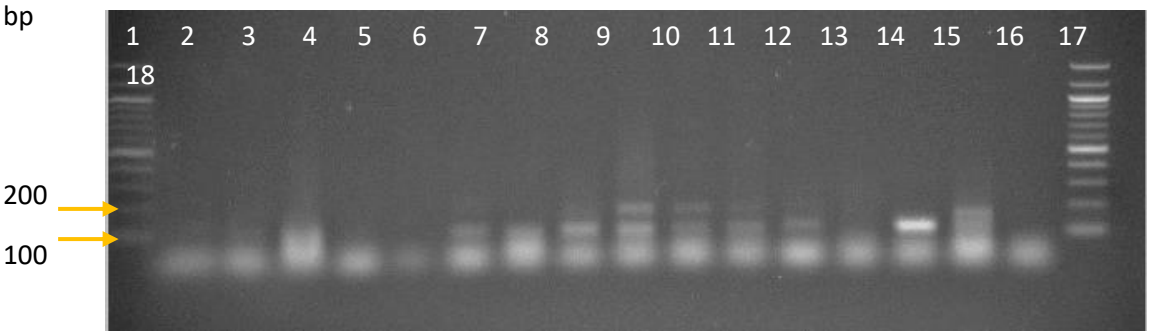
*For somatic cell extraction from raw milk, 50 ml of milk is centrifuged at 2,500 x g for 15 min to pellet somatic cells and separate cream fraction. Raw milk sample FR83 (**A**) was discoloured and translucent in opacity compared to the off-white opaque supernatant of all other milk samples (**B**). FR83 had a large pellet after centrifugation, uncharacteristic of all other milk samples. DNA from this sample also showed signs of inhibition on MTBC specific qPCR and was not detected using RD4 specific endpoint PCR.*

6.2.3.5. Isolation of individual cows to identify MTBC within herd

At the next compulsory SICCT testing time, decisions were made based on the results of the milk screening and the latest SICCT results. Negative animals were introduced into the herd that contained all non-reactors, and the Jersey herd was also split into a 'clean' group and a 'reactor' group, again based on the reaction to the bovine PPD antigen alone. Milk samples from any reactors at this time of testing were again sent for Actiphage® assay testing.

For this round of testing, 40 milk samples were taken. Two samples were from bulk milk groups: Jersey 'clean' group and Jersey 'reactor' group. Thirty-eight samples from individual cows were also tested from both Friesian and Jersey cows. Actiphage Rapid™ assay was performed on all samples after 50 ml milk was sampled to extract somatic cells. Presence of MTBC within samples was confirmed by IS*6110* endpoint PCR. Twenty gave a positive MTBC test result using IS6110 end point PCR, and 10 samples gave a negative MTBC result (Figure 6.8). All samples that gave a positive IS*6110* PCR result were confirmed to be due to detection of *M. bovis* by RD4 deletion PCR (data not shown).

Figure 6.8 IS*6110* PCR detection of MTBC from raw bovine milk samples subjected to Actiphage Rapid™ assay



Somatic cells were extracted from individual milk samples from reactor cattle and tested using the Actiphage Rapid™ assay. Detection of MTBC from samples was performed using IS6110 endpoint PCR. The detection of a 123 bp PCR product indicates detection of MTBC DNA. The samples loaded on the gel were: Lane 1: 100 bp molecular size marker. Lanes 2-14 contain the results of the PCR assay for the following milk samples: Lane 2; cow F95, Lane 3; cow F278, Lane 4; cow F242, Lane 5; cow J731, Lane 6; cow F217, Lane 7; cow F164, Lane 8; cow J655, Lane 9; cow F105, Lane 10; cow F228, Lane 11; cow J661, Lane 12; cow J636, Lane 13; cow J717, Lane 14; cow F269. Lane 15 contains the result for DNA extracted from 10^2 M. bovis cells (positive assay control) and Lane 16 contains the result for DNA extracted from the Media Plus sample (negative assay control); Lane 17 shows the result for the water PCR no-template control; Lane 18: 100 bp molecular size marker.

6.2.3.6. qPCR detection of MTBC from raw bovine milk

After detection of MTBC from bovine milk using IS*6110* specific endpoint PCR, DNA was used to attempt qPCR detection of MTBC DNA using the Bio-T Kit MTBC (Biosellal; France). Despite Media Plus negative control showing no signs of contamination in IS*6110* PCR and RD4 PCR, the negative control recorded a Cq value of 30.86. As the QPCR kit positive control (EPC) had a Cq value (Cq = 31.07), again the ΔCq methods could not be applied. Since the EPC Cq was within the expected range for the control, positive samples on qPCR were identified as anything with a Cq earlier than that of EPC. Using this approach, 55 % of samples had concordant results for both endpoint and qPCR (data not shown). Overall, 29 samples produced a positive test result using IS*6110* end point PCR compared to 21 samples with qPCR.

6.3. DISCUSSION

The aim of the work presented in this Chapter was to investigate whether Actiphage Rapid™ could provide a suitable alternative to the Actiphage® Core assay for testing milk samples, while maintaining similar levels of sensitivity but offering a quicker turnaround time from sampling to test results. In addition, components of milk that could potentially be causing inhibition of Actiphage Rapid™ assay were also investigated.

Despite methods implemented to improve cream fraction removal using strainers and filters to aid in separation of cream fraction from the rest of the milk, spiked milk experiments suggested that there was not a great difference in detection of MAP from cells between the different strainers and filters used. It must be noted that the MAP cells were inoculated into the milk so were not internalised by the somatic cells and were therefore not truly representative of natural infection where mycobacteria would be persisting within somatic cells. However, as these initial experiments were only interested in the MAP-Actiphage® kinetics and the influence of milk components on Actiphage® productivity, this was not deemed to be an issue. The interest of these experiments was directed at Actiphage® inhibition and there was shown to be a large biological significance between detection of MAP spiked into milk and MAP not spiked into milk (ΔCq 7.21 cycles), which is over 100-fold difference in detection level. This suggests that inhibition was causing a less sensitive detection of MAP from milk. Nevertheless, it was not investigated further which milk component was causing this inhibition. As efficient cream removal was achieved, this reduces the possibility that the main inhibitory factor of Actiphage®

productivity was fat globules as this is the main component of milk cream fraction (Perna *et al.*, 2022). This suggests that inhibitory components of Actiphage® assay remained in the skim milk fraction, such as the milk proteins casein and whey. Despite casein making up 80% of all milk proteins in bovine milk (Choi *et al.*, 2011), a study by García-Anaya *et al.* (2020) showed that casein had the least inhibitory effect on *Listeria* bacteriophage activity compared to fat globules and whey. This study showed that fat decreased bacteriophage counts by 2.6 log_{10}PFU ml^{-1}, compared to 2.1 and 0.8 log_{10}PFU ml^{-1} for whey and casein respectively. Gill *et al.* (2006) studied inhibition kinetics of whey on staphylococcal bacteriophage and found that there was a significant positive correlation between bacteriophage binding inhibition and the cow's history of clinical mastitis. In addition, this study noted that bacterial cells exposed to whey and thus preventing phage binding, phenotypically had copious material binding to cell surfaces and had a strong tendency towards agglutination. Despite the bacteriophage in these studies not being mycobacteriophage, the reports still provide evidence that whey protein may be a large contributing factor to Actiphage® inhibition. On the contrary, Angela *et al.* (2006) found that binding DNA to a silica matrix in the presence of a chaotropic agent effectively removed milk inhibitors and allowed for isolation and amplification of mycobacterial DNA. Given that the DNA binding buffer used for the DNA purification step of the Actiphage® Rapid assay contains guanidine chloride, which is a chaotropic agent, it could be assumed that at least some of the proteins that may be contributing to PCR inhibition are denatured. Without the data, it cannot be determined which components from milk are inhibitory and to what extent any reagents currently used throughout the assay are aiding in denaturation

of these proteins to minimise inhibition. More experiments would need to be performed to determine whether whey proteins are affecting Actiphage® binding and to determine the extent of denaturation of proteins during DNA purification. This can be achieved through isolation of whey protein and performing minimum inhibitory concentration assays.

As an alternative approach to remove cream layer, a smaller volume of milk was used. Results showed that cream fraction removal was easier with a smaller volume leaving less residual cream in tubes, however it was since shown that that efficient cream removal was not the main solution to improving the Actiphage® assays. Nevertheless, using a smaller volume of milk did not affect sensitivity of PCR in terms of being able to detect MTBC from samples in comparison to Actiphage Core assay and endpoint PCR. For these experiments, milk was provided from naturally infected cattle therefore number of MTBC cells were not quantified prior to experimentation, so these experiments cannot be used to determine whether there is still reduced sensitivity of milk assay due to inhibitors present in milk. For quantifiable results for this experiment, these results were also compared to MTBC-specific qPCR detection of MTBC. Results were variable, as with some samples different volumes of milk or assay type (Core or Rapid Actiphage assay) did not make a difference to detection, whereas for other samples, there was a difference, with 10 ml milk being less sensitive. Logically, a smaller volume of milk would mean less somatic cells, assuming an equal distribution of somatic cells within milk, however, this wasn't seen as the overall trend from these results. If removal of milk components inhibitory to Actiphage® activity can be optimised to improve

detection of mycobacteria from raw milk, then the need for a smaller volume of milk when testing bovine samples would be unjustified. However, using a smaller volume of milk would be beneficial for smaller ruminants who don't produce as much milk as cows. This requires more evidence to be provided to demonstrate that smaller volumes of milk still maintain similar sensitivity compared with using 50 ml samples.

Despite these issues that are yet to be overcome to optimise Actiphage Rapid™ assay for detection of mycobacteria from milk, the assay has shown to be capable for aiding in disease herd management for MTBC in dairy cows. Through regular testing of both bulk milk samples and individual cattle that had presumptive positive tests using SICCT testing and splitting the herd accordingly after each round of testing, Actiphage Rapid™ assay was successful in aiding in achievement of TB free status for a large dairy farm. This case study shows that Actiphage Rapid™ is an efficient method for aiding in herd management, although more work needs to be done to optimise detection methods, as there was only a 55 % agreement rate between IS*6110* endpoint PCR results and MTBC specific qPCR results. The lower reported number of positive samples for qPCR compared to endpoint PCR may be due to a lack of sensitivity, or specificity, combined with inhibitory milk factors. When using the commercial qPCR kit (Biosellal; France), the target region of MTBC genome is unknown, therefore understanding whether this had an impact on sensitivity or specificity remains unfulfilled. Despite this, using endpoint PCR with primers of known sequence showed that MTBC detection is possible, therefore this experiment could be repeated with the Empirical qPCR mix from Chapter 3, as these

primers are the known T4/T5 primer pair, which were the same ones used in endpoint PCR assays, to see if this improves overall detection of MTBC from milk by qPCR.

Results have shown that Actiphage Rapid™ can be presented as an alternative to using Actiphage Core assay, however sensitivity of the assay is still relatively low, and therefore more optimisation of qPCR assays are required. Nevertheless, this chapter has demonstrated useful practical applications in using Actiphage Rapid™ assay to aid in disease herd management.

In summary:

1. Actiphage Rapid™ assay was successfully used to detect *Mycobacterium* from milk
2. Despite efforts to remove inhibitory cream fractions, inhibition was still observed, and more work needs to be done to develop this further
3. Actiphage Rapid™ proved effective for herd-level tuberculosis management, helping a dairy farm regain TB-free status through routine milk testing

CHAPTER 7

GENERAL DISCUSSION AND FUTURE WORK

7.1 DISCUSSION

Mycobacterium species infections are of large global importance, affecting both humans and animals. Often due to severity of disease of both tubercule *Mycobacterium* species infections and non-tubercule infections, there is a need for rapid and sensitive diagnosis. There are currently many established methods for detection of Mycobacteria including culture-based approaches, immunological assays and histology. These diagnostic methods can often lack sensitivity (Slana *et al.,* 2009) hindering diagnosis and delaying treatment plans for humans, or herd management plans for animals.

Actiphage assays were designed to overcome the issues often presented with current methods of detection, whilst having the appeal of only infecting viable mycobacteria, thus also providing information on infection status of subject. The Actiphage Core assay was the first Actiphage assay developed and was designed to provide a rapid and sensitive detection of *M. bovis* and *M. avium* subspecies *paratuberculosis* in animal blood and milk samples. Although this assay reduced the turnaround time that was a given for culture-based methods, due to the broad range Mycobacteriophage used in the Core assay, strict identification of species of *Mycobacterium* detected required subsequent analysis of DNA extracted from plaques formed at the end of the assay. Whilst this assay provided a quicker and more specific alternative to culture, the assay was later improved further into the Actiphage Rapid™ assay format, reducing turnaround time from sampling to results from 48 h to 8 h (Swift *et al.,* 2013). With a limit of detection of 10 cells (Swift *et al.*, 2020) Actiphage Rapid™ assay was designed to also overcome the issue of

sensitivity posed with many immunological approaches which only detect more advanced stages of infection. Whilst Actiphage Rapid™ assay has been used successfully with endpoint PCR for detection of a range of Mycobacteria from blood of humans and animals, to enhance the development of Actiphage Rapid™ assay as a diagnostic kit, moving from an endpoint PCR format to a qPCR format would provide a further improvement, that can provide results in real-time and offers a higher throughput, less labour-intensive approach.

7.1.1. Development of Actiphage-qPCR assay

There are many variables and factors to consider when designing and optimising a qPCR for diagnostic use. The result from this study shows that there is a lot of variability in the ability of these assays to detect mycobacteria between different PCR assays. Peripheral blood mononuclear cell extraction method, qPCR assay kit, PBMC lysis method and number of Actiphage® particles used per assay were all factors that induced variability in the effectiveness of qPCR assays. Svec *et al.* (2015) highlighted other key variables that can contribute to qPCR variability noting that even choice of qPCR machine can contribute to variability in detection of target, something that cannot be controlled for when designing a diagnostic kit, but that must be acknowledged as source of variability. Other factors that can influence the output of qPCR assays include consumables, including the degree of transparency of qPCR tubes used and plastic material used to manufacturer tubes, as these can influence fluorescence reflection and sensitivity (Reiter and Pfaffl, 2008). Whilst it can be extremely difficult to control for every one of these variables,

acknowledgment of their contribution and influence on the diagnostic assay is essential when trying to develop a robust commercial assay.

The qPCR assay designed in-house to rival those of commercially available qPCR kits showed promise. There was comparability between in-house and commercial kits however, the in-house qPCR still requires more optimisation before there is confidence in using the kit for every Actiphage Rapid™ assay from blood and milk samples. Issues with fluorescent probe choice for detection of MAP has been highlighted, as the probe had an annealing temperature lower than that of the primers for the assay. The implications of this primer-probe combination reduce the sensitivity of the qPCR, however, this is an issue that can be addressed with further experiments exploring different fluorescent probe options.

Furthermore, an essential component of a diagnostic qPCR assay is the inclusion of an internal amplification control (IAC) to monitor qPCR efficiency and detect any signs of qPCR inhibition. Despite efforts to incorporate an IAC into the qPCR assays, trying both a competitive and non-competitive format, this was never successfully achieved. Solutions to this problem could be as simple as trying a wider range of concentrations of primers, probe and IAC DNA combinations, assessing the efficiency of each in the presence of target Mycobacterial DNA. Alternatively, although the IAC used in current experiments was designed previously with the intent of containing the same primer sites as those used for detection of *M. avium* subspecies *paratuberculosis* IS*900* (P90/P91 primer pair), *M. tuberculosis* complex IS*6110* (T4/T5 primer pair) and *M. avium* subspecies *avium* IS*901* (MK7/MK8 primer pair) to enhance the ease of use of the diagnostic assay, a total redesign of IAC can

be implemented. A redesigned IAC construct to contain primer and probe recognition sites that share no homology to any *Mycobacterium* species reduces the risk of preferential binding of target primers to IAC instead of target DNA. The use of IAC to monitor inhibition in the current experiments could not confidently be established, therefore other methods had to be implemented.

Experiments to monitor qPCR inhibition often involved spiking PBMCs or milk with target mycobacteria, however variability between qPCR reactions and their susceptibility to inhibitory substances copurified with DNA often occurs (Huggett et *al.,* 2008). MIQE guidelines state, for monitoring of inhibition in qPCR reactions, the preferred method is to use serial dilutions of known concentrations of DNA as a reference standard to compare against Cq values from sample results (Bustin *et al*., 2009). This is a practical consideration for the Actiphage Rapid™ assay when used as a diagnostic tool, as including serial dilutions of reference standard in each qPCR run will provide insight into any inhibition coming from blood or milk that may be a threat to qPCR reaction efficiency. However, to include such a quality control check on every qPCR run limits the number of samples that can be tested at a time, reducing the high-throughput ability that is desirable with qPCR. Results using the Actiphage core assay format indicate that often low numbers of Mycobacterial pathogens are found in these samples, and therefore dilution may reduce the level of DNA below a clearly detectable level.

7.1.2. Exploiting nucleotide polymorphisms in *M. avium* complex

Species within the *M. avium* complex show high genetic diversity however SNPs in conserved regions allow for speciation and sub-typing of MAC species. Whilst WGS offers great depth and insight into this genetic diversity, the approach is expensive, inaccessible in many labs and can often require specialist training in bioinformatics, making WGS an unrealistic tool for diagnostic purposes. Results from Chapter 5 supported the notion that WGS is inappropriate for rapid diagnostic use when Actiphage® was used as the cell lysing agent. Although Actiphage® is capable of being used as a biological lysing agent to prepare mycobacterial DNA for sequencing, abundance of target mycobacterial DNA within samples remained questionable. Whilst it was expected that WGS would produce a high number of reads for Actiphage®, target mycobacterial DNA was not always in high enough abundance to confidently recognise this as the target DNA that was sequenced. For these experiments, pure mycobacterial cultures were used, however abundant reads were recorded for multiple species. This solidifies the conclusion that WGS is undesirable for use with the Actiphage Rapid™ assay as mycobacterial DNA from this assay would also be mixed with eukaryotic DNA within the sample further reducing the effectiveness of this approach in confidently identifying *Mycobacterium.* Nevertheless, WGS is a useful tool to use alongside Actiphage Rapid™ and could complement the diagnostic assay if there was a need to get an entire genome profile of mycobacteria after diagnosis to give information on drug resistance.

Exploiting polymorphic variation between strains by development of a successful qPCR which includes high resolution melting to discriminate, would allow monitoring of prevalence of specific RFLPs within regions and potentially allow the spread of mycobacterial diseases through populations to be traced. Although this was not fully achieved, experiments show that there is potential in the method if optimised. At present, experiments focused on polymorphic variation of IS*1311* at 2 locations within the insertion sequence. Although different qPCR melt curve analysis peaks for sub-lineages were obtained, melting temperatures for these peaks were within 1 °C of each other, making it hard to determine whether these were true melt peaks, or arising because of qPCR variation. Currently, it is unclear whether IS*1311*-HRM is effective in subtyping MAP strains, nevertheless, this presents an opportunity to explore the applications of using HRM with Actiphage Rapid™ assay to obtain more targeted information about mycobacterial infection of animals and humans. Elucidation of RFLPs within IS*900* to study spread of infection by gel electrophoresis techniques has previously been successful for cattle in Australia (Whittington *et al.*, 2000), therefore by using genetic information on this polymorphic variation other Actiphage Rapid™ assay HRM analyses can be explored to determine if these are more suitable targets for this assay. Success in design and optimisation of such an assay would allow for a high-throughput approach for large scale screening and herd surveillance.

7.1.3. Applications of Actiphage-based PCR methods

Actiphage Rapid™ assay is a versatile product that not only can diagnose Mycobacterial infection from blood, sputum and milk, but also provide more information on disease and be used successfully to isolate *M. bovis* positive animals from herds for disease management.

Single intradermal comparative cervical tuberculin (SICCT) testing is routinely used to monitor *M. bovis* infection in farmed cattle. However, this test often lacks sensitivity and can also result in false-negative results when animals are in later stages of infection, due to anergy as a result of a weakened immune system (de la Rua-Domenech *et al.,* 2006). Zarden *et al.* (2013) have previously highlighted that SICCT tests are not sufficient in managing disease outbreaks and identifying all infected cows in a herd, however this has been achieved with Actiphage Rapid™ assay. Through repeated testing of bulk milk and individual cattle, as well as separating herds based on Actiphage Rapid™ results, Actiphage Rapid™ assay proved to be a useful tool in aiding to eradicate MTBC from a large dairy farm.

Further applications of Actiphage Rapid™ assay include detection of multiple-strain infections using endpoint PCR targeting two insertion sequences sequentially. *M. avium* complex are opportunistic pathogens that are an ever-increasing public health risk, creating a need for rapid detection and surveillance of disease cases. Chapter 5 showed that Actiphage Rapid™ assay can identify and speciate MAC infection isolated from a human, whilst also providing evidence of mixed strain infection through IS*901* and IS*1311* targeted PCR assays. Furthermore,

using these two PCR methods, Actiphage Rapid™ assay was able to discriminate between strains of pathogenic and non-pathogenic *M. avium* subspecies *avium.*

7.2. FUTURE WORK

Despite these issues with qPCR development, if a probe-based qPCR for detection of mycobacteria can be optimised there are many further applications of the Actiphage Rapid™ assay that could be explored. The qPCR can further be developed as a multiplex PCR by tagging primers for multiple targets with different fluorescent probes. This concept could also be applied to targeting and identifying multi-drug-resistant MTBC in one assay, to allow for a quicker decision on treatment management. Multiplexing would also aid in understanding mixed strain infection of MAC and allow for elucidation of pathogenic and non-pathogenic MAC infection by targeting multiple insertion sequences within one qPCR reaction. Similar multiplexing is already in use to identify and speciate NTMs with high sensitivity and specificity (Park *et al.,* 2023), although using Actiphage Rapid™ with this qPCR assay instead of the DNA preparation as described in the study, would eliminate the need for harsh decontamination treatments of samples.

Exploiting mycobacteriophage to identify and profile drug resistance of mycobacteria is important for quick treatment and effective control of disease. *Mycobacterium* drug resistance profiling is already being used by incubating *Mycobacterium* with antibiotics prior to exposure to lytic phage D29 and change in phage DNA monitored by qPCR (Pholwat *et al.*, 2012). Methods such as this can be

investigated to determine whether they can be applied to Mycobacteria isolated from PBMCs and extracted by Actiphage Rapid™ assay to further increase the versatility of Actiphage Rapid™ assay. Again, providing another option for drug resistance profiling of MTBC, something which is of large clinical importance.

7.3. CONCLUSION

Actiphage Rapid™ assay has proved to be a powerful tool in diagnostic testing of both animals and humans. Further development of Actiphage Rapid™ assay for detection of mycobacteria moving from endpoint PCR to qPCR allows for a rapid, sensitive and high-throughput diagnostic assay. Whilst further applications of Actiphage Rapid™ have demonstrated the versatility of Actiphage Rapid™ being more than just a diagnostic assay.

CHAPTER 8

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CHAPTER 9

APPENDICES

frontiers in Veterinary Science

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

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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**

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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^{TM}) 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 maker of disease for infections caused by both *Mycobacterium bovis* and *Myocbacterium 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 Johne'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 $\sim 10^2$ 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

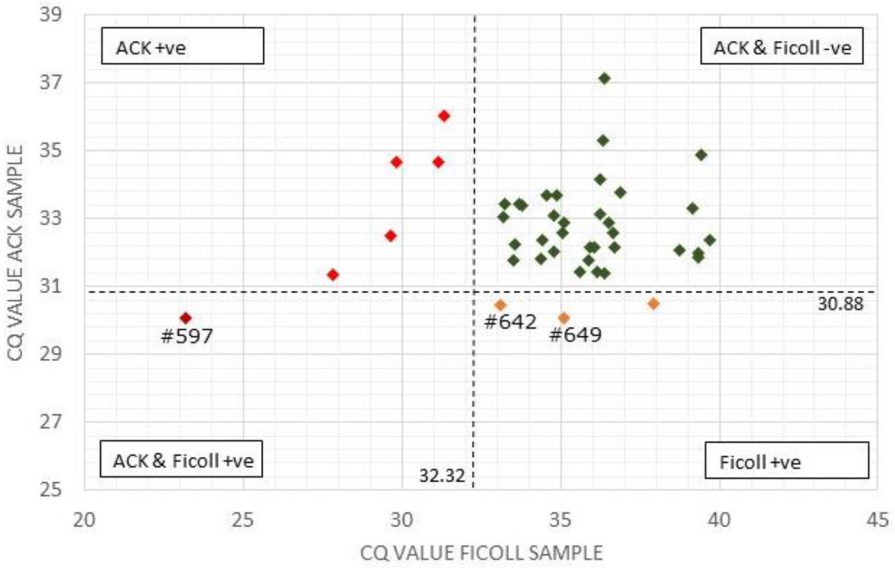

FIGURE 1 | Comparison of Actiphage pPCR test results using Ficoll and ACK methods to purify white blood cells. The Cq-values for the paired blood samples are compared using a scatter plot. Vertical and horizontal dashed lines are set at the cut off values for the Ficoll and ACK samples, respectively. 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 (ΔCq $\geq$2). A sample producing a Cq-value between 1.5 and 2 units lower than the negative control was scored as weak positive (ΔCq =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 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 ~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 ~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

[a] *Actiphage positive control = Media Plus inoculated with ∼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 = ΔCq = ≥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.*

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 is 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

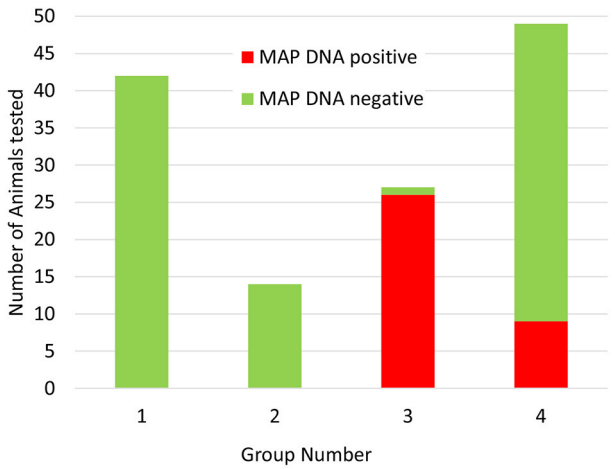

FIGURE 2 | Distribution of Actiphage MAP test results in commercial deer herd production groups. Blood samples were taken from 132 female deer prior to the rutting season. The samples were supplied to the lab blinded to production group. After the test results were available, the samples were unblended 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 (ΔCq > 5), and the remaining 10 samples were scored as weak positive (ΔCq 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 (ΔCq 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	ΔCq[c]	Test result[b]	Cq-value	ΔCq[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).

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

[b] *Test result; Positive = ΔCq = ≥2; Positive weak (w) = ΔCq 1.5-2.*

[c] *ΔCq; 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 (~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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Optimisation of IS*900* and IS*1311* PCR for detection and subtyping of *Mycobacterium avium* complex to improve specificity of Actiphage® assay

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Abstract

Actiphage® exploits the lytic properties of bacteriophage to extract DNA from low levels of MAP cells isolated from blood samples. MAP is typically detected by PCR amplification of conserved IS*900* sequences, however early primer design was based on a limited number of cattle type genome sequences. Using primers P90 and P91, it was found that PCR sensitivity was poor when samples from host species other than cattle were tested. Analysis of MAP genome sequences found a sequence mismatch at the 3' end of the P90 primer in many non-cattle strains. P90 primer was re-designed to improve specificity and was successfully used to detect MAP in the blood of farmed goats. The method was further developed to allow direct determination of subtype based on restriction enzyme analysis of amplified IS*1311* (PCR-REA) and it was shown that this can be used to both detect infection and subtype MAC organisms in the blood of infected bison, humans and other exotic species.

Introduction

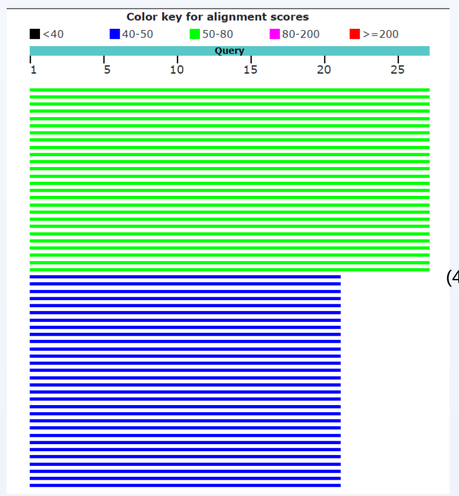
The *Mycobacterium avium* complex is a group of related organisms that cause a range of diseases. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a member of this complex and causes a chronic enteritis of adult cattle and other ruminants (Johne's disease). Genome analysis has identified different sub-types of this organism which have different host tropisms[1]. MAP is typically very slow growing and very long incubation periods are required for conventional culture (typically 40 days to 18 weeks). Therefore DNA-based methods of detection and identification are normally used to diagnose infection. Actiphage® Rapid assay utilises mycobacteriophage D29 to infect and lyse Mycobacteria to allow DNA to be used for PCR identification[2].

Method & Results

BLASTn analysis through the NCBI database of P90 primer shows a mismatch at the 3' end of many MAP genome sequences. The majority of sequences with this mismatch are non-cattle strains of MAP (figure 1a, 2).

The primer was redesigned, and this new primer was found to be able to detect lower levels of MAP genomic DNA (Figure 1b), with an decrease in the LOD of approximately 100-fold.

A



B

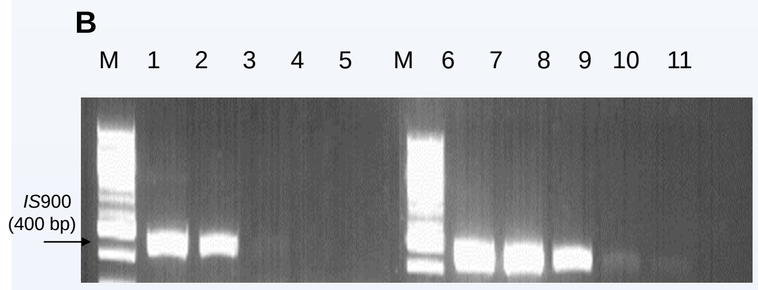


Figure 1A. Graphical representation showing 6 bp mismatch identified at 3' end of P90. **Figure 1B. Comparison of sensitivity of P90 primers.** Genomic MAP DNA was diluted in 10-fold steps from 45 ng and amplified using either the original 90 (lanes 1-5) or redesigned P90 (lanes 6-10). Lane 11 is a no template control.

A

B

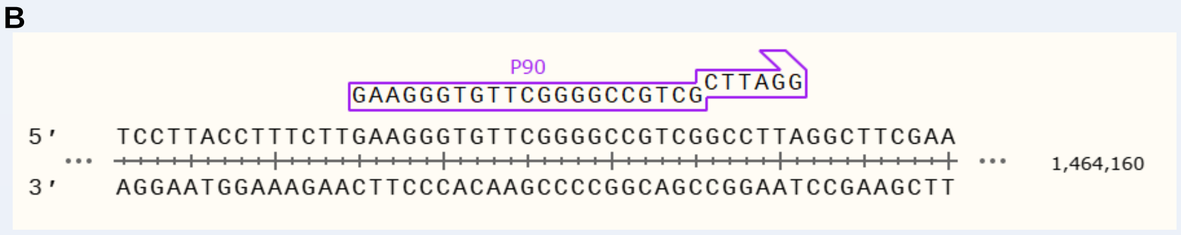
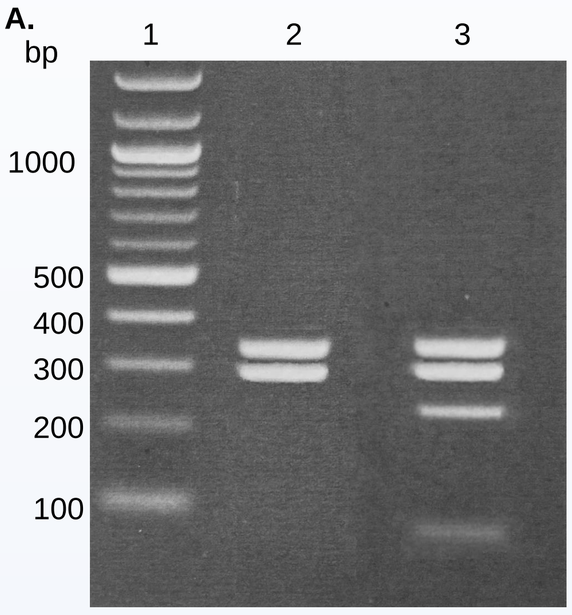


Figure 2. Analysis of the genome sequences of many non-cattle strains including **A.** MAP Telford strain from host *Ovis aries* and **B.** MAP MAP4 strain from host *Homo sapiens* show a 6 bp mismatch at the 3' end of the primer sequence.

Method & Results

MAP DNA was extracted from blood samples of animals using Actiphage® Rapid assay. Primer pair M56/M119 were used to amplify a region of IS*1311* insertion sequence found in *M. avium* complex. After amplification, restriction enzymes *Mse*I and *Hinf*I were used to digest PCR products. Products of restriction digest were run on a 2 % (w/v) agarose gel. MAP subtypes can be differentiated based on the band patterns after digest which detects variation in gene sequence

A.



B.

MAC subtype	band pattern (bp)
M. avium	285, 189, 134
MAP Sheep (S) strain	323, 285
MAP Cattle (C) strain	323, 285, 218, 67
MAP bison strain	323, 218, 67

Figure 3A. Electrophoresis gel image of MAP subtypes identified after IS*1311* PCR-REA from the blood of a parakeet. **Lane 1;** 100 bp ladder, **Lane 2;** MAP Sheep (S) strain, **Lane 3;** MAP Cattle (C) strain.

Figure 3B. Base pair band patterns of different members of the *M. avium* complex which allow for differentiation through IS*1311* PCR-REA

Conclusions

Sensitivity and specificity of IS*900* targeted PCR were improved for the detection of MAP from blood samples of non-cattle hosts following adjustment of P90 forward primer to remove non-homologous 3' end.

IS1311 PCR-REA[3] was successfully used to supplement the Actiphage® Rapid assay to both detect infection and subtype MAC organisms from blood samples of parakeets, bison, humans and other exotic species. In some cases co-infection of two different MAP types has also been found using this method.

References

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