# Preliminary validation of *Mycobacterium avium* subspecies *paratuberculosis* detection in ovine pooled faecal culture and species verification by PCR

**By**

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# Declaration

I declare that the thesis which I hereby submit for the degree of Masters of Science at the University of Pretoria is my own work and has not previously been submitted by me for a degree at any other university or institution of higher education.

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# List of abbreviations

| | |
|---|---|
| AFB | Acid fast bacilli |
| ANQAP | Australian national quality assurance program |
| C3 | Complement component 3 |
| CD4 | Cluster of differentiation 4 |
| CFT | Complement fixation test |
| CFU | Colony forming units |
| CPC | Cetylpyridinium chloride |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| ELISA | Enzyme-linked immunosorbent assay. |
| ESP | Extra sensing power |
| JD | Johne's disease |
| HPC | Hexadecylpyridinium chloride |
| LED | Light-emitting diode |
| LECD | Light emitting ceramic device |
| IgG | Immunoglobulin G |
| IGRAs | Interferon-Gamma Release Assays |
| IFN | Interferon |
| IS900 | Insertion sequence 900 |
| MAA | *Mycobacterium avium* subspecies *avium* |
| MAC | *Mycobacterium avium* complex |
| MAH | *Mycobacterium avium* subspecies *hominissuis* |
| MAS | *Mycobacterium avium* subspecies *silvaticum* |
| MAP | *Mycobacterium avium* subspecies *paratuberculosis* |
| MGIT | Mycobacterium growth indicator tube |
| MOTT | Mycobacterium other than tuberculosis |
| mRNA | Messenger ribonucleic acid |
| MTBC | Mycobacterium tuberculosis complex |

| | |
|---|---|
| NADIS | National Animal Disease Information Service |
| OD | Optical density |
| OIE | International Office of Epizootics |
| PCR | Polymerase chain reaction |
| PPD | Purified protein derivative |
| P90 | Primer 90 |
| P91 | Primer 91 |
| qPCR | Quantitative polymerase chain reaction |
| PT | proficiency testing |
| RNA | Ribonucleic acid |
| SA | South African |
| SANAS | South African national accreditation system |
| TAE | Tris-acetate-EDTA |
| Taq | Thermus aquaticus |
| TB | Tuberculosis |
| tRNA | Transfer ribonucleic acid |
| TTD | Time to detection |
| µl | Microliters |
| UV | Ultraviolet |
| VPTB | Validation paratuberculosis |
| ZN | Ziehl Neelsen |

# 1. Summary

## Preliminary validation of *Mycobacterium avium* subspecies *paratuberculosis* detection in ovine pooled faecal culture and species verification by PCR


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Paratuberculosis interchangeably referred to as Johne's disease (JD), is a condition in ruminants due to infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The infection is acquired by animals feeding on food contaminated with faeces of an excreting infected animal. The term "wasting" describes the condition with which is marked by progressive emaciation and weakening of the infected animal. The disease is incurable and culling is currently the only solution. The infected flocks are worthless to the national and international trade and cause a strain to the agricultural sector. Paratuberculosis is a controlled disease in South Africa, yet remains an unsolved and increasing problem. MAP is currently only diagnosed serologically, but the tests are ineffective due to low sensitivity. Asymptomatically infectious animals are the major stumbling block to manage the disease. It is equally difficult to diagnose it and to date no one method gives a 100% detection rate. Culture is the gold standard

method not withstanding its lengthy (16 weeks or longer) turnaround time, but liquid culture medium can reduce this time. This study was designed to evaluate an internationally recognised method (automated liquid culture) that is used as a tool to combat the disease in a South African setting.

The ability of the liquid culture method to detect SA MAP strain was assessed by collecting 284 samples of either tissue, faecal or both samples from herds that are known to be infected with MAP. While the ability to distinguish faecal samples not infected with MAP was assessed by collecting 50 faecal samples from herds that have not had occurrence of MAP infection. The samples were processed and cultured in the Versa Trek liquid culture system for a maximum of 70 days, growth signals or no growth signals were further assessed with molecular method to confirm the signal. The detection limit of the method was evaluated using the spiking and serial dilution method. The reproducibility of the liquid culture method was assessed through proficiency testing and precision. Finally, the sensitivity and specificity of the liquid culture method was determined by comparison with the standard reference method, histopathology.

In this study 16 MAP isolates (7 faecal and 9 tissue samples) were isolated and confirmed of the 284 cultured samples from flocks with an existing MAP infection. Thirty-five growth signals were detected from flocks that have not had an occurrence of MAP infection and none of these were MAP DNA positive. The detection limit in individual faecal samples and pooled faecal samples was $1.08x10^{4}$(2) MAP/ml and pool of 10 faecal samples respectively. The threshold for the detection limit is reduced when the recommended antibiotic supplement is excluded. Reproducibility of the method on average was 79.1%% and precision was inconclusive due to the shortage of original samples. Lastly, the sensitivity & specificity of the liquid culture method

against the standard reference method was determined to be 33% & 25%, 97% & 98% for faecal and tissue culture, respectively. On the other hand, for tissue culture, the sensitivity increased to 100% when we excluded the antibiotic supplement.

In addition, the study demonstrated that there might be a strong inhibitory effect posed by the antibiotic supplement on the SA MAP faecal isolated strain. This needs further assessment using larger sample size and antibiotic profiling.

In conclusion, the study did demonstrate that the method can detect SA MAP strain but this is limited for the faecal isolates. By this, the study has emphasised the importance of validating a diagnostic method from different geographic regions. When dealing with bacteria, the behaviour of type species may differ from one location to another and we have demonstrated that this can possibly fall short as a method in one region while it may succeed in another, therefore, validation of a method for its intended use is imperative. The determined pool size will be valuable in a screening process part of a control program, offer reduced cost associated with individual sample processing. Validation is a continuous process and the information attained in this study will form a foundation for this and the validated method will be applied routinely as a diagnostic service offered by the Tuberculosis laboratory.

# *Chapter 1*

## Literature review

### *1.1.* Historical review of *Mycobacterium avium* subspecies *paratuberculosis*

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) as a pathogenic organism is not a new phenomenon; it has been dated back in time, with most of the cases reported in animals (Manning & Collins, 2010). Literature records the first clinically demonstrated incident of MAP infection as early as 1807, where a cow presented with wasting. The same symptoms were to later on appear by the year 1829 in a different case (Chiodini, 2006; Manning & Collins, 2010). Even though there were many cases at the time, there was no evidence connecting the symptoms or lesions with the Mycobacterium species. It was not until 1895 when post-mortem analysis by Johne and Frothingam on a cow from Germany that provided a breakthrough (Thorel. 1984, (Cocito et al., 1994; Harris & Barletta, 2001). The cow presented with a loss of weight, milk production failure, thickened intestinal mucosa lining and enlarged mesenteric lymph nodes (Manning & Collins, 2010) The tissue samples from this cow were stained and acid fast bacilli resembling that of *Mycobacterium tuberculosis,* the etiological agent responsible for tuberculosis in humans was observed (Kreeger, 1991; Verdugo et al., 2014). The possibility that the observed acid-fast bacilli could be *Mycobacterium tuberculosis* was quickly ruled out by the failure to isolate it, as with *Mycobacterium tuberculosis,* and that tissue samples containing these microorganisms failed to cause disease in guinea pigs, as *Mycobacterium tuberculosis* would (Behr & Collins, 2010). These observations by Johne and Frothingam marked the groundwork of connecting

the disease with Mycobacterium species. Johne and Frothingam made the first diagnosis as *Mycobacterium avium*, the etiological agent of tuberculosis in birds and proposed the name of the disease as pseudotuberculosis enteritis (Behr & Collins, 2010). The name paratuberculosis was proposed by Bang in 1906 after he differentiated the different infections caused by Mycobacterium species. Bang added to the classification of MAP by demonstrating that cattle with symptoms described for paratuberculosis responded highly to an antigen prepared from *Mycobacterium avium,* he further related it to the *Mycobacterium avium* identified in birds and this finding led to the development of diagnostic methods for the condition (Cocito et al., 1994). The disease paratuberculosis today is commonly known as Johne's disease, named after Heinrich Albert Johne by John Mcfadyean. The first isolation of the bacterium responsible for the cause of this devastating disease was in the year 1912; it happened unintentionally when Twort observed satellite colonies around that of *Mycobacterium phlei,* for which he then proposed the name *Mycobacterium enteriditis chronicae pseudotuberculosis bovis johne*. In the same year, a successful intended isolate of MAP by Holth was made, which he named *Paratuberculous bacillus* (Bakker et al., 2000; Chiodini et al., 1984; Cocito et al., 1994). An important discovery that Twort and the latter made was mycobactin a sidephore used by members of the genus Mycobacterium to transport free extracellular iron into the cytoplasm of mycobacterial cells and it is an important component in the isolation of MAP by culture (Morrison, 1965). Mycobactin is a sidephore that most members of the genus Mycobacterium produce, it serves to bind and transport iron from the surroundings into the cytoplasm of the mycobacterial cells. MAP and some members of the genus lack the capacity to produce mycobactin and require mycobactin supplement for growth in the laboratory (Thorel 1984). The successful isolation was a doorway to study and experiment on the

subject. In 1923 the disease was officially named paratuberculosis with *Mycobacterium paratuberculosis* as the official name for the etiological agent. Today the etiological agent is accepted as *Mycobacterium avium* subspecies *paratuberculosis*, accredited to the work of Thorel and co-workers. They proved that *Mycobacterium paratuberculosis* belongs to the *Mycobacterium avium* complex. They then proposed to rename it to *Mycobacterium avium* subspecies *paratuberculosis* and their proposal was accepted and made the official name (Chiodini, 2006).

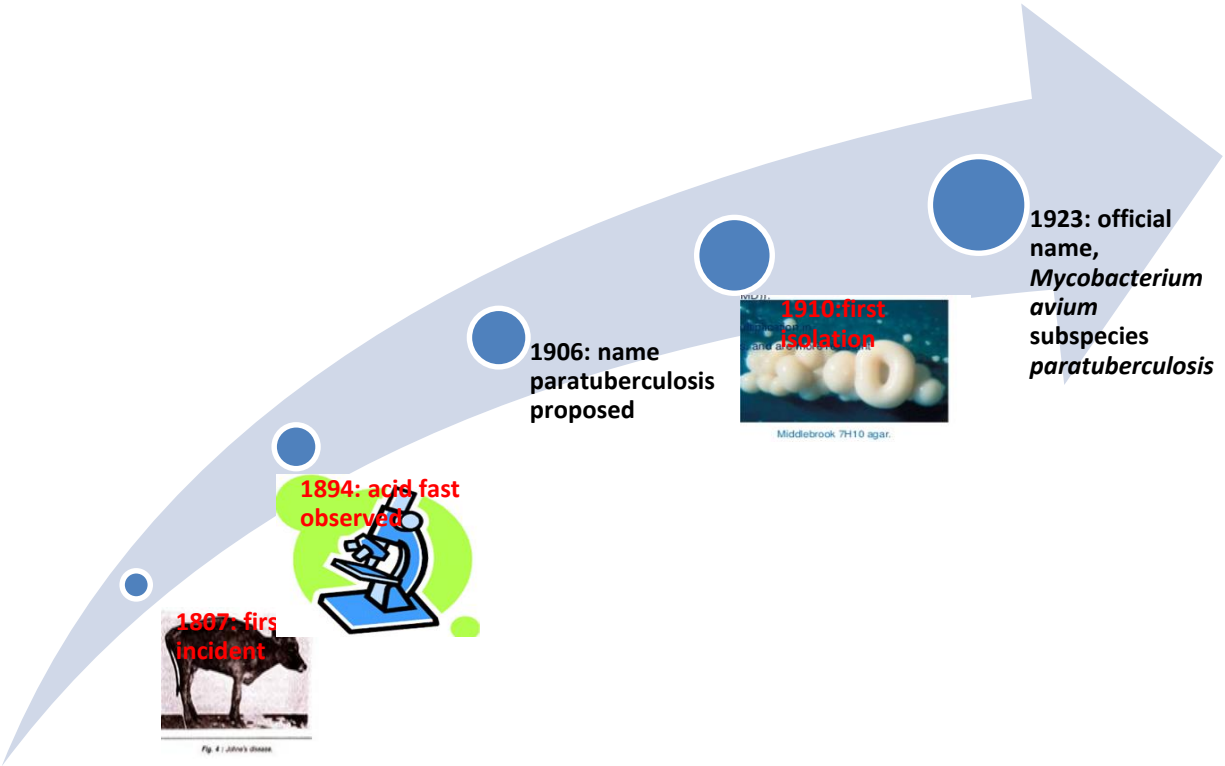



**Figure 1: historical events of *Mycobacterium Avium* Subspecies *Paratuberculosis* that lead to its identification, images adopted from : http://cdn.biologydiscussion.com/wp-content/uploads/2016/03/clip_image011_thumb2_thumb.jpg, https://i.pinimg.com/originals/47/3c/64/473c64a896704e3879118e3a35f95345.jpg, Shahini.2016,**

## 1.2. Taxonomy and classification of *Mycobacterium avium* subspecies *paratuberculosis*

Conventional methods of classification and grouping of microorganisms were according to pathogenicity, host, and growth rate together with pigmentation properties initially described by Runyon and co-workers (Clark et al 1993). *Mycobacterium avium* subspecies *paratuberculosis (*MAP) is a member of the Mycobacterium genus. The Mycobacterium genus is grouped under prokaryotic organisms, classified within the phylum Actinobacteria and the family *Mycobacteriaceae*. Two broad distinctions are made between the Mycobacterium species, achieved by conventional methods and ribosomal gene analysis. One class is that of the fast growing mycobacteria and the other being the slowly growing mycobacteria, with the latter considered mostly as the pathogenic species (Harris & Barletta, 2001). The slow growing mycobacteria consist of photochromogenes, characterised by formation of yellow to orange-pigmented colonies when exposed to light. Another class of slow dividing mycobacteria is those termed scotochromogenes, and their pigmentation is exhibited in the dark. The third group characterised by no pigmentation is termed non-chromogens (Rastogi et al., 2001). Runyon designates the fourth group as the rapidly dividing mycobacteria, in his classification method according to growth rates and pigmentation properties (Madkour, 2003). Designation according to difference or similarity to *Mycobacterium tuberculosis* complex can easily be identified in the nomenclature of the different groups of Mycobacterium species, except for the leprosy agents (Inderlied et al). Mycobacterium tuberculosis complex (MTBC) is a class of Mycobacterium having the same pathogenic trait of causing tuberculosis in animals and humans, for example, *Mycobacterium bovis* and *Mycobacterium africanum*. Mycobacterium other than tuberculosis (MOTT) consists of Mycobacterial species that do not cause tuberculosis,

but may cause a disease that resembles tuberculosis. Examples of these are the *Mycobacterium kansasii* and *Mycobacterium avium,* where we find the subspecies of interest in this study, the MAP species. The group Leprosy agents consist of the species *M. lepromatosis* and *M. leprae,* and these are characterised by causing a skin disease known as leprosy.

Within MOTT, species are further divided into *Mycobacterium avium* complex (MAC) and non-tuberculosis other than *Mycobacterium avium* complex. *Mycobacterium avium* subspecies *paratuberculosis* falls under MAC, which consists of two species, *Mycobacterium intracellulare* and *Mycobacterium avium*. *Mycobacterium avium* species is divided into four subspecies, including *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Mycobacterium avium* subspecies *hominissuis* (MAH), *Mycobacterium avium* subspecies *avium* (MAA) and *Mycobacterium avium* subspecies *silvaticum* (MAS) (Everman, 2014; Soininen, 2014; Sykes & Gunn-Moore, 2014).

## 1.3. *Mycobacterium avium* subspecies *paratuberculosis* strains and phylogenic analysis

Designation of MAP to the *Mycobacterium avium* complex and assignation of MAP within the complex is achievable through phenotypical analysis (Turenne et al., 2008). In addition, there are pathogenic studies observing the disease pattern and their ability to cause disease in ruminants (Svastova et al., 2002). Genetic analysis detects specific insertion sequences in the genome of the organism to designate it to species level. For the speciation of MAP, a high copy number of insertion element IS*900* was

discovered to be specific for the species (Harris & Barletta, 2001), and it is now used to speciate (Turenne et al., 2008). Multi locus sequencing analysis and whole genome sequence analysis of the MAP genome studies suggests that MAP has evolved from MAH (Bryant et al., 2016; Mobius et al., 2015; Turenne et al., 2008). Though genetically common, *Mycobacterium avium* subspecies *avium*, *Mycobacterium avium* subspecies *paratuberculosis* and *Mycobacterium avium* subspecies *silvaticum* have evolved independently of one another from MAH (Figure 2) (Hsu et al., 2011; Vluggen et al., 2016). In early studies, the MAP subspecies genome has always suggested a much closer relationship to MAA (Thorel, 1989), with most of the genes studied at the time presenting with more than 95% homogeneity (Bannantine et al., 2003; Harris & Barletta, 2001; Manning & Collins, 2010) . *Mycobacterium avium* subspecies *paratuberculosis* has three molecular types that differ at nucleotide level and these are differentiated by using molecular methods. In early studies, these molecular types were termed Cattle strain (C strain), Sheep strain (S strain) and Bison strain. However, as research advanced, it was established that the molecular types are not directly related to the host (de Juan et al., 2006) and are now mostly denoted to as type I, type II and type III molecular types respectively (Sulyiman, 2014; Windsor, 2015a). Type III was previously recognized as the intermediate type between type I and type II molecular types (de Juan et al., 2006) , and was initially identified to be closely related to molecular type I (Möbius et al., 2009). However, recent studies have identified type III as a subtype of type II (Stevenson, 2015). Josephine and coworkers (2016) in their recent study, they used single nucleotide polymorphism methods to discard the trend that type III is an intermediate molecular type and instead concluded that both type I and type III are subtypes of type II (Leao et al., 2016). Insertion elements in a genome are studied, with reference to the number of copies present in

the genome and variations in base composition of the specific insertion element, to differentiate the molecular types of a subspecies (Turenne et al., 2008). Insertion element IS1311 in the paratuberculosis genome is observed for variations in the base position 223. Molecular type I at this position in the copy of this element is identified by having a T base, molecular type II has a C base and molecular type III interchanges T and C bases at this position from one copy of the element to the next in the genome (Bryant et al., 2016) . In addition to the molecular difference exhibited by molecular type I, culture and isolation of type I is more challenging compared to the two (Stevenson, 2015). Animal model studies suggest that the virulence abilities of the identified strains have a lot more to do with the host susceptibility or resistance to the strain infection as compared to the strain's ability to cause disease. But not to disprove that the different strains appear to be well adapted to their apparent host preferences (Stevenson, 2015).

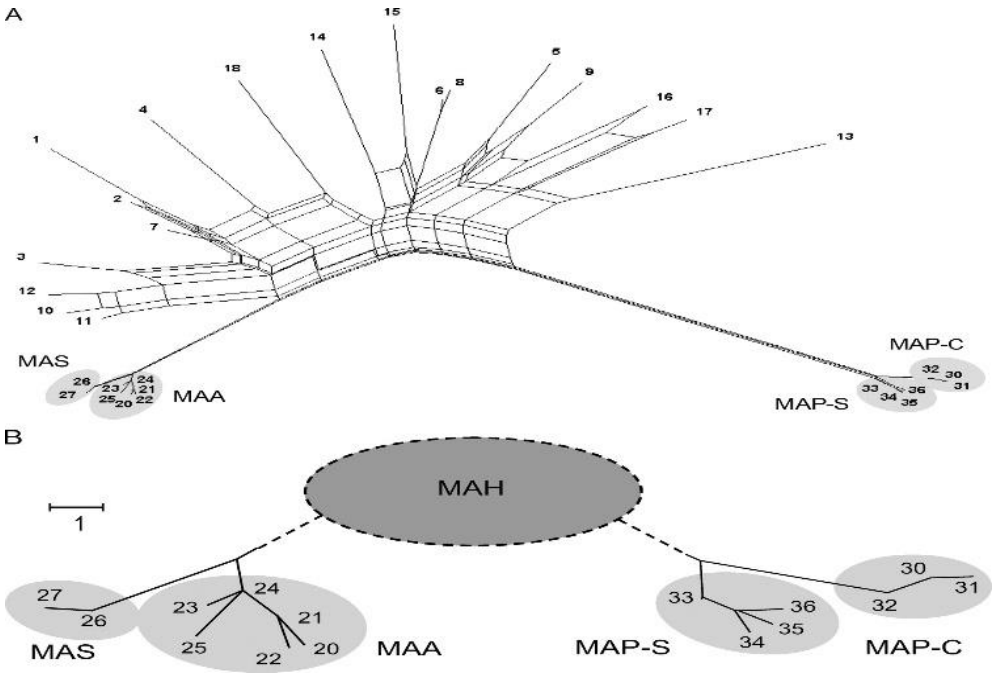



**Figure 2 Turenne et al 2008: phylogenetic tree demonstrating the evolutionary relationship between the Mycobacterium avium species and subspecies**

## 1.4. Microbiological characteristics of *Mycobacterium avium* subspecies *paratuberculosis*

Aligning with other Mycobacterium species, MAP is a gram-positive acid-fast bacillus and appears as straight or curved rods that at times may be branched when viewed under the microscope. In nature, MAP organisms tend to attach to one another by intracellular filaments and form clumps (Sulyiman, 2014). They are non-motile, non-spore forming with no capsule, though surrounded by a capsule-like material (Eckstein et al., 2006) and their typical size is 0.2 to 0.6 µm wide and one to 10µm in length. The bacterium size of MAP is approximately 0.5-µm wide and 2µm in length, MAP can be as small as 0, 5 by 1.5 micron (Manning & Collins, 2010) . The Mycobacterium genus is also a very fastidious group of organisms. Like most typical bacteria, mycobacteria have a cell wall covered by a cell envelope made of proteins, carbohydrates and lipids (Inderlied et al.,1993) . A characteristic that distinguishes the Mycobacterium genus from other genera is the lipid rich cell wall. The cell wall has three major components. A mycolylarabinogalactan sandwiched by covalent bonds between the peptidoglycan (bottom layer) and long chains of fatty acids called mycolic acids (upper layer). The wall is said to be a lipid rich wax layer (figure 2), because in addition to these attached mycolic acids making up more than 30% of the envelope (Eckstein et al., 2006; Niederweis et al., 2010) , additional mycolic acids are present as free monomycolates, dimycolates, surfaced lipids like the sulfolipds and diacyl trehaloses (Chopra & Gokhale, 2009). Fatty acid profiling has demonstrated abundance of palmitic acid and oleic acids in MAP (Alonso-Hearn et al., 2017). Alonso-Hearn et al. reported that the fatty acid profiles of MAP differ with strains and that this profile is altered once MAP has established an intracellular infection. This characteristic gives them the benefit of

being hydrophobic, thermo-tolerant, chlorine resistant and surviving acidic environments (Manning & Collins, 2001a; Niederweis et al., 2010).

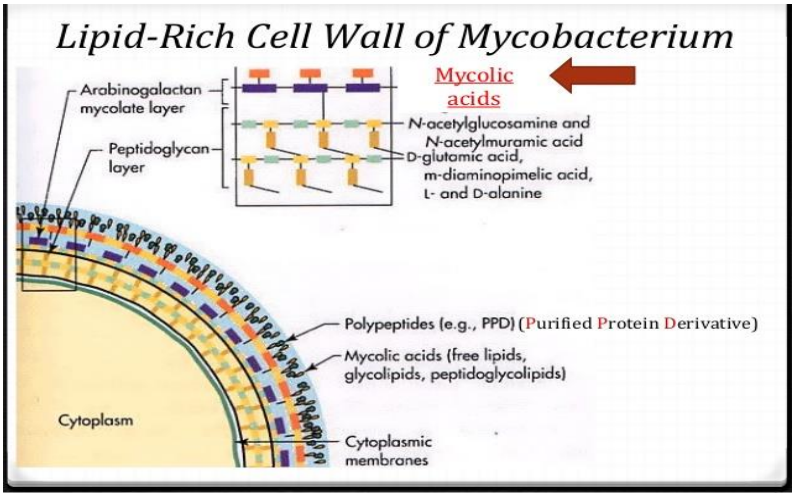



**Figure 3: labelled schematic diagram of Mycobacterial lipid rich cell wall (Anuj et al., 2015)**

Below the peptidoglycan, the cytoplasmic plasma membrane follows and is divided into two layers that differ in their thickness and composition (Chopra & Gokhale, 2009). The plasma membrane surrounds the cytoplasm containing a single copy DNA molecule (Inderlied et al.,1993) .

The DNA molecule of MAP consists of coding sequences and non-coding sequences (Li et al., 2005). At nucleic acid level, base composition of the mycobacteria genome predominates with G+C base pairing and the pairs are evenly distributed across the genome, comprising between 62% and 70% of the genome (Harris & Barletta, 2001; Li et al., 2005; Rastogi et al., 2001). Furthermore, the genome size of MAP has been estimated at 4.4 to 4.7 Mb, however, in sequencing studies the genome size is estimated at 5 Mb (Harris & Barletta, 2001; Li et al., 2005).

Linling Li and co-workers (2005) sequenced a MAP strain and reported the genome as a closed circle linear chromosome consisting of 4.8Mb (figure 3). Additionally, the

MAP genome contains repetitive DNA sequences like the ISMAP02 and IS*900,* occupying about 15 % of the total genome (de Silva et al., 2013; Li et al., 2005) and some repeated housekeeping genes (Li et al., 2005). IS*900* is further discussed under the diagnosis section.

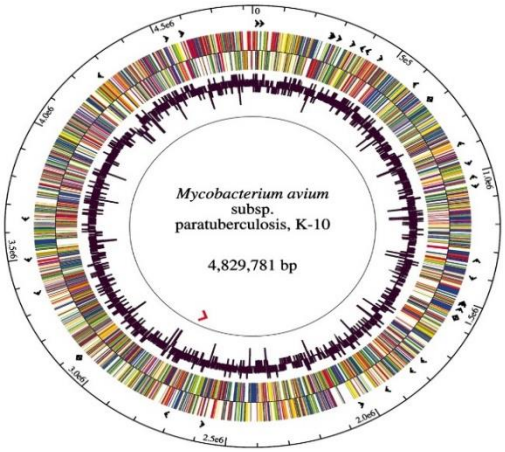



**Figure 4: MAP genome representation (Li et al., 2005)**

Linling Li and colleagues went further to do a comparative analysis with some other Mycobacterium species and found the MAP genome to be highly homologous to the genome of the human *Mycobacterium tuberculosis* (Li et al., 2005). High homogeneity is also reported with *Mycobacterium avium* subspecies *avium* (Manning & Collins, 2001b). Genomic comparison of different isolates of MAP reveals genetic variability in certain regions of the genome (O'Shea et al.,2011). The MAP genome consists of some and not all gene clusters present in other mycobacteria, example, the urease production genes are present in other mycobacteria but not found in the MAP genome (Li et al., 2005). Another notable difference is the gene cluster coding for synthetic enzymes responsible for mycobactin siderophore, in the MAP genome, this gene is shortened compared to in other mycobacteria (http://hdl.handle.net/1969.1/ETD-TAMU-2612). The MAP genome appears to be well adapted for its lipid metabolism pathways,

with Lingling Li and co-workers reporting 80 more additional genes in the MAP genome as compared to the MTBC genome for this function (Li et al., 2005).

In contrast with most typical bacteria, in a growth medium, mycobacteria prefer to use fatty acids such as palmitic acid and oleic acid as a source of carbon, instead of carbohydrates like glucose and glycerol (Inderlied et al., 1993, Li et al., 2005). The *Mycobacterium avium* complex cell growth is categorised in three stages. During the first stage of growth, there is cell elongation accompanied by the rapid uptake of fatty acid and protein synthesis. In the second stage of growth, there is an intracellular break down of triglycerides for the supply of carbon and energy. The third stage is characterized by the formation of heterogeneous cells consisting of filamentous, rods and coccobacilliary forms (Inderlied et al., 1993). When cultured, MAP colonies exhibit either a pigmented (yellow color) or a non-pigmented phenotype depending on the strain. Additionally, the colony morphology formed by MAP strains appears to be similar with small hemispherical colonies with a glossy surface and well-defined ends (Whittington et al., 2011).

## 1.5. *Mycobacterium avium* subspecies *paratuberculosis* and Johne's disease

Certain Mycobacterium species are pathogens to both humans and animals. MAP in humans is a suspect in the cause of a disease known as Crohn's disease, where the bowel becomes inflamed and thickened. However, the pathogenesis is still not yet fully understood (J & EL-Zaatari.F.A.K, 2004; Sulyiman, 2014). In animals, MAP is proven responsible for the cause of Johne's disease. Johne's disease is a contagious chronic infection that primarily affects the small intestine of ruminants. It infects both domestic and wild ruminants; of which the most affected ruminants are sheep (ovine) and cattle (bovine) (Ayele et al., 2001).

### 1.5.1. *Pathogenesis of Johne's disease*:

Subsequent to the oral consumption of MAP, MAP colonizes the mucosa of the small intestine, and associates with lymph nodes, infrequently the tonsil or pharyngeal lymph nodes. *MAP* in an infected mother crosses the placenta to reach the foetus (Sockett. 2000). Bacterial replication begins at the terminal part of the small intestine and the large intestine (Ayele et al., 2001). MAP employs several virulence factors such as fibronectin. Fibronectin is a secreted surface protein that binds and forms a bridge with the integrins on the M cells, which are cells localised in the follicle associated epithelium, forming an inside layer of the Peyer's patches in the intestines. The bridge formed facilitates the bacterium crossover and penetration of the intestines (http://www.paratuberculosis.info/web/images/stories/pdfs/128). *Mycobacterium avium* subspecies *paratuberculosis* also employs digestive enzymes and takes advantage of the mucus in the payers' patches. Additionally, MAP demonstrates to form an active interaction with enterocytes, the cells lining the intestine that facilitates

its entry (Koets et al., 2015). Through these virulence factors, MAP passes through the epithelial cells down to sub-epithelial cells infiltrating the intermediate epithelial layer, reaching the adjacent lamina propria and establishes an intracellular infection (Sulyiman, 2014). Once the infection is established, MAP has a number of proteins recognised as antigens by the host immune system. These proteins include the heat shock proteins, the cell wall antigens, and the secreted antigens, resulting in host's immune system response being triggered (Harris & Barletta, 2001). Antigens used with immunological diagnostic methods include the polysaccharide bacterial component known as the Lipoarabinomannan. It gives high specificity and sensitivity, suggesting dominance in provoking immunity during an infection (Reichel et al., 1999). Recognition of foreign material results in local macrophages and dendric cells engulfing the MAP particles (Koets et al., 2015). Initial exposure to MAP triggers a T-cell mediated immune response. Proinflammatory cytokines such as gamma interferon, interleukin-6 and interleukin-2 are released during this response to the infection. It is suggested that animals that do not progress to the clinical disease stage, control the infection at this level. Inability of the cellular immune response to control the infection will pass on to humoral immune response, which predominates with immunoglobulin GI (Coussens, 2004). MAP is generally considered a poor immune response-stimulating organism (Purdie et al., 2011). MAP alters host immune responses such as macrophage processes and cell apoptosis (the programmed cell death) that fight infections. Additionally, MAP circumvents $CD4^+$ based antigen presentation and opts for the $CD8^+$ antigen presentation based pathway. These are all done to ensure its survival (Purdie et al. 2011). Events occur that lead to activation of macrophages and the macrophage activation leads to phagocytosis. Macrophages use the general nonspecific receptors, the mannose receptors and that of the

complement 3 (C3) pathway to engulf MAP (Alonso-Hearn et al., 2017). During phagocytosis, the bacterium is engulfed forming a phagosome. In the phagosome, acidification, lysozyme and iron limited environment are employed, which all act to kill and inhibit the growth of MAP, which employs more strategies to survive. It prevents phagolysosome fusion and acidification. Lipoarabinomannan is recognised as one of the major virulence factors employed by MAP to initiate the prevention of lysozyme fusion and plays a big role in the fate of the infection. Moreover, the fatty acid profile alteration and remodeling that occurs in the phagosome is suspected to play a role in the survival of MAP (Alonso-Hearn et al., 2017).To survive the limited iron environment, Mycobacterium species produce siderophore mycobactin and exocholin (Harris & Barletta, 2001). However, MAP is deficient in the genes required to produce the above-mentioned iron chelating agents, it appears to use alternative ways, dependent on neither the mycobactin pathway nor the host to acquire iron from the intracellular environment (Wang et al., 2016). These abilities allow MAP to multiply and grow in the macrophages, through which they are released in large numbers (Singh et al., 2013b). Additionally, MAP is shown to manipulate the host lipid pathway, such as the cholesterol pathway, to create favorable conditions that will allow it to survive and persist. Thirunavukkarasu and coworkers (2013) demonstrated that cholesterol accumulates during MAP infection, suggestive of host manipulation to create a favorable environment (Thirunavukkarasu et al., 2013).

Granuloma formation isolates the focus of infection, contains the bacteria and prevents the propagation to new sites. The granulomatous lesions formed expand due to the continued growth of MAP and damage the intestinal tissue, which leads to inflammation of the tissue. These events lead to malabsorption and thickening of the intestinal wall. Malabsorption leads to the observed clinical signs such as loss of

weight, diarrhea and other signs (Ayele et al., 2001; Sulyiman, 2014). Pathogenicity of MAP is greatly enhanced by the inflammation of the infected tissue (Coussens, 2004).

Disease progression of Johne's disease is divided into four phases (figure 5). The first phase is the latent Johne's infection; at this stage, the infected animal shows no clinical signs of the disease. The first line protective immunity induced is the cell-mediated immune response (Steven, 2006). During this stage, most diagnostic methods can give false negative results. What follows is the early subclinical phase when the animal may show no clinical signs but may be shedding the bacteria with its faecal excretions. Humoral immune response is associated with this stage, though faecal shedding may occur before this immunity is induced. The clinical phase of the disease is associated with profuse diarrhea, loss of weight and weakening of the infected animal. A large number of MAP are excreted in the faecal matter of the animal and during this stage, MAP is detectable by most diagnostic methods. The fourth stage, usually considered as the advanced stage, the animal is obviously sick. During the third and fourth stages of the disease, cell mediated immune response drops. The serum antibody based immune response develops with little effect on the disease outcome (Steven, 2006). Some animals suppress disease progression to advance stages; however, it is not certain if animals are able to clear the infection (Hayton, 2007).

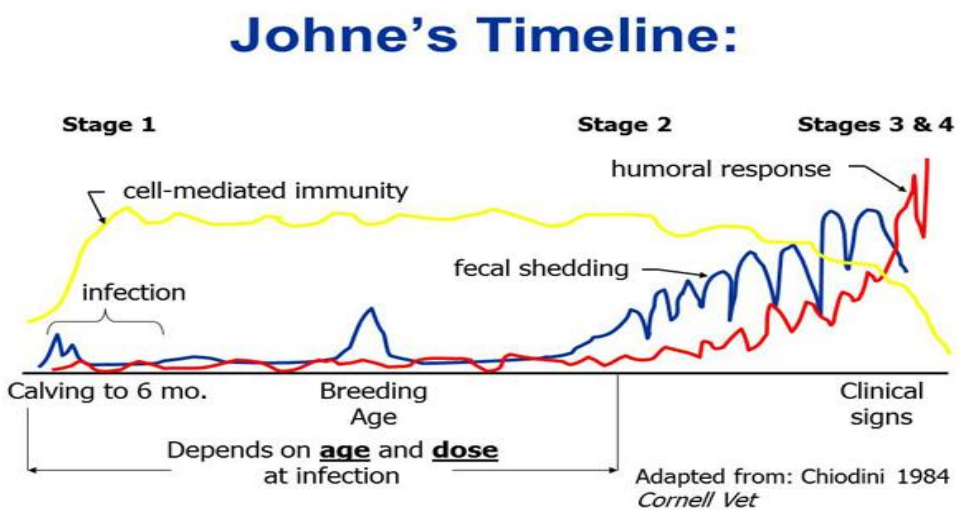



**Figure 5. Disease progression, the different stages and the associated immune response (Steven, 2006)**

### *1.5.2. Epidemiology of Johne's disease*

#### 1.5.2.1. ***Host range***:

Johne's disease is common in some domesticated ruminants, however, the disease is not limited to them but also occurs in ruminants, whether in captive or free living (Clarke, 1997). Cases in non-ruminants are reported and this includes horses and rabbits.

#### *1.5.2.1.1. Johne's disease in cattle*

**Figure 6: Cow presenting with loss of weight and wasting, resulting from MAP infection (Rathnaiah et al., 2017)**

MAP infection was first documented in cattle (Chiodini, 2006) and since then, bovine paratuberculosis is common worldwide. Clinical symptoms common in cattle involve wasting, loss of weight, diarrhea (Plattner et al., 2013) and decrease in milk production is also an associated symptom (McAloon et al., 2016). Bovine paratuberculosis is endemic in areas such as Australia, Canada, Netherlands and United States. Dairy industries experience high incidence rates of bovine paratuberculosis (Pritchard et al., 2017). Risk factors for cattle infection include age; young cattle are more susceptible to MAP infection and progression to disease as compared to adult cattle. Genetic associated risk factors and heritability of infection are reported in cows (Pinedo et al., 2008; Ruiz-Larrañaga et al., 2017). Different resistance among different breeds is reported, the Shorthorn breed shows higher incidence rates (Chiodini et al., 1984), whilst in the United Kingdom, the Channel Island breed is reported to have a higher incidence (Kirkpatrick et al., 2011). Clinical disease in cattle after infection may not develop until the age of 2 to 5 years. Cattle are considered more resistant to paratuberculosis compared to sheep and goats, moreover to the type II strain (Windsor, 2015a).

#### *1.5.2.1.2. Johne's disease in sheep.*

**Figure 7 : Sheep presenting with Johne's disease symptoms, with the rare observed diarrhea (www.nadis.org.uk)**

Bovine paratuberculosis has received more attention as compared to the smaller ruminants like sheep and goats. However, ovine paratuberculosis occurrences has been increasingly reported in Australia, South America, South Africa and other parts of the world (Dhand et al., 2016). The first case of ovine paratuberculosis was documented in the year 1911 (Juste & Perez, 2011). The different susceptibility according to breeds is not thoroughly studied; we have reports by farmers stating a higher infection and clinical cases with Merino breeds as compared to Romney sheep (Kirkpatrick et al., 2011). Abendano and coworkers (2014) suggested that sheep are susceptible to cross infection with other ruminants depending on cohabitation and geographic region (Abendano et al., 2014). Age dependent susceptibility in sheep is suggested to be the same as cattle, but it has not been well studied. One study on different infection rates in sheep according to age, showed no significant difference (AusVet Animal Health Services. 2005). McGregor and co-workers did a study across all age groups in sheep. They found that all the age groups are equally likely to be colonized by MAP after exposure to different specific doses. The difference lay in the time it took to being an infectious animal. It took longer for older animals compared to the younger animals (McGregor et al., 2012). It is evident experimentally that sheep

progression to disease after infection is more severe when infected with the Type II strain. The primary clinical sign in sheep associated with Johne's disease is weight loss, oedema (occasional) and diarrhea is not considered a frequent clinical feature in sheep. Hypoproteinemia, wool shredding and reduced fleece are some of the clinical symptoms in sheep and these are associated with the advanced stage of the disease (Stehman, 1996). Sheep can start to show clinical signs at the age of two, whilst in other animals can take three to five years before showing clinical symptoms (Begg et al., 2011).

#### *1.5.2.1.3. Johne's disease in goats*

A goat showing symptoms of Johne's disease.

**Figure 8: Symptomatic Caprine paratuberculosis (http://adga.org/johnes-disease/)**

Caprine paratuberculosis is reported in Turkey, France, Canada, USA and other parts of the world (Windsor, 2015a). Australia experiences high prevalence of the sheep strain (type II) in goats that produce fiber, while the C strain is more common in dairy goats (Windsor, 2015b). Goats are considered more susceptible to MAP infection as compared to sheep and cattle (Windsor, 2015a). As with sheep and cattle, infection may be in the form of faecal-oral route, ingestion of contaminated milk, colostrum and

other sources that are contaminated by faeces of a shedding infected animal (Angelidou et al., 2014). Higher intestinal parasitic loads in goats are a risk factor of MAP infection. Parasitic loads are amplified by anthelmintics resistance, which is worsened by the continual use of the same drug (Angelidou et al., 2014). As with sheep, profuse watery diarrhea is not common in goats (Rawther et al., 2012).

### *1.5.2.2. Transmission and the spread of Johne's disease:*

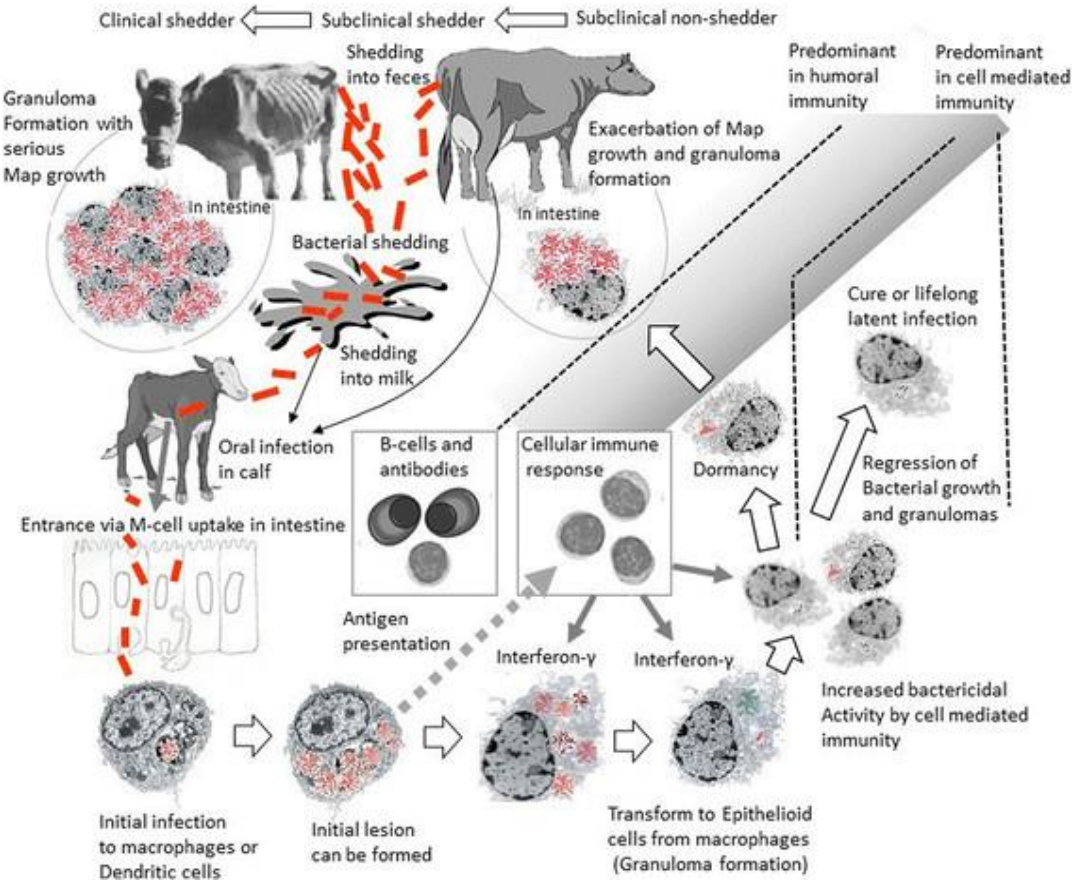



**Figure 9: An adopted diagram representation of MAP transmission and events within a host after infection occurs (Koets et al., 2015)**

MAP survives on grass and soil surfaces, where most grazing animals acquire the infection through ingestion of the pathogen (Singh et al., 2013b). Water, milk and feed that have been contaminated by faeces of an infected animal are the source of infection for most animals (Ayele et al., 2001). MAP contains a lipid layer made of mycolic acid on their cell wall. This characteristic, together with its ability to enter a dormant phase and the possible uptake of the bacterium by other microorganisms that feed on bacteria, such as nematode larvae enables the pathogen to survive for a long period of weeks (Eppleston et al., 2014), or up to even more than a year in the outside environment (Khol et al., 2010; Singh et al., 2013a; Whittington et al., 2005). Other

factors that can enable the organism to survive for longer periods in the environment are its ability to infect free-living amoeba and to form biofilms, giving them protection from the harsh environmental conditions (Sykes & Gunn-Moore, 2014). The organism can grow and survive in protozoans (Biet & Boschiroli, 2014). Infection rates are high during or before the winter season when plants enter a period of vegetative inactivity and are overgrazed (Biet et al., 2008). Infection can occur vertically where MAP uses the placenta as the route to infect the foetus (Sweeney, 2011). Primary infection of calves occurs during feeding on the milk of the infected mother, as MAP can also be excreted in the milk. The pathogen can also be found in the semen of an infected animal as it is able to colonize the reproductive tract of infected animals (OIE. 2014, Singh et al., 2013a), thereby transmitting from one adult animal to the next during mating (Magombedze et al., 2013).

The ability of MAP to spread through wildlife such as birds and rabbits cannot be disregarded. Wildlife may ingest MAP on one grazing land and excrete it in a different grazing land, though the amount of MAP shed and the significance to transmission and cause infection is not well-studied (Clarke, 1997; Corn et al., 2005).The major source of new infection in a herd is the purchase of sub-clinically infected animals. It is has been found that the purchasing of younger animals is more risky as compared to adults (Collins, 2011; Collins & Morgan, 1991) as these animals will have an extended period for disease progression and/or shedding MAP into the environment without clinical symptoms (Vilar et al., 2015). Flock level risk entry includes co-grazing with other flocks, common water sources such as streams or shallow wells; these increase the risk of infection introduction in a herd (Angelidou et al., 2014).

### *1.5.2.3. Distribution of Johne's disease*

The nature of Johne's disease progression has made it difficult to determine the true prevalence. In a given infected herd of animals showing symptoms, it is said they may be representing far less of the true number of infected animals, this being called the "tip of the iceberg phenomenon ''. It is evident that the animals beneath the tip are not easily detectable; hence the difficulty in getting a closer estimate of the disease in a given farm (Magombedze et al., 2013).It is this reason that many studies on prevalence are merely estimates (Lombard, 2011).

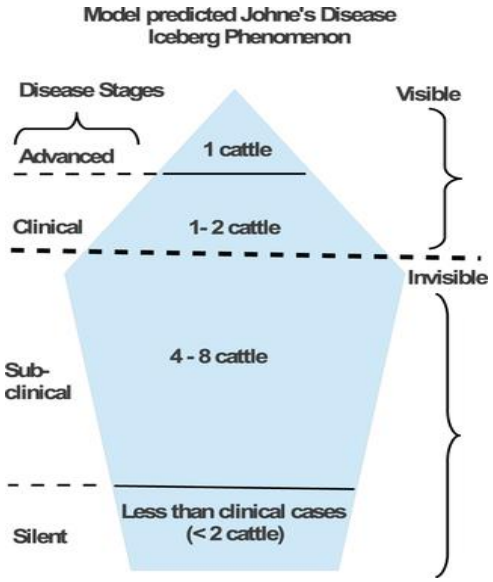


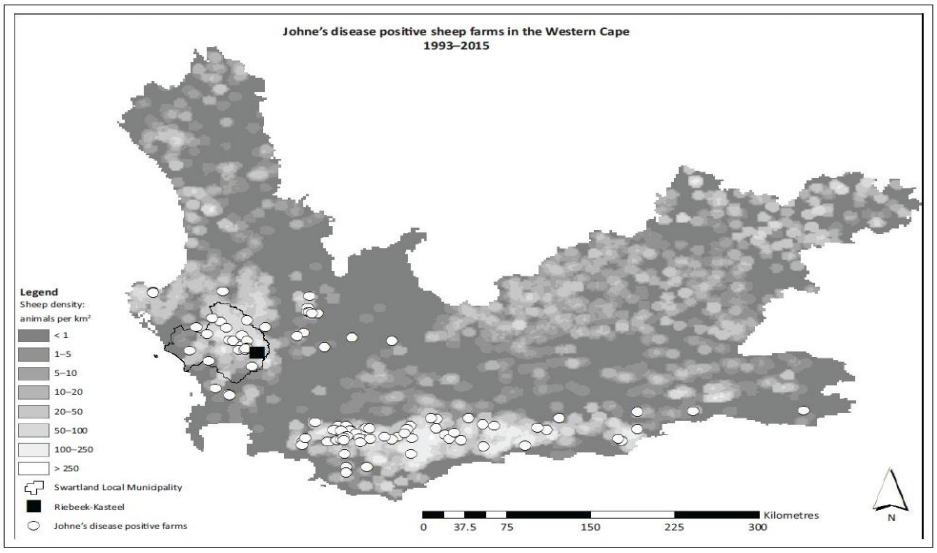

**Figure 10; Tip of the ice-burg phenomenon, demonstrating a likely prevalence in an infected cattle herd. (Magombedze et al., 2013)**

Since the first description of MAP as the cause of Johne's disease in Germany, the disease is now of global prevalence (Windsor, 2015a). Johne's disease is a disease of the ruminants; evidently so, domesticated ruminants are the main targets and wild ruminant cases are on the rise (Windsor, 2015a). MAP occurs particularly where the

water and soil of the area has an acidic pH, low dissolved oxygen content and high organic matter (Eppleston et al., 2014). Mostly affected areas include North America, Europe, and Australia; showing a high prevalence in the cattle industries. Prevalence of infected herds in European countries varies. In certain countries such as Belgium; prevalence is reported to be as low as 0.52 % (Garcia & Shalloo, 2015). Other countries have reported more than half of their herd as infected, with Netherlands reporting 54.7% and Germany reported 67% by the year 2008. Switzerland reported more than three quarters (83%) of their herds as infected (Garcia & Shalloo, 2015; Kupper et al., 2012). In North of America, the United States estimates 68% (Biet & Boschiroli, 2014) and a 70% estimate of dairy herds are infected (Sweeney et al., 2012). The Mexican dairy herds estimated an overall sero-prevalence of 5% in the year 2015 (Milian-Suazo et al., 2015). By the year 2010, Canada estimated 83% in their dairy goats and 66.8% in the dairy sheep infection rate at the farm level (Bauman et al., 2016a). Paraiba (North-Eastern of Brazil), in South America reports an average of 33.25% across the four regions studied (Vilar et al., 2015). India, part of Asia, conducted a study of animals, Bangalore and the study reported a prevalence of 10% on symptomatic animals (Anvita et al., 2012). Endemically infected countries include Australia, Canada, the Netherlands, the United Kingdom and the United States (Geraghty et al., 2014).The most prevalent type reported in the United States is type I, with most cases occurring in the cattle industry (Manning & Collins, 2001a). India reports the most prevalent type to be a type III (Sonawane et al., 2016).

Okuni, in his review study, highlighted the lack of information concerning this disease in Africa and revealed information indicating a significant number of diagnosed cases occurring across the continent. These cases occurred as sporadic incidents (Okuni et al., 2013).

In 1923, the first case of bovine paratuberculosis in South Africa was reported, while the first case of ovine paratuberculosis was confirmed in 1967 (Okuni et al., 2013; Van Niekerk & Van Der Walt, 1967). Johne's disease is a notifiable disease in South Africa. During the early years, it was seen as a rare disease in the country (Okuni et al., 2013). However, in a previous study, Michel and Bastianello described it as an emerging disease with many ovine cases in the areas Eastern Cape, Western Cape, Orange Free State and Mpumalanga Provinces of South Africa (Michel & Bastianello, 2000). South Africa has recently had a positive symptomatic paratuberculosis in a domestic dog (Miller et al., 2017).




*Source:* Western Cape Department of Agriculture

**FIGURE 5:** Map of Johne's positive sheep farms in the Western Cape 1993–2015.

**Figure 11: Geographical distribution map representation of MAP within the Western Cape Province (Miller et al., 2017)**

### *1.5.3. The control and economic impact of Johne's disease*

To measure the financial impact of Johne's disease (JD) in an infected area is difficult and time consuming. One study conducted in Southern New South Wales, did an analysis on the JD impact on financial turnover and found an annual reduction in turnover profit of AUD $13,715 in a sheep farm (Bush et al., 2006). The study reports

on the impact resulting from mortality and the average mortality rate on the studied farms were estimated to be at minimum 5% (Bush et al., 2006). Other factors that contribute to the negative cost impact of JD is the early culling of infected animals. This is done to eliminate an infected animal and to prevent spread of MAP or if an animal has lost its fertility. An infected animal may still be able to give in profits due to milk production, removal and culling of this animal will then result in the loss of the income it generated and their unrealised future profits. Additionally, there might be a need to replace the removed animal (Chiodini et al., 1984; Garry, 2011; Hasonova & Pavlik, 2006). Animals infected with MAP show a decrease in milk production, further decreasing the profit of a farmer (Richardson & More, 2009). The slaughter approach may be more practical in low JD prevalence farms (Robbe-Austerman, 2011). It was estimated that an animal showing clinical symptoms has an average cull price €516 lesser compared to those culled without clinical signs (Richardson & More, 2009). In higher prevalence areas, they might have to consider the isolation of infected animals from uninfected animals. The cost associated with Quarantine involves building of long demarcating fences (Robbe-Austerman, 2011). Vaccination is another control measure that can add to the cost of controlling Johne's disease, estimated at $12/calf (Groenendaal et al., 2015). Vaccination can also add indirect cost due to cross reactivity between the MAP antibodies and that of the tuberculosis antibodies, hence an animal infected with tuberculosis would require more test than the unvaccinated one (Groenendaal et al., 2015). Johne's disease is an incurable condition and it is best to prevent the infection. However, due to the nature of the disease, it makes it close to impossible to prevent an infection. Farmers to maintain a Johne's disease free status follow strict expensive measures.

#### *1.5.3.1. Vaccination against Johne's disease*

Vaccination against paratuberculosis is available and is a control measure used mostly in sheep (Juste & Perez, 2011). It is evident that vaccination does not offer full prevention and instead prevents the progression to severe clinical cases and reduces the amount of MAP shed into the environment. Immunized animals have longer life in the herd; with substantial levels of MAP they shed, thus reducing the economic losses (Juste & Perez, 2011). Dhand (2016) and his coworkers conducted a longitudinal study on the efficacy of the Gudair™ vaccine. The overall impact was a reduction in positive animal's faecal pools from 50.3% to 3.1% and animal level prevalence in flocks reduced from 7.6% to less than 0.5% in a period of 10 years (Dhand et al., 2016).

#### *1.5.3.2. Treatment of Johne's disease*

Mycobacterium species are susceptible to macrolide drugs such as the azithromycin (Krishnan et al., 2009) and their treatment usually requires a combination of drugs to overcome the development of drug resistance during the course of treatment (Krishnan et al., 2009). Still on that point, there are no conclusive cures for MAP infection (Fecteau & Whitlock, 2011). It is considered not economically worthwhile to treat infected animals, as the treatment period is extremely long since they have a slow growth rate, the regimens treatment are expensive and the animal has minimal chances to be cured from the disease (St-Jean & Jernigan, 1991). In vitro studies of MAP susceptibility show MAP to be susceptible to macrolides, especially clarithromycin (Krishnan et al., 2009). On the other hand, drugs such as the Ethambutol and Isoniazid are not as effective as they are against *Mycobacterium tuberculosis* (Krishnan et al., 2009). In vivo studies indicate that treatment does not eradicate the infection but rather slows down disease progression, prolongs the life of

the infected animal and minimizes the shedding of MAP (Fecteau & Whitlock, 2011). St-Jean (1991) showed that to maintain the above conditions, an animal has to be given 20mg/kg of isoniazid daily, for an animal at an acute stage of the disease, isoniazid together with rifampicin at 20mg/kg has to be given. He further advises that aminoglycosides being added in the initial stage of treatment if an animal is in a life threatening stage (St-Jean & Jernigan, 1991). But currently in South Africa, treatment of infected animals is not permitted and there are no registered drugs.

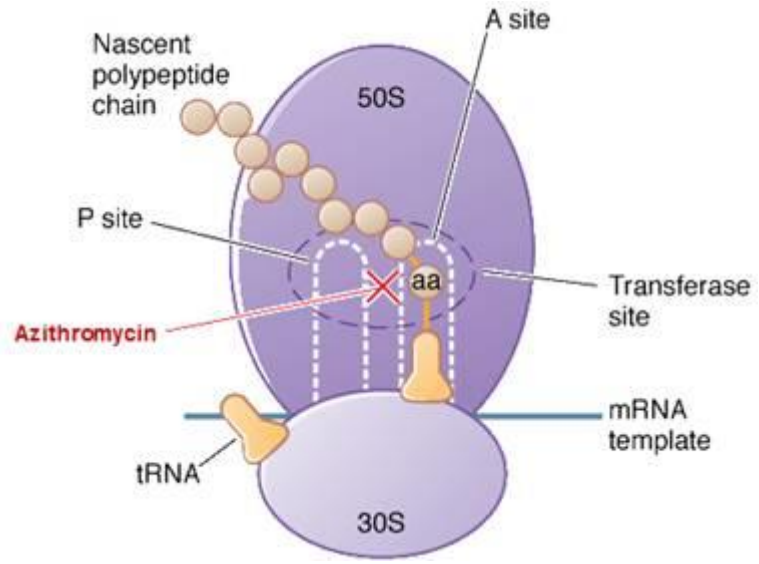



**Figure 12: Azithromycin inhibiting the process of translation (http://www.antibiotics-info.org/azithromycin.html)**

Isoniazid a hydrazide of isonicotinic acid, acts against actively dividing bacteria by acting against the synthesis of mycolic acids, which is an important component of mycobacterial cell wall. Azithromycin prevents bacterial replication by disrupting protein synthesis, it binds to the 50S subunit of the bacterial ribosome (figure 12) and there by inhibiting translation (http://www.antibiotics-info.org/azithromycin.html) Rifampin is a semi-antibiotic derived from rifampicin. It inhibits bacterial DNA replication by inhibiting the action of DNA dependent RNA polymerase.

Aminoglycosides such as kanamycin and streptomycin act by inhibiting protein synthesis (figure13) and decrease the activity of mRNA during translation (Fecteau & Whitlock, 2011; St-Jean & Jernigan, 1991).

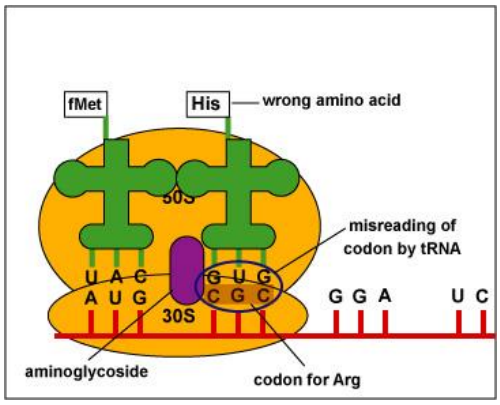



**Figure 13: Aminoglycoside-inhibiting protein synthesis.**
**http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit2/control/images/agmiscode01_illus.jpg**

## 1.6. Laboratory diagnosis of *Mycobacterium avium* subspecies *paratuberculosis*

MAP infection can be identified by demonstrating the presence of the bacteria from a specimen that represents the animal in question. Presence of bacteria can be detected microscopically, by selective culture isolation or by demonstrating the presence of antibodies specific for MAP. It can also be detected by checking for a cell-mediated response, or by identifying components of the bacterium such as protein and its antigens or genetic material by molecular methods (Bhattarai et al., 2013a). The specimen of choice depends on the type of test to be carried out and the stage of clinical disease. The most commonly used specimens are faeces, tissue, milk and blood (Timms et al., 2011). Of these specimen types, intestinal tissue culture is more

specific and sensitive of the test methods as MAP is readily available for isolation from these sites (Britton et al., 2016; Gwozdz et al., 1997).

### *1.6.1. Staining and microscopic examination*

The cell wall characteristics of MAP have excluded the use of Gram stain method for their characterization. The thick lipid layer on the outermost surface of their cell wall is not penetrable by the easy stain application used in Gram staining. A different staining procedure is used, where harsh conditions such as heat are applied to allow the dye to penetrate the lipid layer and reach the cell wall. An example of such staining processes is the widely used Ziehl Neelsen stain (figure 14). Carbol fuchsin is used as the primary stain. The stain binds to the mycolic acid in the mycobacterial cell wall. Subsequently, an acid decolorizing solution is applied. This eliminates the red colorant from the background cells, tissue fibers and any other bacteria in the smear except the mycobacteria which retain the dye and are therefore referred to as acid-fast bacilli (AFB). As they retain the dye, malachite green or methylene blue is then applied as a counterstain to stain the background material, providing a distinction color against which the red AFB can be seen. Results are interpreted as positive when red, straight or slightly curved rods, occurring singly or in small groups are observed. Staining and microscopic examination is not specific, meaning it does not speciate the Mycobacterium species.

Staining and microscopy are usually used as a screening tool. The disadvantage of this is that the tool has very low sensitivity in the early and intermediate stages of the disease (Salem et al., 2013) and as many as $10^6$ bacteria/g of a sample may be necessary for detection (Thoresen et. al., 1994)

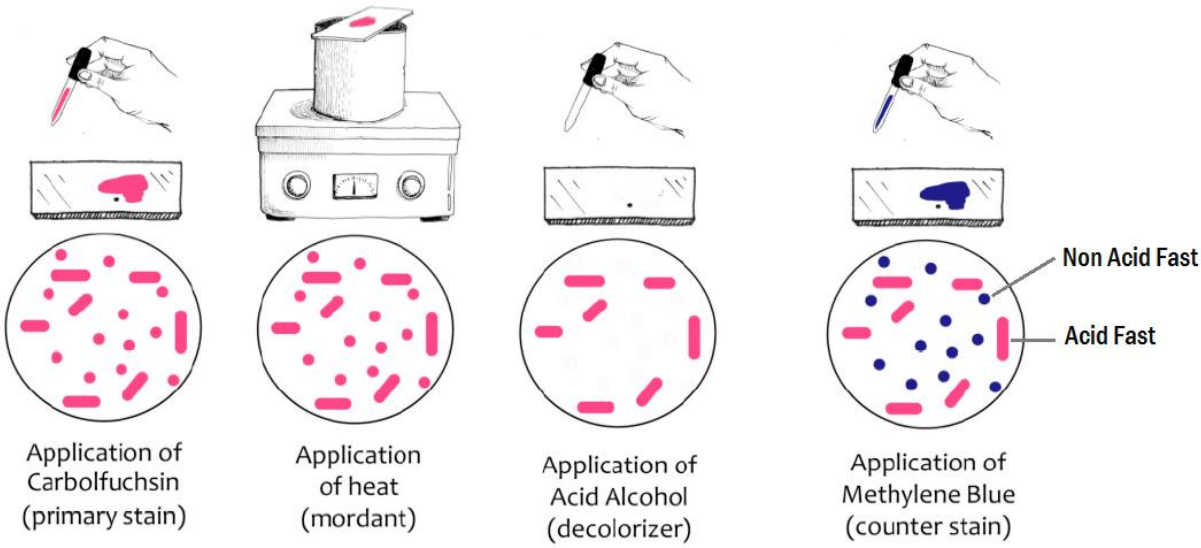



**Figure 14: Acid-fast principle demonstration (https://laboratoryinfo.com/zn-stain/)**

### *1.6.2. Culture Methods*

Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from excretions such as faecal matter, bodily fluids such as milk or tissue samples has been demonstrated to be the most reliable (provided there is shedding in the case of faecal samples) and most specific method for accurate diagnosis of the disease (Salem et al., 2013). Culture, in addition to its specificity and accuracy, allows isolation of the organism, which additional studies can be carried out on the isolate, such as pathological studies, molecular studies and epidemiological analysis (Collins, 1996).

At least 100 MAP organisms per gram of faecal sample can be detected by culture and with the possibility of detecting as low as 1 MAP organism per gram of a sample (Bhattarai et al., 2013a). Conventional culture method has approximately 50% sensitivity, meaning in a community of infected animals, only about half of the infected animals can be detected and the other half will go undetected 9 (Collins, 1996). Faecal, tissue and milk samples can all be used for culture and faecal culture is used most

often. However, tissue samples have shown to detect infection even in animals that are not yet shedding and having no clinical symptoms; as a result, tissue samples are considered more sensitive (Britton et al., 2016; Gwozdz et al., 1997). Greater sensitivity of tissue culture over faecal culture is concluded based on the ability to detect a wider spectrum of infected animals and it varies from one study to the next. One study reports 16% and 4% of tissue and faecal culture sensitivity, respectively (Bhattarai et al., 2013a), while another reports 32% and 17% of tissue and faecal culture sensitivity respectively (Whittington et al. 1999). The disadvantage with faecal culture is that it greatly depends on shedding of MAP by the animal, it is reported by Weber and coworkers (2010), that in a herd of high prevalence an animal can start shedding at an early age of two years or below, whilst in low prevalence shedding mostly occurred during a productive life span of an animal (Weber et al., 2010). It is possible for an animal to ingest large amounts of MAP from the environment, resulting in what is termed a 'pass through detection' in its faecal excretions, without the replication of MAP in the system of the animal (Collins, 1996). Detecting in faecal samples suggests a high possibility that MAP will be found in the milk and colostrum and that there is a horizontal transmission as well (Collins, 1996). Pooling of faecal samples of individual animals may decrease the high costs of faecal culture, reducing cost associated with single specimen processing and testing (Collins, 2011; Schaik et al., 2007; Serraino et al., 2014).

Faecal sample pooling, depending on the ratio of non-infected to infected and also on the shedding patterns of the animals represented in the pooled sample, can result in reduction of the number of MAP in a sample, which is an effect of dilution. The sensitivity of the faecal pools is poorer in pools with low shedders than in pools with moderate or heavy shedders (Schaik et al., 2007). Kalis and co-workers report a

dilution of 1 MAP containing positive faecal sample with four other faecal samples not containing MAP to reduce the Colony Forming Units (CFU) MAP per gram below detection level (Kalis et al., 2000). It is therefore important to determine the number of individual samples per pool that will not decrease the sensitivity of detecting MAP infection in a community of animals. Samples from the environment can be collected to test for infected herds at large, however, Wolf and his team (2014) warns about the influence of sample location, season and herd size on the culture results (Wolf et al., 2014).

Since MAP has been proven a sluggish growing bacterium and varies according to the strains (Collins, 2011) and this is another added disadvantage of isolation by culture as it is a timeous process, generally requires 12 to 16 weeks (Collins. 1996). Decontamination is a very important step in specimen processing. It is most likely that specimens to be used are contaminated by other microbes from the site of collection with faster generation time and will multiply on the media, depleting nutrients of the medium, hence outcompeting any mycobacteria present (Salem et al., 2013). Decontamination methods used involve the addition of antibiotics in the medium and incubation at 37 ºC. These have proven to be effective in hindering the growth of other microorganisms besides mycobacteria (Gwozdz, 2006; Pinedo et al., 2008; Timms et al., 2011). The decontamination methods can result in a loss of some of the mycobacteria present in a sample (Botsaris et al., 2010), making the decontamination method a crucial parameter to the sensitivity of the test. The use of hexadecylpyridinium (HPC) has evidently served the purpose of killing other microorganisms that are present in a sample while remaining less harmful to mycobacteria (Collins, 1996).

Culture in a solid phase medium such as the Herrold's egg yolk medium with the addition of mycobactin J, a siderophore necessary for MAP isolation. This growth requirement is used to differentiate the mycobacteria infection (Bhattarai et al., 2013a; b). Costs associated with labour and reagents needed to isolate MAP using conventional culture method are another listed disadvantage (Collins, 1996).

Liquid media have been developed and described and it is apparent that they are more sensitive than the solid media (Laurin et al., 2015a). Compared to solid phased media, liquid phased media provide results within a shorter span of time, requiring 8 weeks at most, whilst solid phased media can take up to 16 weeks (Debroy et al., 2012). Liquid media have also led to manual and advanced automated systems (Bhattarai et al., 2013b). Examples of these are the Mycobacteria growth indicator tube (MGIT), the Bactec 460, which uses a radiometric culture medium, and the Bactec MGIT 960, a non-radiometric culture system. The result is achieved automatically depending on the release of carbon dioxide gas during metabolism. Detection of the gas is achieved by adding a carbon source that is labeled, so that when it is utilised, it releases carbon dioxide that fluoresces. These methods have been proven more sensitive than the conventional culture systems (Bhattarai et al., 2013b).

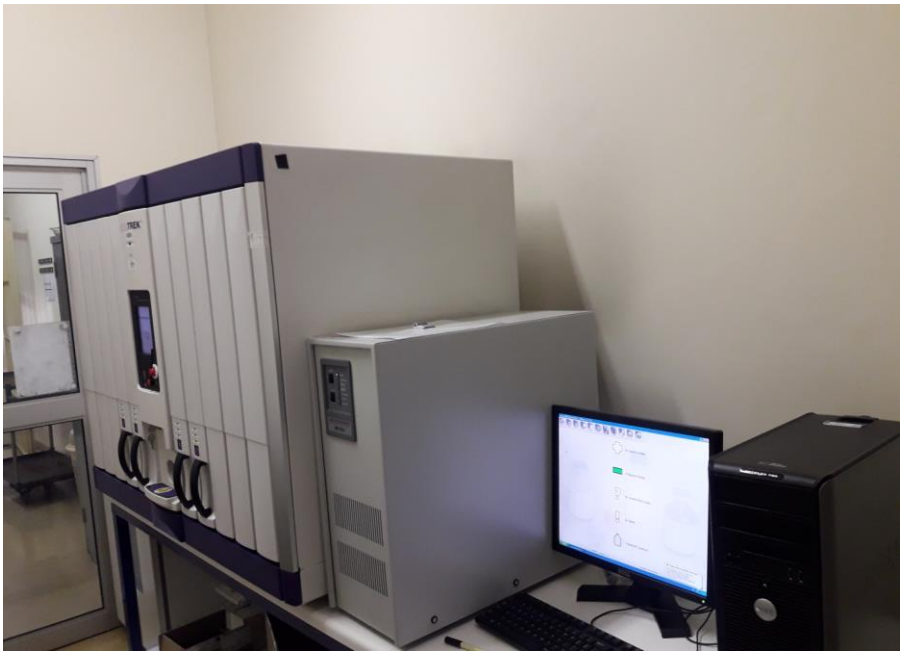


**Figure 15: The Versa Trek automated liquid culture system machine with the connected Desktop computer (Picture taken at the ARC OVR TB-Laboratory**

The automated Extra Sensing Power (ESP) technology based culture systems II. Versa Trek system (figure 15) is an automated liquid based culture system that uses the ESP technology to detect microorganisms.

### *1.6.2.1. Physical nature of the Versa trek liquid culture machine:*

The machine is a set of drawers (Figure 16) aligned in a vertical order; each of the drawers has 24 numbered incubation spaces divided in three horizontal lines.


**Figure 16 one of the Incubation drawers of the Versa Trek automated liquid culture system, with incubated bottles (Picture taken by Nomawethu Masina at the ARC OVR TB-laboratory**

Each of the incubation spaces is a round mouth opening designed to fit the exact designed culture flask/bottle. Directly above the bottom opening that fits in the bottom of the culture bottle is an opening that fits the upper part of the bottle attached to what is termed the Versa Trek connector. The connector has a sharp needle that pierces through the rubber lid of the air tight culture bottle. Each of the incubation space has an LED indicator light that indicates the status of the incubated bottle. Changes in the incubation status of the bottle are also communicated outside the LED light also present on the outside of each drawers. Each incubation drawers has three LED lights attached to them, one light indicates positive detection (red color), another (green color) indicates for negative culture bottles and the last LED (orange) indicates for any error that is occurring at the time. At the center of the machine is an LECD screen that displays all that is in the machine. During incubation, this screen allows input and

selection of the desired conditions according to the requirements of the targeted organism. These range from incubation period, oxygen conditions and whether it's aerobic or anaerobic. Without opening the drawers, information regarding the status of the incubated bottles, the location, and graph direction, period in the instrument, identification number, available spaces, and reserved locations and occupied spaces in the machine can be seen.

The desktop connected to the machine communicates directly with the machine; any action performed on the machine is transferred to the computer and vice versa. However, the computer allows additional work to be done that cannot be done directly on the machine. These include status override, determining shedding level where applicable, more information regarding the history and data of the sample can be entered and saved in the computer for desired periods. It allows easy data retrievals and test history of even finalized samples.

### *1.6.2.2. Detection method of the machine*

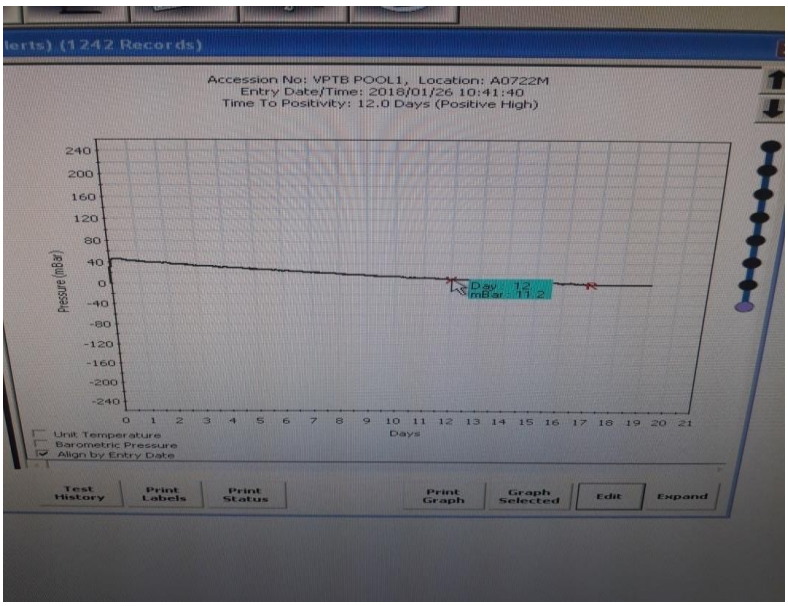



**Figure 17 Computer screen shot displaying the Bar Graph pressure of a positive sample. Sample identification number, date entered, direction of the graph, shedding level and other options are displayed here (Picture taken by Nomawethu Masina at ARC-OVR TB-laboratory 14.2.2018)**

Briefly, the technology takes advantage of microbial metabolic processes that lead to gas consumption and production. Natural reactions that lead to gas production or consumption are manipulated by the addition of special media that maximize the gas reactions. The system incorporates sensitive technology, a pressure transducer that measures the change in the headspace pressure of the incubated bottle due to gas production or consumption continuously. The change in the headspace pressure of the incubated bottle is indicative of active dividing microbes. The Mycobacterium detection platform, measures a net decrease in headspace pressure that occurs due to oxygen consumption. It also incorporates a graph displaying the relationship between pressure and number of read data points in 12 minutes intervals (figure 17). The direction of the graph (increase or reduced pressure) can indicate if the positive signal is likely to be a mycobacterium (reduced pressure) as typical mycobacteria will

consume oxygen, resulting in a reduced pressure (VersaTrek™ Automated Culture System User Manual. 2013).

The many reported advantages to the use of this system include elimination of costly specialized media, a broader scope of specimen sources and to reliable detect the presence of microbes. Antimicrobial susceptibility testing is one of the added advantages to this system. A Windows software program is included that links the machine to a desktop. The advantage of this software is that it allows input of sample or patient information, easier tracking and easy data retrievals (Thermofishercom. 2016).

The limitation to the system lies in the fact that it covers broad range recoverability. Therefore, a positive signal can be given off by the presence of any other microorganism surviving the procedure and able to use the medium. Of interest, most pathogenic mycobacterial species equally survive the decontamination process. The inability of the system to be specific for MAP creates a need to verify the positive samples from the Versa Trek system. (Laurin et al., 2015a; Lui et al., 2000; Thermofishercom. 2016).

### *1.6.3. Immune response based diagnosis*

There are two broad types of immune responses against MAP infection. The first and early response is the cell-mediated response characterised by the release of pro-inflammatory cytokines, followed by the humoral immune response which is characterised by antibody production. However, other studies have observed the humoral immune response preceding cell mediated immune response (Lybeck et al., 2011). Under cell-mediated immune responses, assays developed for the diagnosis of MAP include an intradermal skin test and a gamma interferon test. Under the arm

of the humoral immune response, assays developed include Enzyme linked immunosorbent assay (ELISA), the agar gel immunodiffusion and the complement fixation tests (CFT). The mentioned immune responses are considered an advantage in that they can detect infection in the early stages before an animal becomes infectious. However, Lybeck and co-workers report faecal shedding preceding the early immune responses (Lybeck et al., 2011).

#### *1.6.3.1. Cell-mediated immune response based detection methods*

Intradermal skin test: The test is based on prompting a delayed-type hypersensitivity reaction to the intradermal inoculation of tuberculin, a crude protein extract from supernatants of mycobacterial cultures. *Mycobacterium avium* subspecies *paratuberculosis* antigen filtered protein derivative is inoculated in the skin. Inoculation in the skin of an animal not exposed to tuberculin antigens will result in no substantial local inflammatory response. However, if tuberculin is inoculated into an animal whose immune system has been sensitised by infection with MAP, it triggers an inflammatory response resulting in an enlargement at the injection site that reaches its extreme intensity 48–72h (post-injection) and reverts speedily subsequently. The test is known as the single intradermal test (SIT) and it is applied on the skin of the mid-cervical region or in the base of the tail (Kalis et al., 2003). The skin fold thickness is measured with callipers to the nearest millimetre prior to the injection with tuberculin and 72h post injection. Cross reactivity reduces the specificity of the test and greater cross reactivity was observed between *Mycobacterium tuberculosis* and MAP. Reaction to the avian purified protein derivative (PPD) is stronger than to bovine PPD in

paratuberculosis diseased animal. This factor can be used to screen and distinguish an infection as either paratuberculosis or tuberculosis. The disadvantage to this method is that it has low specificity resulting from the majority of common antigens of MAP and other environmental mycobacteria (Collins, 1996), the test is used only in screening test (Bhattarai et al., 2013a).

Interferon gamma (IFN-γ): The release of cytokines plays a vital part in modulating cell-mediated immune responses to mycobacterial infection, including interferon gamma. Interferon gamma release assays (IGRAs) are in vitro tests of cell-mediated immune response. *Mycobacterium avium* extract is applied as a stimulating antigen to measure interferon (IFN) -gamma released by stimulated T cells in blood samples from an animal infected with MAP (Kalis et al., 2003).

Both the mentioned cell mediated methods have low specificity. Cytokine release is an early response that eventually is replaced by a humoral immune response. At this stage, Cytokines release seizes and drops to undetectable levels. There is no constant interpretation method to conclude results (Kalis et al., 2003). The sensitivity of the test methods is lower than that of the ELISA, approximately 17, 5% (Collins, 1996). The test requires fresh samples, which then takes away the advantage of allowing storage offered by culture (Plain et al., 2012). This then gives a challenge to the service providers and the farmers who may require the test for their animals (Kalis et al., 2003).

A very important advantage of this method is the ability to identify an infected animal in the very early stages of infection, before the infected animal becomes a source of infection (Kalis et al., 2003). It is then an important method in breaking a cycle of

infection and a crucial aspect in managing Johne's disease. Results can be obtained in a period of days and it is not costly (Jungersen et al., 2012).

#### *1.6.3.2. Humoral Immune Response*

The Enzyme Linked Immunosorbent Assay: The aim of this test is to detect MAP antibodies from serum and blood samples. During a course of an infection by MAP, IgG class antibodies proliferate, these IgG class antibodies are later predominated by IgG1 subclass antibodies directed to the MAP antigens (Fosgate et al., 2009).

The principle behind this method is the specificity of antigen-antibody reactions during immune response. The MAP antigen is coated on to well plates and serum samples are then transferred into these antigen-coated wells (Berger et al., 2007). MAP specific antibodies present in the sample due to MAP infection will form a so-called Antibody-Antigen immune complex. Subsequent steps of washing unbound material and the addition of enzyme conjugate that binds exclusively to the Antibody-Antigen complex follow. After a wash that removes unbounded conjugate, an enzyme substrate is added. The added substrate will then react with the bound enzyme; the reaction formed is signalled by colour change measured by wavelength filter. This, indicates the presence of MAP antibodies in the sample (Berger et al., 2007), as summarized in figure 13.

The specificity of this test is enhanced by the application *Mycobacterium phlei* as an absorbent that serves a purpose of removing non-specific cross-reacting antibodies

(OIE. 2014, Sockett et al., 1992). Cross reactivity can result from other mycobacteria, norcadial and corynebacterial species (Reichel et al., 1999).

MAP antibodies are only produced at a later stage of infection; because of this, an infected animal cannot be identified in the early stages of infection by this method. The overall sensitivity of the ELISA method is 45% and can vary depending on the clinical stage of the infected. It can be up to 87% in animals that are at a clinical stage and as low as 15% in animals that asymptomatic (Collins, 1996).However, the advantage of this method is that it takes few hours to complete the test, it is affordable and the test also affords the laboratory a storing advantage as the samples can be frozen for a later analysis. (Aly et al., 2014, Jark et al., 1997, Laurin., 2015). Antibodies against MAP can in some cases be detected before an animal begins shedding or becomes infectious. If applied regularly to monitor Johne's disease, farmers can have a probability of removing an animal quicker before it becomes infectious (Nielsen, 2008).

### *1.6.4. Histopathology*

Histopathology evaluates freshly preserved tissue samples by observing the cell morphology in the tissue samples (Britton et al., 2016). Johne's disease is the infection of the gastrointestinal tract systems. An ideal specimen for histopathology can be obtained from the distal part of the ileum, ileocecal valve, adjacent lymph nodes and biopsy or scraping of the rectal mucosa (McGregor et al., 2015).

Histopathology observes for specific pathogenic thickening and corrugation lesions within the tissue samples, as well as accumulated macrophages, epithelial cells and Langhans giant cells that occur in tissues due to MAP infection (Sulyiman, 2014). Intestinal lesions develop as early as the subclinical stage of the disease (Coussens, 2004).

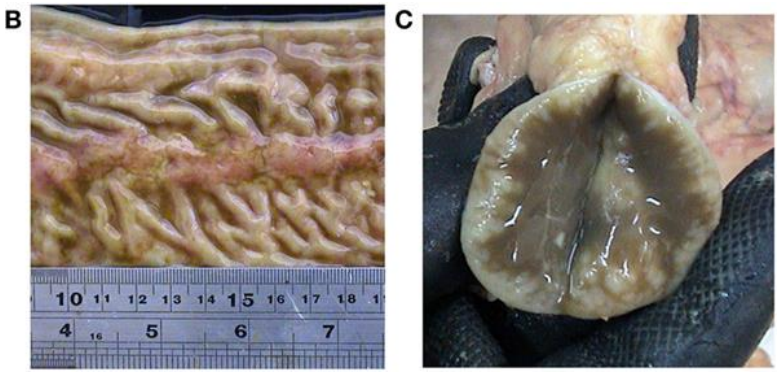



**Figure 18 Thickened and corrugated intestinal tissue from post mortem analysis of an animal infected with paratuberculosis. (Rathnaiah et al., 2017)**

Specimens are examined for thickened intestinal walls infiltrated by epithelioid macrophages, plasma cells and lymphocytes due to the immune system response to MAP infection (Huda & Jensen, 2003; Leão et al., 2013).

There are two types of lesions, as described by Huda and Jensen (2003), which characterise MAP infected tissue. Typical lesions may occur with well-defined granulomas or lesions may present with widespread disseminated lesions not resembling focal granulomas. (Sikandar et al., 2013; Sulyiman, 2014). Kurade and his team described and classified lesions occurring due to MAP infection into four categories based on the stage of the disease. The first grade, are lesions found solely in the ileal or ileocecal Payers patches or both, consisting of disseminated micro granulomas in the follicular and interfollicular areas. Acid-fast bacilli at this point of lesion are rarely demonstrated. The grade 2 lesions were those with small focal granulomas found also in the mucosa associated with Payers' patches, the villi and underlying areas infiltrated with moderate number of lymphocytes, macrophages, and a few plasma cells. In this type of lesions, a few acid-fast bacilli are demonstrated. The third type of lesion (grade 3), has many granulomas of different sizes and infrequently diffuse granulomatous lesions in the Payers' patches and jejunal and ileal mucosae associated or not associated with the Payers patches. The mucosa is diffusely infiltrated with lymphoid cells and few to abundant acid-fast bacilli are demonstrated. The fourth type of lesion described for severe conditions; is described by more severe payers' patches lesions, granuloma enteritis, abundant macrophages and epithelioid cells. Abundant acid-fast bacilli are easily seen with this type of lesions (Kurade et al., 2004).

Histopathology is considered as the most definitive diagnostic test for ovine paratuberculosis (Huda & Jensen, 2003), as histopathological lesions are commonly observed in sheep with paratuberculosis (Windsor, 2015b).

The benefit of the histopathological analysis is that it permits identification of animals with focal lesions related to subclinical phases whose excretion, such as faeces or

milk, does not release adequate MAP for bacterial culture or Polymerase Chain Reaction (PCR) tests (Gulliver et al., 2015). Lesions associated with paratuberculosis are observable through histopathology, seen as early as 45 days post infection in experimentally infected sheep (Kurade et al., 2004). Confirmatory test demonstrating the presence of MAP is done and considered necessary (Sulyiman, 2014).

### *1.6.5. Molecular diagnosis*

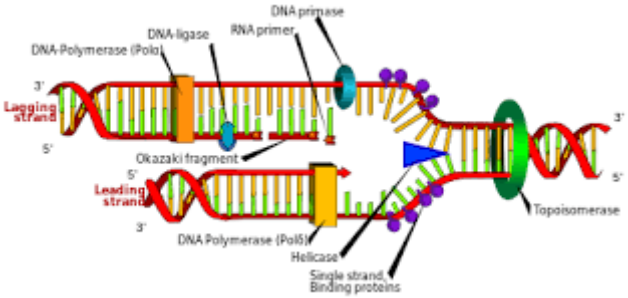



**Figure 19: Cartooned demonstration of DNA replication process (https://upload.wikimedia.org/wikipedia/commons/8/8f/DNA_replication_en.svg)**

Polymerase chain reaction (PCR) was the original work of Kary Mullis that he developed in the 1980s and he was awarded the Nobel Prize for it in 1994 (Britton et al., 2016). PCR functions to multiply DNA, adapted from the natural process of DNA replication (figure 19). It enables the multiplication of specific DNA fragments. It uses repeated cycles, individually consisting of three steps: First the denaturation step, second the annealing step, third the elongation steps and these three steps are repeated to attain a high copy number of DNA. For the reaction to be successful, the solution should comprise the target DNA molecules to be copied, heat-stable polymerase called Taq polymerase, primers that serve as starting DNA to which the polymerase enzyme will begin the elongation step by adding bases complementary to the copied strand (figure 20 ) (Valones et al., 2009). Thermal cyclers have been developed; they heat and cool the reaction tubes automatically to achieve the temperatures required at each step of the reaction.

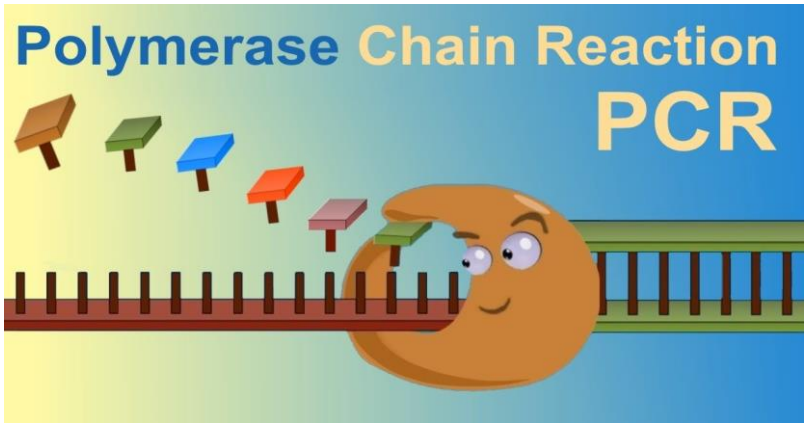



**Figure 20: cartooned representation of Polymerase adding complementary bases to the elongated strand (http://www.fenw.org/img/fb/pcr.htm.)**

Insertion sequence *900* (IS*900*) is a small transposable genetic element found in mycobacteria. The sequence is reported in *Mycobacterium cookii* (Valones et al., 2009) and is present in up to 20 copies in the MAP genome (Englund, 2003). Most field mycobacteria are found to contain an insertion element closely resembling the IS*900* (Englund et al., 2002). The sequence is summed up by 1451 base pairs of which most of the bases are G+C pairing (Harris & Barletta, 2001). It has the same origin as the insertion sequences IS901, IS*902*, and IS*1110*, which have been identified and described in *M. avium* subsp. *avium, M. avium* subsp. *silvaticum*, and *M. avium* subsp. *avium*, respectively (Herthnek et al., 2006). IS*900* being the first insertion sequence to be observed in MAP, has been the choice of target for identification of MAP infection in PCR (Green et al., 1989).

Specific primers targeting the *IS*900 sequence region of the MAP genome are used in the protocols for *Mycobacterium paratuberculosis* (Englund, 2003). The advantage of this discovery is that it can differentiate whether infection is due to *Mycobacterium avium* or *Mycobacterium paratuberculosis* since they share a great homology (Garrido et al., 2000). The PCR product (known as amplicon) is allowed to run on a 1-2%

agarose or 6% acrylamide gel electrophoresis (figure 21) alongside the DNA ladder also known as molecular weight marker constituting DNA fragments of known band sizes (Manning & Collins, 2001b).

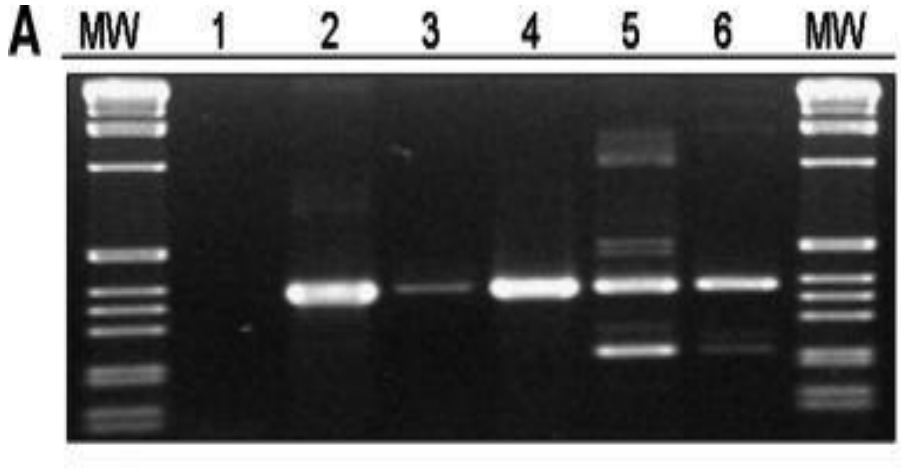



**Figure 21: PCR gel image showing the molecular weight marker and IS900 amplicons of the MAP genome. Adopted from (Alonso-Hearn et al., 2009)**

The advantages of PCR are that it is quick while achieving some of the desires in culture, such as determining an excreting animal. It is more attractive than culture in that no additional tests are needed to confirm the identity of the organism and it is 100% specific (Collins, 1996). The disadvantages of PCR are that it does not isolate the whole organism; as a result, no additional studies can be done on it. PCR is costly and is in all probability to be contaminated (Collins, 1996), PCR failure that can require repeated testing and further increasing the costs. In addition, the sensitivity of PCR is high; it can detect 94.1% (Garrido et al. 2000)

# *Chapter 2*

## Comparative evaluation of histopathology and liquid culture of ovine faecal and tissue samples

### 2.1 Introduction

An ideal and perfect method would be one that reliably identifies or isolates solely the targeted organism, within a short space at any given time. Then the method would be specific, sensitive and practically acceptable. If a method can isolate the targeted organism in its live form, not only is it favorable in diagnostics but it forms the gold standard in research, as further studies can be performed on the organism. Enriched selective culture media can allow just that (Collins, 2006; Salem et al., 2013), with most bacteria. Important aspects are omitted in the culture of MAP and this is due to the fastidious nature of the bacteria. To culture MAP is an extensively prolonged process (Collins, 2011; Salem et al., 2013) and by this, the turnaround time, laboratory capacity and business at both farm and diagnostics level are challenged. Solid phased medium relies on colony formation for a successful detection of MAP. For a colony to be visible, it can take up to 16 weeks and even longer with the sheep strain (Münster et al., 2013). Liquid culture medium can produce results in half the time taken when using solid phased media.

As technology and research advance along the years, more efficient culture systems have been introduced based on the liquid phased medium. Examples are the radiometric detection based culture system, the Mycobacteria growth indicator tube (MGIT) and the Bactec 460. The none-radiometric detection culture system, the

Bactec MGIT 960 fluorescence detection and the MB/BacT reflectance detection (Bhattarai et al., 2013a).

An automated Extra Sensing Power II (ESP II) technology based culture systems is one of the recent promising technology for the diagnosis of paratuberculosis. The method is known as the Versa Trek ™ Automated Culture system (figure 13). Briefly, the ESP technology takes advantage of microbial metabolic processes that lead to gas consumption and production. Depending on the infectious dosage, shedding level and the stage of the disease, results from this system can be obtained within days (www.thermofisher.com). Tissue samples are more sensitive and reliable for the isolation of MAP by culture. The sensitivity of tissue culture over faecal culture and milk culture is due to the pathogenic traits of MAP. *Mycobacterium avium* subspecies *paratuberculosis* first invades the intestinal tissues of the infected animal, colonizes them then penetrates to establish an intracellular infection. This is where bacterial replication begins and perpetuates (Ayele et al., 2001; Sulyiman, 2014). Sampling from these sites makes MAP readily available for isolation. Shedding of MAP in the faecal excretions depends on the stage of the disease.

The ESP automated Liquid culture system is not mycobacteria specific, through its bar pressure graph, it is only indicative of the likely growing pathogen. Polymerase chain reaction (PCR) is therefore used as a species confirmation molecular method (Motiwala et al., 2004; Plain et al., 2015; Whittington et al., 2013). The success of identifying a genetic element in the MAP genome, the IS*900,* marked the groundwork for PCR application in the molecular identification of MAP. The sequence is present in a reasonably high copy number in the MAP genome. The major contribution by the discovery is that it can differentiate between *Mycobacterium avium* subspecies *avium* and MAP, since the two subspecies are not readily differentiable. The presence of

IS*900* is also reported in *Mycobacterium cookie* and similar sequences are found in environmental mycobacteria such as the *Mycobacterium scrofulaceum* reported by Cousins et al. (1999). Specific primers targeting the IS*900* sequence region of the MAP genome are used in the protocols for MAP detection (Englund, 2003).

Bacterial culture of MAP is accepted as the gold standard method because of high specificity (Bogli-Stuber et al., 2005; Plain et al., 2015; Woodbury et al., 2008). While this may be true, histopathology is considered the most definitive diagnostic test for ovine paratuberculosis (Huda & Jensen, 2003), by way of histopathological lesions common in sheep with Johne's disease (Windsor 2015). Besides the common slow growth rate challenge in isolation by culture across all molecular types of MAP, the sheep type is even more fastidious (Plain et al., 2015). Histopathology prevails over faecal culture because it is able to identify infections in early stages of the disease (Sukantu et al., 2014) and has tended to identify more positives than culture in the past (Coelho et al., 2017; Younus et al., 2012). Due to stated reasons, histopathology is taken as the standard reference method (Coelho et al., 2017; Hailat et al., 2012; Sikandar et al., 2013; Younus et al., 2012). The only disadvantage with histopathology is that it is postmortem and cannot be done of live animals.

If the liquid faecal culture method is to be accepted as a reliable method for the diagnosis of MAP, it is to be evaluated against the standard reference method, which in this case is histopathology (Younus et al., 2012). As according to the requirements of South African National accreditation system (2014) and the international methods committee guidelines (Feldsine et al., 2002), a method to be validated and credited has to demonstrate adequate comparable evidence with the standard reference method. This study aimed to culture faecal and tissue samples in the automated liquid culture system and confirm the culture isolates as MAP species by amplification of the

IS*900* insertion sequence in the MAP genome by the use of conventional PCR and to determine the sensitivity and specificity of confirmed liquid culture results against the standard reference method, histopathology. The focus study areas for sample collection were in regions where ovine paratuberculosis in South Africa is prevalent (Eastern and Western Cape Provinces). As for the negative controls, collection was from farms with which we have not had a positive identification of Johne's disease, the Gauteng Province. The objective was to determine the ability of the culture system, the Versa Trek™ Automated Culture System to detect MAP in a South African setting including to evaluate any differences in the time to detection and cultivability characteristics exhibited by the South African originating MAP type when the internationally accepted method was used. Confirmation of the ability to detect South African isolates by the internationally recognized method for the control and management of the disease, will lay the foundation to compute a control program in the country. This is important because it was already established early in the century that the disease is emerging in the country (Michel & Bastianello, 2000) and this disease can be economically devastating (Plain et al. 2015).

## 2.2 Materials and Methods

### *2.2.1. Study areas*

Twenty one different sheep farms with previous MAP infection from either the Western Cape or Eastern Cape provinces of South Africa were sampled. As negative controls, nine different sheep farms with no previous positive diagnosis of paratuberculosis from Gauteng province of South Africa were sampled.

### *2.2.2 Sample collection*

During collection, two sets of tissue (pooled-ileocecal valve area, ileum, ileocecal Lnn and ileum) samples and one faecal sample were collected each from 108 slaughter sheep from 11 different farms of the Western Cape Province. One of the set of the tissue samples were preserved in a 10% formalin buffer and immediately sent for histopathology. The second set of the tissue and the faecal samples were kept cold with an ice-pack and transported to the laboratory, where it was stored at -20 ºC until processing.

From 5 animals, paired samples of tissue (pooled-ileocecal valve area, ileum, ileocecal lnn and ileum) and faecal samples were collected from 3 different farms from the Western Cape Province. An additional 4 tissue samples were received from slaughter animals without any corresponding faecal samples from 3 different farms. From live animals, 60 faecal samples were received from 3 different farms. These samples were all collected from the farms with previous MAP infection. A total of 50 faecal samples were received from five different farms around Gauteng Province. The farms were considered free of MAP infection due to their flock histories being negative for MAP.

All samples were kept cold with an ice-pack and transported to the laboratory, where they were stored at -20 ºC until processing.

### *2.2.3. Sample identification and storage*

Upon receipt at the ARC- OVR Tuberculosis laboratory, samples were assigned with sequential identification numbers named 'Validation paratuberculosis number', abbreviated VPTB1, VPTB2 and so on. The letter 'A' was used to identify faecal samples, for example VPTB1A and the letter 'B' was used to identify tissue samples

### *2.2.4. Sample processing*

#### 2.2.4.1 *Double decontamination*

##### 2.2.4.1.1. Faecal samples

On the day of processing, samples were first allowed to sit at room temperature to defrost. Decontamination method of samples was done according to Cornell double incubation decontamination for isolation of MAP from faeces as described by Bonhotal et al (2011) and Kim et al (2004). This method is also recommended by the Trek diagnostic-system. (http://www.trekds.com/techinfo/posters_abstracts/files/poster_no1.pdf).

First day: At least two to two and half grams of the faecal sample at a time was weighed for each sample. This quantity was suspended in a fifty ml centrifuge tube prefilled with 35 ml of distilled water. Followed by vigorous shaking to pulverize and blend the pelleted form of the faecal sample. The homogenate was then allowed to incubate at room temperature for 30 to 60 minutes to allow sedimentation. About 5 ml drawn from the upper portion of the visibly settled two phased sample was added into a 25 ml of 0.9% cetylpyridinium chloride monohydrate (CPC). The blend was then incubated at 37ºC for a period of 18 to 24 hours as according to Kim et al. (2004).

Second day: After the 18 to 24 hours of incubation period had elapsed; the blend was centrifuged with a Heraeus Labofuge© 400 centrifuge for 20 minutes at 3500 rpm at room temperature (18-25ºC) for pellet harvesting. After discarding the supernatant, the pellet was re-suspended in 1ml of an antibiotic supplement and incubated for a period of 18 hours to 24 hours at 37 ºC.

#### 2.2.4.1.2. *Tissue samples*

Sheep intestinal tissue samples from the ileocecal valve area, ileum, ileocecal Inn and ileum (all pooled during collection) were received. A thawed tissue (figure 22) sample was rid of tissue fat using scissors and cut into smaller pieces. The tissue sample was then homogenized in 35 ml of distilled water with a blade homogenizer. The homogenate was allowed to sit at room temperature for 30 to 60 minutes to allow sedimentation. A pipette was carefully used without distorting the sediments, to draw 5 ml from just below the solids that was formed above the liquid phase of the sample and was used for further processing. The remaining sample was stored in a freezer. The drawn 5 ml was transferred into 25 ml of 0.9% cetylpyridinium chloride monohydrate (CPC). The blend was then incubated at 37ºC for a period of 18 to 24 hours. Samples were then processed and cultured as described for the faecal samples.

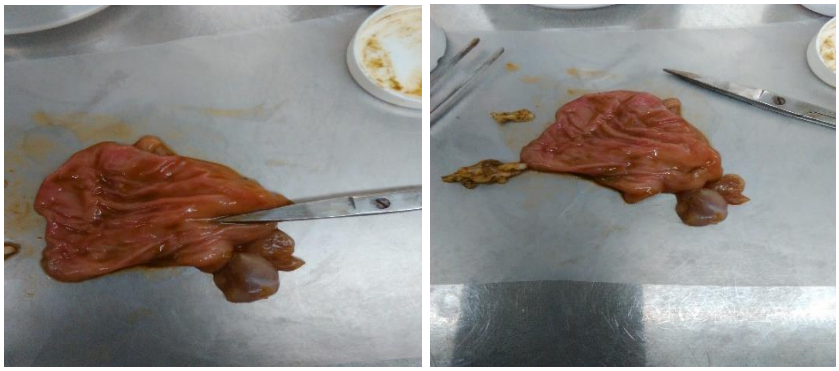


**Figure 22 Sheep intestinal tissue rid of fat, prepared for culture and also showing corrugation, (Picture taken at the ARC-TB Laboratory by Dominic Wagner)**

#### 2.2.4.2. *Mycobacterial culture of tissue and faecal samples*

On the third day, the sample was cultured as described by Kim and co-workers (2004). The decontaminated sample was added to a culture bottle designed especially for the Versa Trek liquid culture system. This contained 11ml of ESP para-JEM Broth from the manufacturer. Prior to inoculating 1ml of the decontaminated sample, the par-JEM broth was supplemented with 1ml of ESP para-JEM growth supplement, 1ml of ESP para-JEM egg yolk supplement, 500µl of ESP para-JEM antibiotic supplement and 50µl of paraJEM Blue. The enriched media was then vortexed/mixed thoroughly to create an even distribution of nutrients.

After inoculating the sample in the supplemented media, the rubber stopper was placed in the opening of the culture bottle which is designed to restrict any air flow movements in or out the incubation bottle. The mixture was mixed thoroughly by inverting the bottle continuously. The outside surface of the stopper was wiped with 1% bleach prior to attaching the versa trek connector. After placing the connector, the culture bottle was incubated in the Versa Trek machine. The Versa Trek Liquid

automated culture system incubation conditions were set at 37ºC for a maximum period of 70 days.

#### 2.2.4.2. *Processing of samples for extraction*

DNA for PCR amplification was extracted from the culture samples identified as positive by the Versa Trek Liquid automated culture system (22 samples from 18 animals) and those that finished the set incubation period but without a positive signal (251 samples from 155 animals) using the automated Maxwell-16- extraction DNA instrument (Anatech instruments (pty) Ltd) (section 2.2.4.2.1) and the boiling method (section *2.2.4.2.2)* were used.

Two milliliters of cultures from the Versa Trek Liquid was transferred into a pre-labelled 2 ml centrifuge tubes using pipettes. The drawn 2ml was centrifuged at 3500 rpm for pellet harvesting.

##### 2.2.4.2.1. *Extraction using the Maxwell® 16 instrument*

After discarding the supernatant, 250 µl of lysis buffer was added to the pellet. The mixture was then incubated overnight at 56 ºC using the Labnet™ heating block.

As described in the user manual: Properly labelled Maxwell® Cartridges (Figure 23) that come with the Maxwell ®-16 DNA Purification tissue kits were assembled in the preparation rack according to the number of samples at the time.

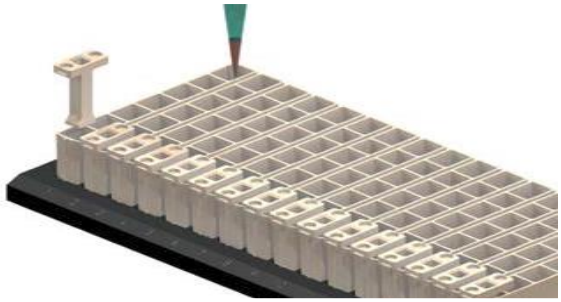


**Figure 23 Maxwell® Cartridges (Technical manual Maxwell® 16 DNA Purification kits)**

Labelled Maxwell® 16 elution tubes were assembled on the Maxwell® 16 tray for each sample. In the assembled elution tubes, 200µl of elution buffer was added. For quality and validity purposes, an extraction MAP positive control culture was included for each run.

After removal of the seal from the cartridge, plungers were placed into well 7 of each cartridge. From the over-night lysed culture sample, 180-200µl of the sample was transferred into well one of the cartridges.

After turning on the Maxwell® 16 instrument (figure 24), protocol for DNA extraction was selected. The cartridges together with the elution tubes were then transferred from the preparation racks (figure 23) to the Maxwell® 16 platforms (figure 24). Instructions on the Maxwell 16 were followed and automated purification was allowed to complete. The technique simply lyses the sample then deposits the lysed sample into paramagnetic particles that bind nucleic acids. The bonded nucleic acids are purified from other matter and serve as the extracted DNA and each cycle took 45 minutes (Operating manual Maxwell® 16 instrument). The eluted material was then transferred into pre-labelled tubes. To avoid DNA evaporation, transfer of DNA was done immediately after the system was done with the extraction. The Maxwell instrument

was cleaned after every run according to the Maxwell® 16 Quick start Guide. The extracted DNA was stored at minus 2 to 8ºC while preparing for PCR test.

**Figure 24 Maxwell® 16 instrument (Operating manual Maxwell®l 16 instrument)**

#### 2.2.4.2.2. DNA *extraction using the boiling method*

After discarding the supernatant and leaving just 250 µl of the supernatant, the pellet was re-dissolved in this. From here, the sample in its tube was submerged in boiling water using a beaker and a Labnet™ heating block for 25 minutes. Samples were left at room temperature to cool down.

### 2.2.4.3. *Confirmation of MAP by PCR*

DNA amplification specific for MAP was achieved by using primers targeting the *IS900* sequence region of the MAP genome (Kim et al., 2002; Moghadam et al., 2010). Amplification cycling conditions for the PCR was carried in a Eppendorf-Netheler-Hinz GmbH, Hamburg© thermal cycler. A 50µl reaction master mix was prepared as according to table 1.

**Table 1: PCR master mix preparations to detect the IS*900* in the MAP genome**

| Component | Quantity |
|---|---|
| $H_2O$ | 28µl |
| 10X buffer | 5µl |
| dNTP (10mM) | 2µl |
| $MgCl_2$ (2mM) | 3µl |
| Forward primer (20mM) | 1µl |
| Reverse primer (20mM) | 1µl |
| Hot Start Tag polymerase (250 units) | 0.25µl |

Primers used were synthesized by Whitehead Scientific (pty) Ltd with the following sequence.

*IS*900 (P90) Forward: 5'-GAAGGGTGTTCGGGGCCGTC-3'

*IS*900 (P91) Reverse: 5'-GAGGTCGATCGCCCACGTGA-3'

The *Taq* polymerase was added last in the master mix; the mastermix was prepared in a master mix preparation laboratory that is specially assigned for this purpose.

Thirty-nine micro litres of the master mix was transferred into pre- labelled thin walled micro-centrifuge tubes.

For inoculation of DNA template, the master mix was carried into another laboratory using a cooler box.

Eleven microliters of extracted DNA sample was added into the master mix, and mixed using the same tip. The combination was subjected to the cycling conditions listed in table 2.

**Table 2 PCR IS900 temperature cycling conditions for the amplication of the IS900 in the MAP genome**

| PCR step | Temperature and duration |
|---|---|
| Hot start | 95$^{o}$C for 10 minutes |
| Denaturation | 95$^{o}$C for 1 minute |
| Annealing | 65$^{o}$C for 1 minute 15 second |
| Extension | 72$^{o}$C for 1 minute |
| Number of cycles | 50 |
| Final extension step | 72$^{o}$C for 10 minutes |
| Holding | 4$^{o}$C |

### *2.2.5. Preparation of 1.5% agarose gel*

Four and half (4.5) grams of agarose powder was dissolved in 300ml TAE and brought to boiling using a laboratory microwave for 5 minutes to make 1.5% of agarose gel. After allowing it to cool down, 6µl of ethidium bromide was added. The melted gel was poured into a tray containing pre-inserted combs for creating wells and then allowed to solidify at room temperature.

### *2.2.6. Loading of PCR amplicons and gel electrophoresis*

The 50µl PCR amplicons were mixed with 4µl of DNA loading dye, and from this mixture, 20µl was loaded into the gel wells. Alongside the sample, 100 base pair DNA molecular weight maker was loaded to allow comparison of the size bands.

The gel was run at 90 volts for 2 hours or until the product bands were clearly resolved when analysing.

The gel image was captured using the BIO-RAD gel doc UV Tran's illuminator system and analyzed using the Quantity One® 1-D analysis software.

### *2.2.7. Detection of MAP from tissue samples by histopathology*

Fresh intestinal tissue samples preserved in 10% formalin were sent for histopathology immediately to either the Western Cape Provincial Veterinary Laboratory or the VETDIAGNOSTIX CAPE for histopathology analysis and diagnosis. As indicated in the test result reports, samples were examined for thickened intestinal walls infiltrated by epithelioid macrophages, plasma cells and lymphocytes due to the immune system response to MAP infection and confirmatory test demonstrating the presence of acid fast MAP was done.

JOB-numbers were used to link the corresponding samples sent for histopathology and those for culture. Histopathology investigates freshly preserved tissue samples by observing the cell morphology in the tissue samples.Results were then sent to the ARC-TB Laboratory for comparative evaluation with the faecal and tissue results.

## 2.3 Results

### *2.3.1 Culture of samples from known MAP positive flocks*

In the Versa Trek™ Automated Liquid Culture System instrument, an audible alarm and visible red LED indicates a positive signal and simultaneously positive signal is indicated by a red hyperlink on the computer connected to the system. A green LED indicates a negative signal, which appeared only after the set maximum incubation period (70 days) had elapsed. A sample was accepted as having a positive growth signal provided that the bar pressure growth indicator was consistent with a typical Mycobacterium species growth indicator, which is a decline in the pressure inside the bottle. An opposite direction of the bar pressure was accepted as a contaminant. Positive PCR results for MAP detection were indicated by a PCR product band size of 400bp, the size was determined by running the PCR amplicons alongside a 100bp DNA

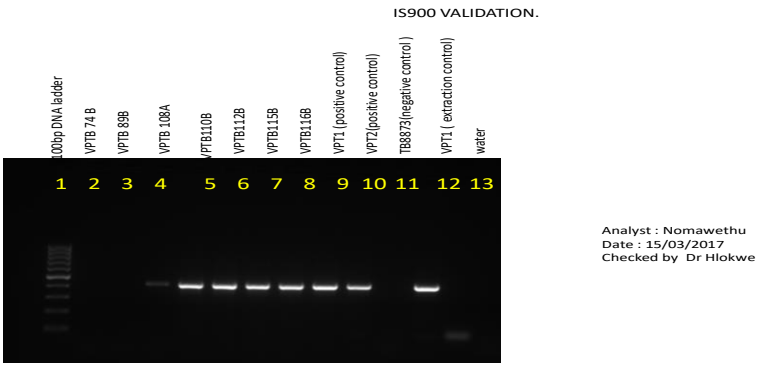



**Figure 25: PCR gel image showing Positive and negative PCR bands for some of the SA strains isolated in the study. Lane 1, 100 Bp DNA ladder, Lane 2 -a negative sample VPTB 74B, lane 3-VPTB 89B , lane 4- VPTB108(positive feint band), lane 5 -VPTB110B, lane 6 -VPTB 112B, lane 7 -VPTB 115B, lane 8- VPTB116B, lane 9-positive control, lane 10- PCR positive control, lane111 –negative control, lane 12 –extraction control , lane 13-water.**

The animals tested from flocks infected with MAP can be divided into main two groups, live and slaughtered animals. From the live animals, 60 faecal samples were cultured and only one sample (VPTB51A) triggered a positive growth signal. PCR results were negative for all the samples.

From the group of slaughtered animals, two subcategories is made, that of animals sampled for both faecal and tissue samples and for those of animals whose only tissue samples were received. From the former, a total of 113 pairs of tissue and faecal samples were tested. Of the 113 pairs, 4 pairs (VPTB105A/B, VPTB263A/B, VPTB265A/B and VPTB269A/B) had correlation on positive growth detection on both sample types. Only one tissue sample (VPTB105B) from one of the 4 pairs was positive on PCR. Seven (VPTB106B, VPTB 109B, VPTB110B, VPTB112B, VPTB115B, VPTB116B and VPTB 270B) of the 113 pairs had positive growth detection on their tissue samples and PCR confirmed only 6 of these samples as true positives, one (VPTB 270B) was a false positive. Lastly, 3/113 pairs (VPTB57B, VPTB60B and VPTB61B) had positive growth detection on faecal culture samples and PCR confirmed only two (VPTB60B and VPTB61B) as MAP. The other 99 pairs had no positive growth detection in the Versa Trek liquid culture method.

In a summary, 173 faecal samples were tested from flocks infected with MAP and the Versa Trek growth detection plus PCR had a test prevalence of 1.12% (2/173). A total of 117 tissue samples from flocks infected with MAP were tested and the Versa Trek growth detection plus PCR method had a test prevalence of 7.69% (9/117). The overall MAP infected farms detected by positive growth detection plus PCR method were 2/20 on faecal culture method and 7/18 on tissue culture method.

**Table 3: Test results from known MAP positive flocks from which a positive growth signal from either of the samples (tissue or faecal) were obtained**

| Sample Identification number | Growth Signal (TTD) | PCR confirmation results |
|---|---|---|
| VPTB51A | Positive (44 days) | Negative |
| VPTB57B | Positive (79 days) | Negative |
| VPTB60B | Positive (12 days) | Positive |
| VPTB61B | Positive (11 days) | Positive |
| VPTB75A | Positive ( 58 days) | Positive |
| VPTB105A | Positive (16 days) | Negative |
| VPTB105B | Positive (9 days) | Positive |
| VPTB106B | Positive (9 days) | Positive |
| VPTB108A | Positive (48 days) | Positive |
| VPTB109B | Positive (64 days) | Positive |
| VPTB110B | Positive (45 days) | Positive |
| VPTB112B | Positive (45 days) | Positive |
| VPTB115B | Positive (43 days) | Positive |
| VPTB116B | Positive (43 days) | Positive |
| VPTB 263A | Positive (9 days) | Negative |
| VPTB263B | Positive (3 days) | Negative |
| VPTB264A | Positive (6 days) | Negative |
| VPTB 264B | Positive (6 days) | Negative |
| VPTB265A | Positive (3 days) | Negative |
| VPTB265B | Positive (9 days) | Negative |
| VPTB269A | Positive (7 days) | Negative |
| VPTB269B | Positive (7 days) | Negative |
| VPTB270B | Positive(12 days) | Negative |

**TTD: Time to detection, A: Faecal samples, B: Tissue samples**

PCR done on negative signal culture samples (155 animals) yielded 6 (VPTB35A, VPTB111A, VPTB113A, VPTB114B, VPTB115A and VPTB117A) additional PCR positive samples. From these 6 PCR positive samples, 5 were from faecal samples and one originated from a tissue sample. Of the 5 PCR positive faecal cultured animals, 1 animal (VPTB115A/B) was positive on tissue culture and PCR confirmation. Table 4 lists the samples alongside the PCR findings for these six samples. This increased the faecal and tissue culture plus PCR confirmation test prevalence to 4%

(7/173) and 8.54% (10/117) when the final diagnosis is determined by PCR findings on cultured samples.

**Table 4: Disparate results for growth signal and PCR observed in tissue and faecal samples**

| Sample identification number | Culture signal | PCR confirmation results |
|---|---|---|
| VPTB35A | Negative | Positive |
| VPTB111A | Negative | Positive |
| VPTB113A | Negative | Positive |
| VPTB114B | Negative | Positive |
| VPTB115A | Negative | Positive |
| VPTB117A | Negative | Positive |

**A: Faecal samples, B; tissue samples**

### *2.3.2 Culture of samples from known MAP negative flocks*

Thirty four of the 50 faecal samples from the farms with no history of paratuberculosis infection gave a positive growth signal. These samples were from four different farms, 10/10 samples from each of three farms and 4/10 samples from one farm. The growth signals were received in less than 24 hours and the graphs were not indicative of MAP. Considering the negative history of MAP infection from these farms, the overall test positive detection rate of the Versa Trek Liquid automated culture system was 68% in faecal samples .PCR done on all these samples were negative together with those without a culture signal. Given the negative histories of paratuberculosis infection in these farms, the specificity of culture method plus PCR is 100%.

### *2.3.4. Histopathology results*

From the slaughter animals, tissue samples from a total of 108 animals were sent for histopathology. Analysis was done by the Western Cape Provincial Veterinary Laboratory or the VETDIAGNOSTIX CAPE. From these 108 animals, twelve (12) animals were diagnosed positive for Johne's diseases and 96 were negative; the calculated test prevalence was 11.1% on histopathology (Table 5). From these 12 samples, faecal culture correlated with only 5/12 (VPTB263A, VPTB264A, VPTB 265A, VPTB269A and VPTB270A) positive signals and tissue culture 9/12 (VPTB57B, VPTB110B, VPTB112B, VPTB115B, VPTB262B, VPTB263B, VPTB265B, VPTB269B and VPTB270B). Faecal and tissue culture positive signal plus PCR confirmation correlated with 0/12 and 3/12 positive samples on histopathology, respectively. The detection rate of growth signals obtained from faecal and tissue samples compared to MAP detection by histopathology was 41.6% and 75% respectively. The sensitivity of faecal and tissue culture (growth signal plus PCR confirmation) compared to histopathology was 0% and 25% respectively. Four faecal samples (VPTB111A, VPTB113A, VPTB115A and VPTB117A) that were negative on culture but PCR positive were also histopathology positive. Faecal culture negative signal plus PCR confirmation correlated with 4/12 histopathology positive animals. Tissue culture negative signal plus PCR confirmation had no correlation with any histopathology positive animals. The determined sensitivity of faecal culture negative signal plus PCR was 33.3%. Table 5 summarizes faecal and tissue growth detection and culture results (including PCR verification) as well as histopathology test methods.

The numbers of test results were determined with reference to the results obtained in histopathology. From the 108 samples sent for histopathology, 96 samples were

diagnosed negative for Johne's disease. Faecal culture plus PCR had 2 (VPTB35A and VPTB75A) false positive compared to histopathology. There were no false positives received on tissue culture plus PCR, only one sample (VPTB114B) that (with PCR) was positive and resulted to a 98% specificity of tissue culture when compared to histopathology. Faecal culture with PCR had 97% specificity when compared to histopathology.

**Table 5: Positive results obtained by either faecal and tissue culture (and PCR verification) as well as histopathology test methods**

| Sample ID | Faecal culture | Tissue culture | Histopathology |
|---|---|---|---|
| VPTB35 | Positive | n. d. | Negative |
| VPTB57 | n. d. | Negative | Positive |
| VPTB75 | Positive | Negative | Negative |
| VPTB110 | Negative | Positive | Positive |
| VPTB111 | positive | Negative | Positive |
| VPTB112 | Negative | Positive | Positive |
| VPTB113 | Positive | Negative | Positive |
| VPTB114 | Negative | Positive | Negative/inconclusive |
| VPTB115 | Positive | Positive | Positive |
| VPTB117 | Positive | Negative | Positive |
| VPTB263 | Negative | Negative | Positive |
| VPTB264 | Negative | Negative | Positive |
| VPTB265 | Negative | Negative | Positive |
| VPTB269 | Negative | Negative | Positive |
| VPTB270 | Negative | Negative | Positive |

n. d. not determined

The study went on an extra mile to evaluate the specificity of the confirmatory method, PCR, because the targeted sequence is reported in other mycobacteria. Figure 26 is a gel image showing results of PCR done on different species of mycobacteria. We had one MTBC isolate amplifying. Two *Mycobacterium tuberculosis* complex (MTBC) isolates amplified with PCR targeting the IS*900* in the MAP genome

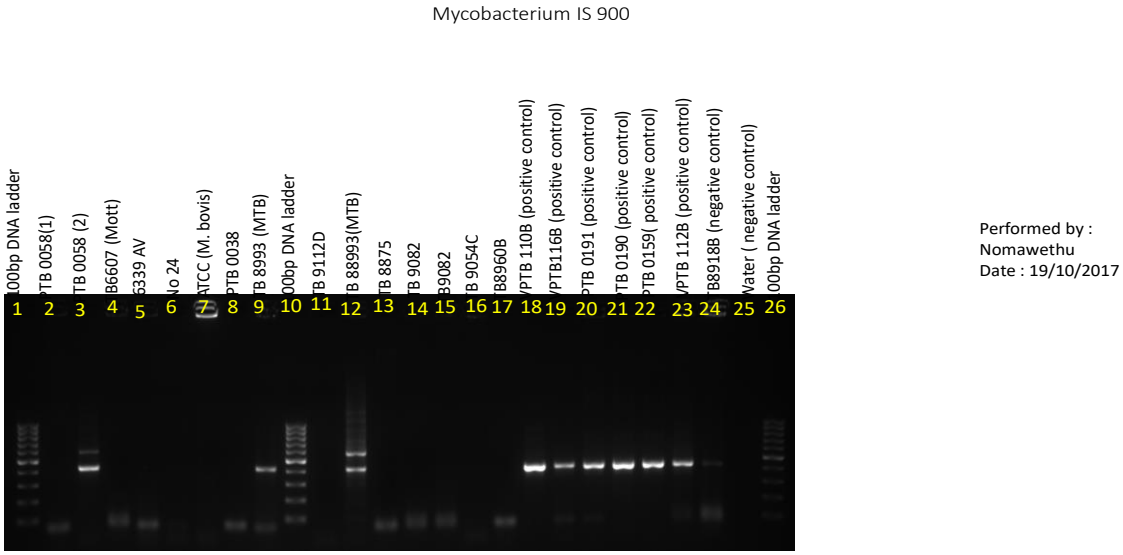



**Figure 26: IS900 PCR gel image of different species of Mycobacteria: lane 1-100bp DNA ladder, lane 2- negative, lane 3-positive, lane4-negative, lane 5-negative, lane 6-negative, lane7-negative, lane8-negative, lane9-positive, lane10-100bp DNA ladder, lane 11-negative, lane 12-positive, lane 13- negative, lane14-negative, lane15-negative, lane 16-negative, lane17-negative, lane 18-positive, lane-19 positive, lane 20-positive, lane 21-positive, lane 22-positive, lane 23- positive, lane 24-positive, lane25-negative, lane 26-100bp DNA ladder.**

Five animals (VPTB263 VPTB264, VPTB265, VPTB269 and VPTB270) that were histopathology positive and culture positive but PCR negative on either of the sample types were further evaluated with PCR targeting the 16S rDNA sequence for the detection of Mycobacterium was performed. Three of the five (VPTB 263B, VPTB269B and VPTB 270B) were 16S positive. The other two animals (VPTB265 and VPTB264) were negative on both sample types.

## 2.4 Discussion

This results analysis does not assume a 100% infection rate from these farms, they are with reference to the number of samples tested compared to histopathology as the standard reference method. The sensitivity of faecal and tissue culture was 33% and

25% respectively. Isolation of MAP from sheep samples has been challenging in different countries in the past when compared to isolation from cattle samples (Whittington et al. 2009). As a result, a higher prevalence in cattle compared to sheep is usually observed and this may be due to the prevalence or the detectability of the very fastidious sheep strain (Plain et al. 2015). Since the liquid culture method was developed, this has changed for many countries such as Australia and Canada which are now able to detect from sheep samples in substantial amounts (Bauman et al. 2016, Plain et al. 2015, Whittington et al. 1999, Windsor. 2015). Isolation of SA MAP strain has improved for both tissue and faecal culture since the early attempt to isolate from sheep samples by Huchzermeyer and Bastianello (1991). From a 11% histopathology test prevalence in a flock infected with MAP in South Africa, they only isolated MAP from 2% of the animals on either tissue or faecal samples (Huchzermeyer and Bastianello 1991, Whittington et al. 1999). This study has 33% and 25% faecal and tissue culture sensitivity using liquid culture compared to histopathology respectively. While a study by Malli in 2010 had a reference of 100% infection rate for the samples analysed, faecal liquid culture detected only 8.6% of the 100% and they were using the MGIT liquid culture system to detect MAP from sheep faecal samples (Malli, 2010). One study on a bigger scale (2050 sample size) and using the same method as in this study (Versa Trek liquid culture), detected MAP in 16% of the samples, however, this study was conducted on cattle rather than sheep (Rothkamp et al., 2009). Because of the fastidious nature and poor cultivability of the S strain that is mostly isolated from sheep samples, prevalence in sheep can appear lower on culture (Plain et al. 2015, Whittington et al. 1999). Tissue culture is known to be more sensitive, detecting more positives compared to faecal culture (Britton et al., 2016; Gwozdz et al., 1997). Consistent with literature review (Ramalakshmi et al.,

2016; Whittington et al., 1999), the current study had more isolates from tissue samples (9 isolates) and seldom from faecal samples (7 isolates). When compared to histopathology, faecal culture had greater sensitivity (33%) compared to tissue culture (25%) in this study. The study has three cases from which growth signals from faecal samples were detected but no growth signals from their corresponding tissue samples. Dukipatti and colleagues (2016) also had a number of animals from which they only detected from their faecal samples but not their corresponding tissue samples. From their study, in which they experimentally infected the sheep and using the Bactec46 culture system, faecal culture had a sensitivity of 92% while tissue culture had a test sensitivity of 84.8%. A total of 8% of animals were detected by faecal culture method only (Dukkipati et al., 2016). One study conducted on naturally infected sheep had similar cases, 2/30 (6%) sheep tested were positive only on faecal samples but not on their corresponding tissue samples. Tissue culture from this study detected 6/30 (20%) infected animals (Coelho et al., 2017). The overlap does demonstrate that from an infected animal, it is possible to isolate MAP from either one of the sample types for an animal, without isolation from the other corresponding sample type. Even though tissue culture is more sensitive than faecal culture, it can fall short in other cases. As a tool to combat the disease, faecal culture method can at times detect animals that tissue culture cannot detect. Though faecal culture does not necessarily demonstrate if an animal is diseased, more importantly, it gives the infection status of a flock and shedding status for an animal. In another study MAP was detected in the milk of the infected animal but not from its corresponding intestinal tissue sample (Giese & Ahrens, 2000). These cases are a typical demonstration of the complexity of the pathogenesis of the disease; moreover, a reminder of why utility of each method is important especially in relevant stages of the disease. Culture growth depends on the

presence of viable bacteria in the sample, which in turn depends on the infection status of the animal and the shedding levels for faecal culture. Shedding of MAP in faecal samples is also age dependent (Weber et al., 2010). The average age of the sheep from which we isolated MAP in their tissue sample but not from their corresponding faecal sample is 2.77 years. Adequate detectable MAP shedding in faecal samples is more likely to occur in older animals that acquired the infection at an early age and have had the time to progress to the clinical stage (McGregor et al., 2012; Pinedo et al., 2008; Ruiz-Larrañaga et al., 2017). With that in mind, the low number of faecal isolates compared to histopathology can be a reflection of the disease stage and less of the incompetency of the faecal culture method in the isolation of MAP.

Time to detection for MAP is documented to be as long as 112 days on solid media (Münster et al., 2013). In liquid medium, a previous study reported a maximum of 4 weeks', with most positive signals detected within the first two weeks of incubation. However, this study reported on the performance of a non-automated 7H9 liquid culture system and the animals used were seropositive high shedders (Pozzato et al., 2011). The longest time it took to receive a signal for this study was 64 days and the least time taken for detection was 9 days, with average time to detection of 35 days. In this current study, for faecal culture alone, an average TTD was 53 days and in tissue culture alone, TTD average was 31 days days. The earliest TTD was 9 days and 48 days for tissue and faecal culture respectively in the current study. The Trek diagnostics reports that TTD for MAP can be as early as 2 to 3 weeks (14 to 21 days) with the Para-JEM system (www.thermofisher.com). From tissue isolates, this study had an earlier (9 days) TTD compared to the time frame as set out by the Trek diagnostics (14 days). This early detection was however not useful in concluding the diagnosis early as PCR confirmation was positive only after 4 weeks from the time of

signal. That means time to detection for the method (culture plus PCR) was 37 days. Similar time frames were found in a study by Okwumabuwa et al (2010) in which their earliest detection was 18 days and 49 days as their longest TTD. Thirty-five days was reported as the maximum TTD by Rajeev et al (2006) with the Versa Trek Liquid culture automated system (Rajeev et al., 2006). It is then this study that reports the longest TTD (64 days) using the versa Trek Liquid culture automated system. This can possibly be due to the different characteristics exhibited by the local MAP type, especially the S type as it has in the past proven to be more sluggish ( Coelho et al., 2018, Collins, 2011). According to the classification of shedding animals described in the study of Laurin et al (2015), this study had two animals that are low shedders as they had time to detection of more than 28 (58 and 48) days (Laurin et al., 2015b).

Culture positive yet PCR negative samples are accepted as a false positive, contaminant or possibly mycobacteria other than MAP (Bauman et al., 2016b). It is important that the diagnosis by liquid culture is based on growth signal plus PCR confirmation because the culture of samples from non-infected farms yielded a high rate (68%) of false positive growth signals. Still on the point of false positives, it is noteworthy that the study received an increased number of PCR positives from culture positive samples after extended incubation period post signal detection was allowed. This was mostly true for samples that had a TTD below 21 days. The clumping effect posed by MAP may be an advantage when cultivating in a solid medium as it can facilitate a visible colony formation quicker than when they are scattered. The clumping effect is at a disadvantage when cultivating MAP in a liquid medium, especially when more than one experiment needs to be done on the same sample and cannot afford to dedicate the entire sample to one procedure. To dismiss the possibility of missing any MAP during the confirmation process, on a positive sample, PCR must be done

on different incubation intervals. This can minimize the possibility of “false PCR negatives”. Similar observation has been reported (Rajeev et al., 2006). These observations suggest that the Versa Trek automated liquid culture system alone can actually give an indication of positive samples at levels that the PCR cannot detect. These findings are contradictory to the results obtained by Kim and his co-workers early in the century. They reported that by the time the ESP II culture system gives a signal there are usually ample MAP cells in the medium to be confirmed by conventional PCR. However, the least time to detection for their samples was 5 weeks and they did not have samples that gave signals at 21 days or less. (Kim et al., 2004).This point is further demonstrated when results from the reference standard method are considered. There are samples that were culture and histopathology positive yet negative on PCR. The confirmation of positive culture resulted in a drop from 41.6% to 0% with the faecal positive signals compared to histopathology. For the positive signals on tissues, PCR confirmation dropped the sensitivity from 75% to 25%. Similar results in what appears to be a drop were obtained by Rothkamp et al., (2009) In their study, confirmation of culture signal by PCR resulted in a drop from 29% to 17% of the number of positives (Rothkamp et al., 2009). The specificity of IS*900* PCR to detect MAP organisms that are present is not questioned but only the sensitivity to detect MAP is, because of the extended time to positive PCR confirmation post the positive culture signal. Secondly, when compared to histopathology, a decreased sensitivity was observed for PCR. . Perhaps this study can corroborate the observation seen by Rajeev and acquaintances. They received positive signals from the Para-Jem liquid culture, stained these samples and observed acid fast typical of MAP, yet these samples were PCR negative and were only positive after several efforts of sub-culture and more molecular work. As with current findings, in addition to the positive growth

culture, acid fast typical of MAP were observed (Rajeev et al., 2006) and for this study, histopathology found these samples positive for MAP infection. This can further be substantiated with additional molecular work and extended culture period for these isolates. Otherwise the specificity of histopathology to detect MAP organisms needs to be considered.

Samples that concluded negative from the liquid culture were then screened with PCR; this was done as a precaution in case of false negative outcomes. By doing this, four MAP positive samples (correlation with histopathology) were detected from faecal culture negative samples and one false positive (Histopathology negative). This can be an indication of the detection limit of the machine or simply dead MAP cells in the culture media. Time to positivity for PCR from culture positive samples lagged behind the TTD for culture. Because of that observation, the samples that had no growth signals, yet were PCR positive, lead to a question of cultivability of circulating MAP types in South Africa rather than the detection limit of the liquid culture method. On the other hand, it could have been that the cells were dead and that is why there was no growth signal triggered. Negative growth signals should also be further evaluated by PCR as this increased the sensitivity of the faecal culture method from 0% to 33%. The specificity of the IS*900* does not give enough confidence as there are other mycobacteria that contain a sequence similar to the IS*900* (Imirzaliogl u et al., 2011) and it is this reason that a sample maybe positive with PCR but negative with histopathology. The positive IS*900* PCR on other mycobacteria done in this study suggests that IS*900* is not exclusive to MAP. IS*900* is reported in other mycobacteria but rarely on MTBC isolates, cross contamination in the lab or co-infection may be a reason. Further evaluation of this finding is recommended.

It is said that liquid culture can detect 30 to 40% of infected cattle (2012). As that is the value most likely associated with bovine paratuberculosis, the value for ovine paratuberculosis can be expected to be below that value, as the sheep type is far more fastidious. This study detected 3.70% of infected sheep by faecal culture and tissue culture detected only 2.75% of infected sheep, infection rate determined by histopathology.

The study has few single out findings worthy to be discussed individually. One sample (VPTB75A) was classified as “culture false positive” by the standard reference method, as the corresponding tissue sample sent for histopathology was negative for this animal. However, the faecal isolate was confirmed as MAP species by amplification of IS*900* in addition to that the animal did not look healthy and was clinically suspected. On another two cases (VPTB114A/B and VPTB117A/B) of culture negatives samples, histopathology concluded positive for one (VPTB117B) of these cases and “no adequate evidence to prove JD” in the second case (VPTB114AB). PCR was positive on the faecal sample from the former case (VPTB117A) and not on its tissue sample (VPTB117B). On the latter case (VPTB114B), PCR was positive on the tissue sample. These results can be a true demonstration of the complex nature of Johne’s disease pathogenesis more than a limitation of the method itself. The first case (VPTB75A), because it was clinically suspected, suggests that there are cases that tissue culture and histopathology can miss that faecal culture method can pick up; this is also supported by other studies outside South Africa (Whittington, 2009). Alternatively, the animal was sick because of other reasons besides that of MAP. Nevertheless, from the presence of live MAP cells in its faecal excretion, it can be reasoned that the animal was exposed to MAP. However, they did not cause any disease (negative histopathology) and culture picked up what is referred to as a pass

through detection (Collins, 1996) as no isolation was made from its corresponding tissue sample. One animal (VPTB117A/B) was positive with PCR from faecal culture sample and negative on tissue culture plus PCR. The tissue analysis with histopathology was positive for this animal. For each pooled tissue samples, MAP may be abundant in one and less concentrated in another (Marquetoux et al., 2018), hence there is a possibility of missing MAP cells present when taking just a portion of a sample for further processing. This can be the reason there was no detection on culture and PCR of the pooled tissue samples for this animal. The infection in the animal was an active infection; because histopathology was positive. MAP DNA without positive growth signal was detected from the faecal excretion of the animal and suggests presence of dead MAP cells. The MAP cell could have lost viability at some stage between sample collection and decontamination. On the other end (VPTB114B), the inconclusive result from histopathology yet positive MAP DNA from the tissue sample suggest that the animal was infected. However, the animal cleared the infection or it was too early in the infection stage to cause damage detectable with histopathology. Either the bacteria died while in the host or at some stage in the decontamination and cultivation process. This can lead to the detection of MAP cells only by PCR and never by culture or histopathology as there was no immune system reaction triggered. No disease and inflammations leading to pathological changes detected by histopathology at the time of sampling (Leao et al., 2016). A simple explanation to this case can again be that the animal was simply resistant to the infection as this is also possible (Sulyiman, 2014), a classical demonstration of infection not necessarily equals to disease. A possible limitation of histopathology is also highlighted, as the histopathological report from this animal for which MAP DNA was detected from its tissue sample did not present enough histological evidence to

make a suggestive diagnosis for Johne's disease. Woodbury et al., (2008), also report lack of correlation between histopathology and bacterial culture. Such results when they are the only source of results may leave the farmers unsure of the measures to take on their farm. State Veterinarians are also in a quandary to decide whether to declare the farms and herds or flocks positive for JD or not. Detected MAP DNA from this sample and the uncertainty of results provided by histopathology does add to the need of using more than one method for the diagnosis of JD.

## 2.5 Conclusion

Based on the findings from this study, it is concluded that the automated Versa Trek Liquid culture system is capable of detecting viable MAP in samples from infected farms. Three of the 8 infected farms were detected by tissue culture; detection with faecal culture is still a challenge. Consistent with literature, histopathology identified more positive animals than the method in comparison (Coelho et al., 2017; Younus et al., 2012). Hence according to the results obtained in this study, histopathology still prevails but has a limitation that it does not differentiate MAP from other mycobacteria. Although the presence of a positive signal can be triggered by other contaminants, the graphical features of the Versa Trek Liquid culture system can help eliminate the suspects. This is a technical aspect built into the machine-specific approach to test result interpretation. Time is a crucial factor and one of the major reasons liquid phase media is preferred over solid phased media. As shown in this study, it is important to note that the slow growth of MAP must be taken into consideration and is not underestimated. In this study one sample took beyond the set incubation period (70 days) to give a positive signal only after 79 days, emphasizing the importance of local validation of a test method. The study demonstrated that though PCR may be negative

for some of the samples detected early by the machine, these do not necessarily suggest a false positive. This point is also suggested by Whittington and his colleagues; they advise that confirmatory of culture signals from liquid culture should be performed at least at the end of the set incubation period even though the growth signal came in earlier (Whittington et al., 2013). With that in mind, thorough investigations, extended incubation period or other confirmatory methods such as Ziehl Neelsen should be employed, with caution of not adding cost to the confirmatory process. Animals that shed MAP are key in the control and management of the disease.

The aim of adapting faecal liquid culture method is in favor of the reduced turnaround time, non- invasive nature of the method and that it is ante-mortem (Imirzalioglu et al., 2011). The study recommends additional experiments to be done on South African MAP to increase the sensitivity of confirmed faecal culture signals compared to histopathology and ultimately optimize the method. The liquid culture method is proven able to detect SA MAP type by this study, especially by the tissue culture method. The faecal culture method has raised some concerns. More so, the interpretation of faecal culture positive results should be done with caution. As it is evident that an animal might be excreting MAP, however, it does not necessarily mean the animal is diseased. This is important because infected animals are usually culled to stop the spread of the disease and considering that the excretion might be temporary. Histopathology cannot detect such incidents and they are equally important because in a community of herd, one animal might resist and just pass through the MAP cells, the next animal might suffer an infection from these. Culture determines not only the disease in animals but also the presence of MAP cells in that community, whether it is causing disease or not. There is no test reliable enough to enable identification of

individual animals as definitely negatives. At present, JD diagnosis has to be at a herd or flock level.

# *Chapter 3*

## The detection limit of the liquid culture method in pooled faecal samples and individual samples

### 3.1 Introduction

The expense that accompanies the testing of a community of animals can be one of the major factors why famers are reluctant to participate in the control and management of Johne's disease. Due to the nature of Johne's disease, the shedding pattern exhibited by an infected animal, sensitivity is problematic in the available diagnostic methods. Shedding of MAP in faeces is not consistent, minimum detectable shedding levels are determined at stages beyond the early phase (Crossley et al., 2005; McNab et al., 1991). In faecal culture, cost savings on a whole herd are possible through the availability of a validated pooled faecal culture method. The condition, disease stage and the age of an infected animal are all factors that determine the shedding pattern. The shedding pattern which describes the number of MAP excreted in the faeces, the regularity of shedding together with the ratio of infected to non-infected animals, all determine the sensitivity of pooling faecal culture (Schaik et al., 2007). Kalis and co-workers report a dilution of 1 MAP containing positive faecal sample with four other faecal samples not containing MAP, to reduce the Colony Forming Units (CFU) MAP per gram below detection level (Kalis et al., 2000). The recovery rate for all types of mycobacteria was reported to be 71.2% in the ESP II Trek Diagnostics Inc Westlake Ohio, by Williams-Bouyer et al (2000) in various samples such as the sputum, urine, tissue and faecal samples and 90.4% by Tholcken et al.,

1997 in blood samples (Tholcken et al., 1997; Williams-Bouyer et al., 2000). It is therefore important to determine the number of individual samples per pool that will not decrease the sensitivity of detecting MAP infection in a community of animals. The aim of this study was to determine the detection limit of the Versa Trek ™ automated culture system, develop and validate a protocol for pooled faecal sample collection and processing for the maximum detection of MAP in infected animals.

## 3.2. Materials and methods

### *3.2.1. MAP isolates – identification and storage*

Two faecal isolates were used for this study. The first isolate designated VPTB108A, was a South African faecal isolate from the Western Cape Province. The animal was previously vaccinated and no tissue sample was sent for histopathology for this animal. Time to detection in the Versa Trek liquid automated culture system was 46 days and the sample was confirmed as MAP by PCR amplification of the IS900 MAP genome (figure27). The Ziehl Neelsen stain of the smear from this culture isolate was acid fast, with very few small clumps typical MAP. The corresponding tissue culture for this animal was negative. The second isolate designated VPTB-PT1 was a non-South African strain isolated from an ovine faecal sample that was received for the purpose of proficiency testing from the ANQAP proficiency testing scheme. The MAP isolates were maintained at 37 ˚C with constant supplement of nutrients for the duration of the study.

### *3.2.2. Faecal Sample collection and storage*

A faecal culture isolate that was confirmed as MAP by PCR was used for this study. Faecal pellets from which no culture signal was detected and were negative on PCR

were all gathered and used as MAP negative faecal samples to achieve this study. The negative faecal samples used in this study were from sheep in the Gauteng province. The gathered faecal pellets were labeled “MAP negative faecal pellets” and were kept in the -20˚C freezer until processing.

### *3.2.3. Sample processing*

#### 3.2.3.1. *Detection limit in individual faecal samples*

For the purpose of this objective, abundant MAP organisms were necessary; therefore, the culture isolates (VPTB108A and VPTB-PT1) were sub-cultured for an extended period of time and constant monitoring of the growth with growth supplements replenished monthly.

##### 3.2.3.1.1. *Enumeration of MAP cell concentrations*

From the liquid culture medium, single cell suspensions were obtained as previously described with some modifications (Shin et al., 2007). One ml of MAP culture in liquid medium was passed through a 5µm pore size filter syringe twice and the sample vortexed for 30 seconds. Dominants of single cells were confirmed by Ziehl Neelsen staining and microscopic analysis. Total cell concentration for the filtered single cell suspension was determined in the following way:

Quantification of viable MAP was achieved by the use of a Luna™ Cell automated counting chamber. Briefly, 1ml from the culture isolate was centrifuged at 3500rpm with Sigma 1-14, Lasec® centrifuge for five minutes. The obtained pellet was then re-suspended in 100µl of its supernatant. Ten microliters (10µl) of the sample was mixed with 10µl of trypan blue dye. Ten microliters (10µl) of mixture was loaded on each side

of the Luna™ Cell counting slides and inserted in the chamber as given in the instruction manual.

The following table (8) summarizes the average cell counts per sample/ isolate obtained from the Luna™ Cell automated counting chamber

#### 3.2.3.1.2 *Serial dilutions and spiking faecal samples*

To determine the detection limit in individual faecal samples, tenfold serial dilutions were created from the quantified two MAP isolates. From an undiluted stock solution, tenfold serial dilutions were created by taking 100ul and adding it to 900 µl of distilled water. In between the dilutions, samples were vortexed thoroughly to maintain single cell suspensions. These serial diluted samples were used immediately.

Faecal pellets that we previously cultured and confirmed negative of *Mycobacterium avium* subspecies *paratuberculosis* infections were used for the purpose of this objective. A series of 2g of faecal pellets equal to the number of dilutions created were prepared. Prior to spiking, the faecal pellets were allowed to dry out in the safety cabinet by leaving them for 1 to 2 hours at room temperature in weighing boats. Each semi-dried ovine faecal pellets (2g) were labelled corresponding to a diluted culture sample. Disposable needle and syringes were used to inject 1000ul diluted culture sample to the corresponding labelled 2g of faecal pellets. Each syringe was used once for each sample. After the samples were spiked successfully, they were transferred into 50ml tubes and allowed to incubate for 15 minutes at room temperature, to allow the bacteria to settle well in the pellet.

#### 3.2.3.2. *Detection limit in pooled faecal samples*

Two sets of experiments were performed for this purpose. The first experiment was the creation of laboratory pools as follows:

Serial dilutions were created and used to spike negative faecal pellets as described for the individual samples. Depending on the pool size, each spiked faecal sample was pooled with different amounts of negative faecal samples to a total of 2 g.

The second set of experiment was done by obtaining a naturally infected faecal sample. Time to detection for this animal was determined to be (6 days) and therefore classified as a high shedder (Laurin et al., 2015a). The corresponding tissue sample for this animal was also positive by culture and the histopathology results diagnosed the animal as positive for JD. The original faecal sample of this animal was combined with non-infected faecal pellets to create pools and determine the detection limit. From this faecal sample, 2 grams was weighed and mixed with 2g of other non-infected faecal pellets in different numbers to represent the groups of pools. A dilution of 1:2, 1:5, 1:10, 1:15, 1:20 were created with negative samples. A plastic homogenizer was used to thoroughly mix the faecal pellets. From the homogenate, 2 grams was weighed and decontaminated as previously described.

## 3.3. Results

It took a period of ten months (March 2017 to January 2018) to get abundant acid fast microorganisms from the SA MAP isolate (VPTB108A). Only then could the spiking experiment be performed. The following table (6) summarizes the average cell counts per sample/ isolate obtained from the Luna™ Cell automated counting chamber. The average live MAP cell concentration was $1.08X10^7$MAP/ml and $1.01X10^7$ MAP/ml for the South African and ANQAP MAP isolates, respectively.

**Table 6: MAP cell concentrations values obtained by Cell automated counting chamber for each of the isolates**

| Isolate number | Total cell concentration | Live cells concentration | Dead cell concentration |
|---|---|---|---|
| VPTB-PT1 | $1.32X10^7$/ml | $1.01X10^7$/ml | $3.1X10^6$/ml |
| VPTB108A | $1.7X10^7$/ml | $1.08X10^7$/ml | $6.26X10^6$/ml |

### *3.3.1. Detection limit in individual sample results.*

Table 7 and 8 list the culture signals obtained at different MAP cell concentrations in individual samples obtained for the two isolates, VPTB-PT1 and VPTB108A. Culture signals of spiked faecal samples for the VPTB-PT1 isolate were detected in all but *$1.01x10^5$(2)), $1.01x10^4$(2), $1.01x10^2$(2), $1.01x10^2$ and $1.01x10^1$* MAP cell concentrations**.** The longest TTD was 48 days at $1.01x10^3$ MAP cell concentrations and this was also the lowest MAP cell concentration detected. The least time taken to detect was 6 days at $1.01x10^7$ and $1.01x10^6$ MAP cell concentrations.

**Table 7 : Culture results of faecal samples spiked with ANQAP MAP strain**

| ANQAP strain cell concentrations of serial dilution | Culture signal ( Signal ) |
|---|---|
| $1.01x10^7X2$ | Positive TTD8 |
| $1.01x10^7$ | Positive TTD6 |
| $1.01x10^6X2$ | Positive TTD8 |
| $1.01x10^6$ | Positive TTD6 |
| $1.01x10^5X2$ | Negative |
| $1.01x10^5$ | Positive TTD21 |
| $1.01x10^4X2$ | Negative |
| $1.01x10^4$ | Negative |
| $1.01x10^3$ | Positive TTD48 |
| $1.01x10^2$ | Negative |
| $1.01x10^1$ | Negative |

**ANQAP: Australian national quality assurance program, TTD: Time to detection**

Culture signals for individual samples spiked with VPTB108A were obtained at only four MAP cell concentrations ($1.08x10^7$(2), $1.08x10^6$(2), $1.08x10^5$(2) and$1.08x10^4$(2) and they all had a TTD of 3 days. No signal was obtained for samples spiked with $1.08x10^7$ to $1.08x10^1$ MAP cell concentrations.

Table 8: Culture results of SA MAP Spiked faecal samples

| VPTB108A<br><br>Subculture dilutions | Culture Signal ( TTD) |
|---|---|
| $1.08x10^7X2$ | Positive( TTD3) |
| $1.08x10^7$ | Negative |
| $1.08x10^6X2$ | Positive (TTD3) |
| $1.08x10^6$ | Negative |
| $1.08x10^5X2$ | Positive (TTD3) |
| $1.08x10^5$ | Negative |
| $1.08x10^4X2$ | Positive (TTD3) |
| $1.08x10^4$ | Negative |
| $1.08x10^3$ | Negative |
| $1.08x10^2$ | Negative |
| $1.08x10^1$ | Negative |

TTD:Time to detection

### *3.3.2. To determine the detection limit in pooled faecal samples.*

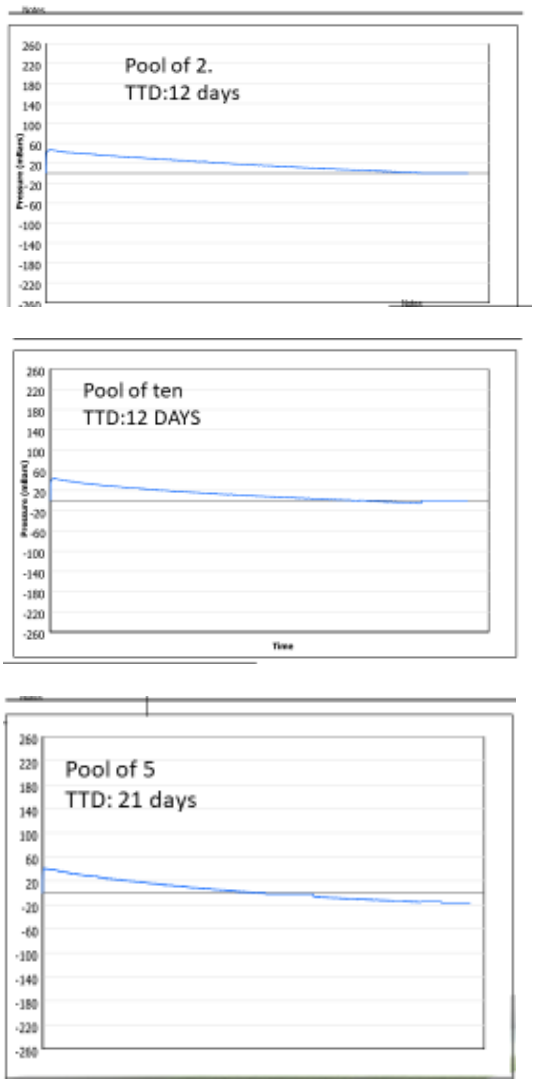
Results from the spiking and laboratory created pools at different MAP cell concentrations are demonstrated in the tables below. Table 9 shows results obtained from the pools created from the laboratory spiked faecal samples. Table 10 list the results from the pools created from the sample that was from a naturally infected sheep. The Versa Trek automated liquid culture system generates a bar pressure graph for each sample incubated. The generated graph displays the direction of the pressure in the incubated bottle; figure 27 is the example of the graph generated for some (pools of 2, 5 and 10) of the pooled faecal samples. From the laboratory spiked and pooled faecal samples, detection was made in all the pools created, pools of 2, 5,

10, 15 and 20 faecal samples at cell concentration of $1.08x10^7X2$ MAP/ml. The lowest MAP cell concentration detected in individual faecal samples was $1.08x10^4X2$. At this concentration, detection was made up until the pool of 10 faecal samples; no detection was made for the pools of 15 and 20 faecal samples. At the lowest MAP cell concentration created ($1.08x10^2$), none of the pools created at this concentrations were detected.

**Table 9: Pool size detection limit at different SA MAP cell concentrations**

| Concentration at which the pools were created | Highest pool size detected ( pool size) |
|---|---|
| **$1.08x10^7$(2)pool** | **100% detection (pool of 20)** |
| **$1.08x10^4$(2)pool** | **Pool of 10** |
| **$1.08x10^2$pool** | **None** |

**Figure 27 Bar pressure growth graphs generated by the Versa Trek liquid culture system for the incubated different pooled faecal samples**




Pools created from the sample that was naturally infected had a 100% detection rate. Time to detection varied for the samples. The least time taken to receive a signal was 2 days and this was the least number of samples pooled (1:2). The maximum TTD (31 days) was received at a pool of 1:3).

Table 10: Results obtained from pools created from the naturally infected animal

| Naturally infected pool | signal | Time to detection |
|---|---|---|
| Pool of 2 | Positive | 2 days |
| Pool of 3 | Positive | 31 days |
| Pool of 5 | Positive | 12 days |
| Pool of 10 | Positive | 12 days |
| Pool of 15 | Positive | 15 days |
| Pool of 20 | Positive | 21 days |

## 3.4. Discussion

Detection limit is defined as the lowermost amount of a certain matter that can be distinguished from the absence of that matter (wikipedia.org). In this case, detection is the lowest number of viable MAP *that* needs to be present at the time of receiving the sample in order to get a positive result. This number will then undergo the decontamination process, then finally culture. Decontamination alone can reduce the number of viable MAP in samples by 2.7 log10 in faecal samples (2012; Reddacliff & Whittington, 2003).

In bovine faeces, Pozzato et al., (2011) obtained a much lower detection limit of (2 X10) MAP/ml and 1MAP/g in both solid and liquid culture media respectively (Pozzato et al., 2011). The effects of strains cannot be taken lightly; Whittington reported a detection limit of $1.1x10^3$ on a sheep strain cultured in a liquid medium and a lower detection limit of 2.4 $X10^1$ on a bovine strain in sheep and cattle faeces respectively (Whittington et al., 2013). For this study, the detection limit was found to be much higher for the SA MAP strain ($1.08x10^4$X2 MAP/ml) compared to the PT strain ($1.01x10^3$ MAP/ml).

Time to detection in faecal samples among other things is determined by the shed amount of MAP cells in the faecal excretions (Crossley et al., 2005; McNab et al., 1991). From the small variations in TTD at higher concentrations for the individual spiked faecal samples in both the isolates, deduction can be made that the Versa Trek Liquid automated culture system approaches a minimum time to detection. This trend is obvious for the SA MAP strain, where all the positive signals obtained at higher concentrations had the same TTD of 3 days. While for the ANQAP strain TTD at higher concentrations were either at 6 or 8 days. For this isolate, TTD began to vary (21 and 48 days) for samples spiked with lower MAP cell concentrations.

Pools created from the sample that was naturally infected and classified as a high shedder according to time to detection, gave 100% detection in all the pools created, pools of 2, 5, 10, 15 and 20 faecal samples. These results are consistent with the results obtained from the pools created by spiking negative faecal samples and thereafter creating pools. Pools created with the faecal sample spiked with the highest concentration and taken to represent a high shedder also gave culture signals in all the pools, pool of 2, 5, 10, 15 and 20 faecal samples. The study defines a manageable pool size detection limit for high shedders at 20 animals per pool, whereby 2g of faecal samples are pooled for each animal. Fifty faecal pellets were weighed individually to get their individual weight size, then ultimately the average pellet size. From this experiment, the average pellet size was 0.5g. At least 4 pellets per animal should be pooled when sampling, however, if the pellets are small, double the pellet number should be pooled.

## 3.5. Conclusion

The detection limit obtained in this study was 1.08 x $10^4$(2) MAP/ml in individual samples and 10 faecal samples/pool for one infected sample containing 1.08x$10^4$(2), for the SA MAP strain under South African conditions. The low detection limit obtained in this study warrants further investigation. The number of MAP defined as the detection limit in individual faecal sample is further diluted in the detection limit of 10 faecal samples per pool, the detection limit obtained in pooled faecal samples. From that perspective, there is a possibility of a much lower detection limit in individual faecal samples and this study recommends further investigation of this value. Further, larger pool size can also be investigated on this basis but also the set incubation time period must be investigated as this can push for an effective and more economic detection in a larger pool size.

# *Chapter 4*

## Reproducibility of the individual and pooled faecal culture method

### 4.1. Introduction

The general definition of validation is verification by providing evidence. The evidence provided must show and support that a method or technique meets the requirements to be declared fit for its intended use (www.sanas.co.za). Reproducibility is an important factor in declaring a method fit for its designed purpose. Reproducibility of a test method can be demonstrated by comparative evaluation (www.eurolab.org). Comparison is done between results obtained by performing the same method on identical test sample in different testing laboratories by different personnel using different equipment (Feldsine et al., 2002). Inter-laboratory Comparison (ILC) study or proficiency testing scheme is a way to achieve and test for reproducibility. Reproducibility, a validation requirement is demonstrated when there is correlation of intended results among the participants.. Precision is another form that can be used to measure reproducibility. One sample can be tested multiple times using the same method under slightly different conditions, to determine the correlation of results between the independent tests. The degree of correlation indicates the reproducibility of the test method (South African National Accreditation System. 2014). The aim of the study was to evaluate the reproducibility of the Versa Trek automated liquid culture method.

## 4.2. Materials and methods

### 4.2.1. Study areas

Selected samples (Table 16) already described and used in chapter 2 were used in this study. These samples were received from the Western Cape and Eastern Cape provinces of South Africa. Lastly faecal and DNA samples were also received from the ANQAP and Molecular Diagnostics Laboratory (MDL) respectively for this study.

### *4.2.2. Sample collection*

The study participated in an Inter-laboratory collaborative scheme, the Australian National Quality Assurance Program (ANQAP) and inter-laboratory comparison (ILC) with Molecular Miagnostics Services (MDS) for verification of MAP by PCR. From the ANQAP scheme, faecal ovine and bovine samples were received during the period February 2016 and January 2017. Samples were delivered kept cold with ice pack, on receipt. The study participated in two rounds, which for we received 18 faecal samples. A set of six ovine faecal samples were received for the first trial and during the second trial, twelve faecal samples were received. A set of six were that of ovine faecal sample and the other six were that of bovine faecal samples. From ILC with MDS, five DNA samples were received. Lastly, selected faecal and tissue samples analysed in chapter 2 were used again for repeat testing purposes. From slaughter animals, we repeated samples that tested positive by culture and PCR in in chapter 2. From this, a total of 37 samples, 17 being tissue and 14 being faecal samples were retrieved. These were chosen on the basis that they were either positive on the first round of test, negative on the first round of test but its corresponding tissue or faecal sample

was positive. These samples were sourced from +-30ml that remained during the decontamination process in chapter 2.

### *4.2.3. Sample identification and storage*

Sample identification previously assigned a sample was used, date was used to differentiate between the samples previously processed and cultured. Samples remained in the -80˚C until processing for the SA samples. As according to instructions on the submission forms, the samples were stored at 4ºC upon receipt if processing was to commence in a short period of time or at -20 ºC if processing was not going to be immediate for the ANQAP faecal samples.

### *4.2.4. Sample processing*

#### *4.2.4.1.* Tissue and faecal samples

Faecal and tissue samples were decontaminated and cultured as described in chapter 2 under section 2.2.4. For the SA repeated samples, results obtained from this experiment were evaluated comparatively against the culture and PCR results obtained in chapter 2 for any deviations. For the ANQAP faecal isolates, culture and PCR results were evaluated against the feedback from the scheme for intended results; this was the same for the ILC with MDS samples.

#### 4.2.4.1. *Pooled faecal samples*

To determine reproducibility in pooled faecal method, only precision was used. The laboratory pools were simultaneously created in chapter 4, to determine precision.

This was done for convenience and the results obtained are evaluated against each other in this study.

Comparative evaluation was done between the results obtained from the naturally infected individual faecal and tissue samples in section 2.3, 3.3 and 5.3. For the spiked individual and pooled faecal samples, results were analyzed against those obtained in chapter 4 section 4.3 with that of those obtained in section 5.3. The results and feedback received from ANQAP in the different rounds were recorded and were used as a basis to evaluate our results.

## 4.3. Results

Table 11 and table 12 illustrate the results of the samples tested from different rounds of the ANQAP that the study participated in and the actual results we received as feedback from the scheme. From the first round of the ANQAP scheme (Table 11), the method under validation correlated with five of the intended results. There was one sample that tested negative with both the culture method but PCR positive while the intended result for this sample was MAP negative. The determined reproducibility for this round was 83.3%. For the MAP type used in the ANQAP scheme in reference to the intended results, sensitivity for this method was determined to be 100% and specificity was 66.6%. There was a 100% correlation of results between the faecal culture signal and the intended results for these samples; a follow up by PCR on these culture signals decreased the correlation.

**Table 11 :Summarizes the results from the first round we obtained and the intended results from ANQAP**

| Identification number | Culture signal | PCR confirmation | ANQAP intended results |
|---|---|---|---|
| Sample1 | Positive | Positive | Positive |
| Sample 2 | Positive | Positive | Positive |
| Sample 3 | Negative | Negative | Negative |
| Sample 4 | Negative | Positive | Negative |
| Sample 5 | Negative | Negative | Negative |
| Sample 6 | Positive | Positive | Positive |

**ANQAP: Australian national assurance program**

On the second trial (Table 12), culture signal alone had a reproducibility of 100% (12/12) and when combined with PCR the reproducibility dropped from 100% to 75% (9/12). Three of the faecal isolates, one from ovine faecal sample and the other from bovine faecal sample amplified for MAP DNA. The liquid culture method has performed exceptionally well for the culture and identification of the MAP type used in the ANQAP scheme.

Table 12: Results obtained from the second trial and the intended results from ANQAP

| Identification number | Culture signal | PCR confirmation | ANQAP Intended results |
|---|---|---|---|
| Sample 1 ovine | Positive | Positive | Positive |
| Sample 2 ovine | Positive | Positive | Positive |
| Sample 3 ovine | Positive | Positive | Positive |
| Sample 4 ovine | Negative | Positive | Negative |
| Sample 5 ovine | Positive | Positive | Positive |
| Sample 6 ovine | Negative | Positive | Negative |
| Sample 1 bovine | Positive | Positive | Positive |
| Sample 2 bovine | Positive | Positive | Positive |
| Sample 3 bovine | Negative | Negative | Negative |
| Sample 4 bovine | Positive | Positive | Positive |
| Sample 5 bovine | Positive | Positive | Positive |
| Sample 6 bovine | Negative | Positive | Negative |

The following table (13) represents the results of the individual South African samples that were retested to determine precision. From these samples, 21/37 samples were positive on culture signal and only three (VPTB109B, VPTB115B and VPTB116B) of these were still positive on culture when they were re-tested. From 21 previously culture positive samples, PCR confirmed 11 as MAP species and on re-test; only the three (VPTB109B, VPTB115B and VPTB116B) were positive on PCR. From the 37 samples, 16 samples were culture negative but on re-test, thirteen (13) of these were still negative, the other three were now positive on culture (VPTB11B, VPTB113B and VPTB117B). From the previously negative culture samples, PCR detected MAP DNA in 3/16 samples, the same samples were still negative on culture but now also on PCR when they were re-tested.

Table 13: Repetitive testing for culture and PCR of selected South African samples

| Sample ID | Culture signal | PCR result | Culture signal | PCR result |
|---|---|---|---|---|
| VPTB57B | Positive | Negative | Negative | Negative |
| VPTB60B | Positive | Positive | Negative | Negative |
| VPTB61B | Positive | Positive | Negative | Negative |
| VPTB75A | Positive | Positive | Negative | Negative |
| VPTB75B | Negative | Negative | Negative | Negative |
| VPTB105A | Positive | Negative | Negative | Negative |
| VPTB105B | Positive | Positive | Negative | Negative |
| VPTB106A | Negative | Negative | Negative | Negative |
| VPTB106B | Positive | Positive | Negative | Negative |
| VPTB108A | Positive | Positive | Negative | Negative |
| VPTB108B | Negative | Negative | Negative | Negative |
| VPTB109A | Negative | Negative | Negative | Negative |
| VPTB109B | Positive | Positive | Positive | Positive |
| VPTB110A | Negative | Negative | Negative | Negative |
| VPTB110B | Positive | Positive | Negative | Negative |
| VPTB111A | Negative | Positive | Negative | Negative |
| VPTB111B | Negative | Negative | Positive | Negative |
| VPTB112A | Negative | Negative | Negative | Negative |
| VPTB112B | Positive | Positive | Negative | Negative |
| VPTB113A | Negative | Positive | Negative | Negative |
| VPTB113B | Negative | Negative | Positive | Negative |
| VPTB115A | Negative | Negative | Negative | Negative |
| VPTB115B | Positive | Positive | Positive | Positive |
| VPTB116A | Negative | Negative | Negative | Negative |

| | | | | |
|---|---|---|---|---|
| VPTB116B | Positive | Positive | Positive | Positive |
| VPTB117A | Negative | Positive | Negative | Negative |
| VPTB117B | Negative | Negative | Positive | Positive |
| VPTB 263A | Positive | Negative | Negative | Negative |
| VPTB263B | Positive | Negative | Negative | Negative |
| VPTB264A | Positive | Negative | Negative | Negative |
| VPTB264B | Negative | Negative | Negative | Negative |
| VPTB 265A | Positive | Negative | Negative | Negative |
| VPTB265B | Positive | Negative | Negative | Negative |
| VPTB269A | Positive | Negative | Negative | Negative |
| VPTB269B | Positive | Negative | Negative | Negative |
| VPTB270A | Negative | Negative | Negative | Negative |
| VPTB270B | Positive | Negative | Negative | Negative |

For the reproducibility of the detection limit in individual samples, dilution and spiking was done in duplicates in Chapter Four to simultaneously achieve this objective. Variations in the detection limit was observed, table 14 summarizes the detection limit culture signals from the samples and their copy thereof. Culture signals were also obtained in repeat tested samples, only time to detection differed for the ANQAP strain duplicates. For the SA MAP strain, time to detection was the same.

**Table 14: Summary of the obtained detection limit in individual faecal samples of the ANQAP and SA strain**

| Isolate number | Culture signal Detection limit 1 | Culture signal Detection limit 2, |
|---|---|---|
| ANQAP strain | $1.01x10^3$ | $1.01x10^3$ |
| VPTB108A | $1.08x10^4$(2) | $1.08x10^4$(2) |

Reproducibility of the detection limit in pooled faecal culture method is shown below. As with detection limit in individual faecal samples, pools created were in duplicates. Table 15 shows a summary of results for the culture signal and pool size of a sample (original and repeat sample). Time to detection of a positive signal varied. Pool size detection limit was only determined for the SA MAP strain.

**Table 15: Detection limit in laboratory created pools, the second round of test (reproducibility)**

| Concentration | highest pool size detected1 (A) | Highest pool size detected2(A) |
|---|---|---|
| $1.08x10^7$(2)pool | Pool of 20 | Pool of 20 |
| $1.08x10^4$(2)pool | Pool of 10 | Pool of 10 |
| $1.08x10^2$pool | None | None |

It was equally important that the reproducibility of the confirmatory method is evaluated, the PCR method. For this, the study further participated in an interlaboratory comparison of results with Molecular Diagnostics Services in DNA testing for *Mycobacterium avium* subspecies *paratuberculosis* and our findings correlated 100% (figure28).

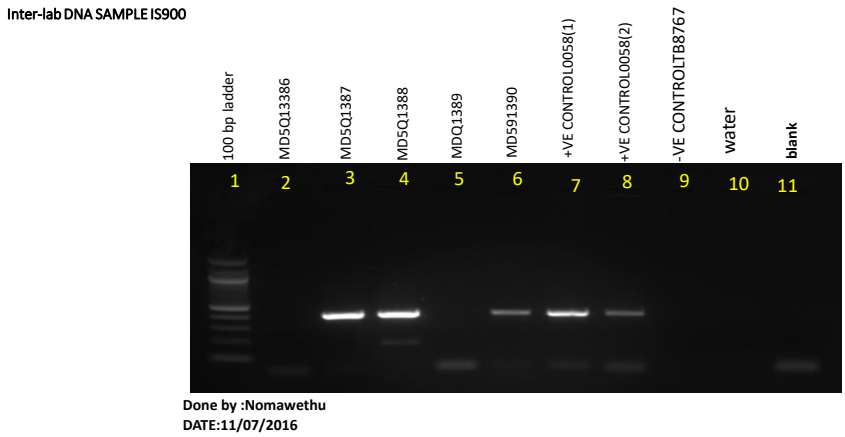



**Figure 28 PCR gel image of MAP DNA samples received for reproducibility testing. Sample 1-lane 2 (negative), sample 2-lane3 (positive), sample 3-lane 4 (positive), sample 4-lane 5 (negative), sample 5 –lane6 (positive). Positive control 1–lane 7 (positive), positive control 2- lane 8 (positive ), Negative control –lane 9 ( negative), water –lane 10 ( negative), blank –lane 11 (negative )**

## 4.4. Discussion

Proficiency testing is one of the methods used to determine reproducibility of a test method. Thus far we have demonstrated that the method is reproducible by proficiency testing. The method's sensitivity and specificity was high when compared to the sensitivity and specificity on the SA MAP strain. The determined sensitivity for faecal culture for SA strain was 0% while the overall sensitivity for the ANQAP strain was 100% in both the trials as no positive sample was missed. PCR confirmation on these samples increased the number of false positives, resulting in a drop for specificity from 100% to 66.6% and from 100% to 25% specificity for the first and second trial of testing. The high recovery rate in Australia is common as studies report high prevalence determined through faecal culture method and this may be because the disease is endemic in Australia (Geraghty et al., 2014). Recovery of 13/16 (81.25%)

from goat samples that were naturally infected from Australia (Eamens et al., 2007) with liquid culture method and these samples contained the both type of strains ( cattle and sheep strain). Another Australian study reports a success (100%) of the liquid culture method in isolating both from cattle and sheep samples (Plain et al., 2015). The high recovery rate received in this study for the ANQAP strain is in consistent with literature.

Reproducibility was determined through precision, where previously tested SA samples were tested again using the same method. The limitation to this experiment was that there were not enough samples to go back and perform the tests on it again. As a result, samples that was left behind (35ml-5ml) and stored at -80C during objective 1 in chapter 2, section 2.2 were used. And a lot of factors influenced the outcome of these results. Storage effects on the viability of MAP in the sample, the initial concentration of MAP is unknown, it is possible that most of the MAP cells present in the samples went through to decontamination when processing the sample for MAP detection. It is important when determining precision that the conditions of the sample are kept the same or only slightly different. The result from this experiment should be used as a future reference but not a true demonstration of precision for the method.

The faecal isolates obtained in this study were far more fastidious, the strains could not be cultivated fast enough to obtain abundant MAP organisms. In addition to the limited space in the Versa Trek automated liquid culture system at the time and that the reagents were imported from outside the country. Time was one of the challenges that hindered the proper reproducibility testing of the detection limit up to PCR confirmation level. As a result, more time is needed in addition to the cultivation period to test the reproducibility completely, from culture to species verification, even though

negative faecal samples were spiked with positive confirmed MAP cells. At the time of the study and signal detection, PCR was done on these samples but they were negative for all the samples and more time is recommended. There are a number of questions left unanswered, such as signal detection limit reproducibility synchronized with PCR. The culture signal detection limit is determined but we have not been able to determine at which stage of culture signals PCR will be positive. The time to detection varied for sample though at same concentrations. This has further made it difficult to determine reproducibility of the liquid culture method as MAP cells clearly behave differently from one sample to the other, regardless of the cell concentration. For example, one of the ANQAP type sample spiked with concentration of *1.01x10$^3$ MAP/ml,* one of the duplicates of this sample had a TTD of 48 days while the other at 10 days. There were samples at higher concentrations that trail behind samples at lower concentrations with regard to TTD, these findings suggest that shedding levels should not only be determined through time to detection of the Versa Trek liquid culture machine. These are further supported by the observation of getting an early signal, yet the sample is PCR negative at that time and is positive later on. If indeed the animal was a high shedder, then PCR should detect it soon enough. Reproducibility of IS*900* PCR for detecting MAP was 100%; this further supports the sensitivity and specificity of PCR

## 4.5 Conclusion

Through proficiency testing scheme the study has determined the reproducibility of the liquid culture method in individual samples. Reproducibility through precision in individual South African originating faecal sample was not truly demonstrated as a result of inadequacy of the sample present and the extended fastidious nature of the

SA MAP strain experienced in this study. Sample re-testing could not be repeated a sufficient number of times to provide statistical power in both the spiking and naturally infected samples. The reason was that the MAP isolate cell was not enough to allow that, as the abundance differed from one sub-culture to the other. We had only one culture containing enough MAP to perform this experiment. The other sub-culture was not thriving well enough even after attempts to rejuvenate the cells. Space in the Versa Trek liquid culture media was limited and the prolonged time set for maximum incubation period (70 days) all hindered the full success of this objective. As a result, we recommend that this experiment serves as a future negative reference to carry out more studies but not a true demonstration of the reproducibility of the method. Validation of a method is an ongoing process and the study has set the foundation for that. One of the research questions raised by this study is at what number of MAP cells present that the method is truly reproducible hand in hand with PCR.

# *Chapter 5*

# The impact of the antibiotic cocktail supplement on the recovery of the South African MAP strain from infected ovine faecal and tissue samples

## 5.1. Introduction

The slow generation time of the pathogenic mycobacteria have made the long in vivo incubation period inevitable (Collins, 2011). Besides having to wait out the incubation period, there is the challenge of controlling other contaminants during this period. The contaminants do not only outgrow the mycobacteria but they deplete the nutrients in the culture media (Salem et al., 2013). As a result, decontamination methods and culture conditions that spare mycobacteria but kill the contaminants are necessary. The inclusion of antibiotics when cultivating mycobacteria have in the past given optimum conditions that fulfill that purpose (Gwozdz, 2006; Pinedo et al., 2008; Timms et al., 2011). Methods to selectively kill micro flora in samples and ways around to ensure maximum detection in samples differ from one laboratory to another (Collins, 1996). Antibiotics can have a negative impact on the growth of MAP and decrease the number of viable MAP in the culture and this in turn decreases the sensitivity of the method (Biet & Boschiroli, 2014,Bradner et al., 2013). The chemicals used during the decontamination process are also reported to decrease the viability of MAP organisms in samples.

This study aimed at investigating the potential adverse effects of the recommended antibiotic supplement on the recovery of MAP from faecal samples. The antibiotic

supplement is listed to contain the PolymxinB, Azlocillin, fosfomycin, Nalidixic acid and Amphotericin B.

## 5.2. Materials and methods

### *5.2.1. Study area*

Samples already received and processed for this study from thirteen sheep farms from the Western Cape or Eastern Cape were selectively chosen.

### *5.2.2 Sample collection*

Faecal and tissue samples were received for the study. During decontamination, weighed samples were suspended in 35 ml distilled water. After homogenization and mixing of the sample with the distilled water, only five ml was drawn for further processing to culture and isolate MAP. The 30ml homogenate and extra samples that remained was properly labeled and stored at -80˚C. From the homogenates, twenty nine (29) faecal or tissue homogenate samples (13 pairs of tissue and faecal sample and 3 tissue only samples) were retrieved from the storage set at -80˚C in the ARC-OVR- Institute TB-laboratory. A total of 19 faecal or tissue extra samples (4 pairs of tissue and faecal sample, 6 faecal only samples and 5 tissue only samples) were retrieved from storage, these were the original samples that remained after weighing the required amount during the first day of processing and decontamination. The criterion used for these chosen samples was that:

1. A sample was positive on at least one of the test methods (Culture, PCR and histopathology) but tested negative on at least one of the other test methods.

2. A sample is one of a pair that tested positive on at least one of the test methods used in the study, which is culture, PCR or histopathology.

### *5.2.3. Sample identification and storage*

For the purpose of this study, sample identification was slightly modified to allow differentiation from the previous experiments. Samples cultured in the presence of an antibiotic supplement were labeled A on the culture bottle; those cultured excluding the antibiotic supplement were labeled NA on the culture bottle. Samples were processed on the same day of retrieval from cold storage and they were not stored any further.

### *5.2.4. Sample processing*

#### *5.2.4.1. To determine the effect of the antibiotic supplement on the recovery of South African MAP strain*

##### *5.2.4.1.1. Processing from the homogenate sample*

From the 30 ml homogenate sample, 5ml in duplicates was drawn with a 5ml pipette and transferred into a 50ml tube containing 25ml of 0.9% of CPC. From here, samples decontamination was carried out as previously described in chapter 2 section 2.2.4 for both faecal and tissue samples.

##### *5.2.4.1.2. Processing from the extra faecal and tissue samples.*

Extra tissue or faecal samples retrieved from the -80˚C were allowed to defrost at room temperature and thereafter processed as originally described.

### *5.2.4.2. To determine the effect of the antibiotic supplement on the detection limit and threshold of the Versa Trek Liquid culture automated system.*

#### *5.2.4.2.1. Detection limit in individual faecal samples cultured in the presence and absence of the antibiotic supplement*

Faecal pellets that have been confirmed negative of MAP infection were spiked with defined MAP cell concentrations as previously described in chapter 3 section 3.2. As in chapter 3, two strains were used for comparative evaluation purposes. Serial dilutions of two MAP strains ($1.01x10^7$(2) to $1.01x10^1$ PT MAP cells/ml and $1.08x10^7$(2) to $1.08x10^1$ SA MAP cells/ml) were created in duplicates and used to spike faecal pellets in duplicate. The spiked faecal pellets were decontaminated and one set of the duplicates were inoculated in culture broth supplemented as described in chapter two. The other replica was cultured in culture broth without the antibiotic supplement but all the other supplements were kept the same as described in chapter 2. The antibiotic supplement contains The formula for the antibiotic supplement given contains 0.02% w/v PolymxinB, 0.0075%w/v Azlocillin, 0.054% fosfomycin, 0.042% w/v Nalidixic acid and 0,015% w/v Amphotericin B for the Versa Trek Liquid automated antibiotic supplement (https://assets.thermofisher.com/TFS-Assets/MBD/Instructions/IFU711242GB.pdf).

#### *5.2.4.2.2. Detection limit in pooled faecal samples cultured in the presence and absence of the antibiotic supplement.*

Serial dilutions were created and used to spike negative faecal pellets as described for the individual samples. Each spiked 2g of faecal pellets was pooled with other

negative faecal pellets to a total of 2g in equal amounts in cell concentrations of $1.08x10^7$(2), $1.08x10^4$(2) and $1.08x10^2$

## 5.3. Results

### *5.3.1 The antibiotic supplement effect on the recovery of South African MAP strain*.

Table 16 (tissue samples) and 17 (faecal samples) list the culture results of the samples re-cultured in the presence or absence of the antibiotic supplement in comparison. PCR confirmation results are also indicated for each sample tested. These are the culture results from the homogenate samples. From the samples that were cultured in the presence of the antibiotic supplement, 0/15 faecal samples yielded positive growth signals and 5/16 tissue samples (VPTB111B, VPTB113B, VPTB115B, VPTB116B and VPTB117B) yielded a growth signal. From the set of samples incubated in the absence of the antibiotic supplement, 6/15 faecal samples (VPTB106A, VPTB112A, VPTB114A, VPTB115A, VPTB116A and VPTB117A) were culture positive and 9/16 tissue samples (VPTB57B, VPTB61B, VPTB109B, VPTB110B, VPTB111B, VPTB112B, VPTB113B, VPTB116B and VPTB117B) were culture positive. Four of the 6 (4/6) faecal culture positive signals were confirmed as MAP and 7/9 positive tissue culture samples were confirmed as MAP by PCR. From the six faecal growth signals cultured in the absence of the antibiotic supplement, 4 were confirmed as MAP species, and 7/9 tissue growth signals were confirmed as MAP species. Samples that did not give a growth signal were negative for MAP DNA with PCR. All the five signals of the tissue samples cultured in the presence of the antibiotic supplement were confirmed as MAP species.

**Table 16: Tissue culture results of samples cultured in the presence and absence of the antibiotic supplement**

| Identification number Tissue (B) | Antibiotic present cultured signal B (TTD) | PCR confirmation BA | Antibiotic absent cultured signal B (TTD) | PCR confirmation BNA |
|---|---|---|---|---|
| VPTB57B | Negative | Negative | Positive ( 6 days) | Positive |
| VPTB60B | Negative | Negative | Negative | N/A |
| VPTB61B | Negative | Negative | Positive ( 12 days) | Positive |
| VPTB75B | Negative | Negative | Negative | Negative |
| VPTB105B | Negative | Negative | Negative | Negative |
| VPTB106B | Negative | Negative | Negative | Negative |
| VPTB 108B | Negative | Negative | Negative | Negative |
| VPTB 109B | Negative | Negative | Positive (5 days) | Negative |
| VPTB110B | Negative | Negative | Positive (28 days) | Positive |
| VPTB111B | Positive (25 days) | Positive | Positive (12 days) | Positive |
| VPTB112B | Negative | Negative | Positive ( 5 days) | Positive |
| VPTB113B | Positive ( 21 days ) | Positive | Positive (12 days) | Positive |
| VPTB114B | Negative | Negative | Negative | Negative |
| VPTB115B | Positive (20 days ) | Positive | Positive | Positive |
| VPTB116B | Positive (20 days) | Positive | Positive (5days) | Positive |
| VPTB117B | Positive (20 days) | Positive | Positive(5 days) | Positive |

**Table 17: Faecal culture results of samples cultured in the presence and absence of the antibiotic supplement**

| Sample identification number Faecal (A) and Tissue (B) | Antibiotic present cultured signal A (TTD) | PCR confirmation antibiotic cultured A | Antibiotic absent cultured signal A (TTD) | PCR confirmation antibiotic absent cultured A |
|---|---|---|---|---|
| VPTB75A | Negative | Negative | Negative | Negative |
| VPTB105A | Negative | Negative | Negative | Negative |
| VPTB106A | Negative | Negative | Positive (3 days) | Negative |
| VPTB 108A | Negative | Negative | Negative | Negative |
| VPTB 109A | Negative | Negative | Negative | Negative |
| VPTB110A | Negative | Negative | Negative | Negative |
| VPTB111A | Negative | Negative | Negative | Negative |
| VPTB112A | Negative | Negative | Positive (5 days ) | Negative |
| VPTB113A | Negative | Negative | Negative | Negative |
| VPTB114A | Negative | Negative | Positive (5days) | Positive |
| VPTB115A | Negative | Negative | Positive (12 days) | Positive |
| VPTB116A | Negative | Negative | Positive (5days) | Positive |
| VPTB117A | Negative | Negative | Positive (5days) | Positive |

**A: Faecal, B: Tissue, TTD; Time to detection**

In addition, 19 faecal or tissue samples were processed and incubated in culture broth excluding the antibiotic supplement and the results compared with culture results of these same samples obtained during the earlier experiment in the presence of the antibiotic supplement (table 3). Table 18 lists the culture results of these samples. The positive signals obtained during this experiment were then confirmed as MAP species to rule out contamination. From this experiment, 4/10 faecal samples (VPTB111A, VPTB113A, VPTB115A and VPTB116A) were culture positive and 2/9 tissue samples (VPTB109B and VPTB114B) were culture positive. Compared to 3/9 tissue culture positive samples and 1/10 faecal culture positive samples previously detected. Lastly, two samples (VPTB111ANA and VPTB 114BNA), from the samples cultured from the original sample were confirmed as MAP species. The other four (VPTB109BNA, VPTB

113ANA VPTB115ANA and VPTB 116ANA) culture signals were PCR negative together with those that did not give a positive growth signal.

**Table 18: Culture signal results of original samples cultured in the absence of the antibiotic supplement**

| Identification number A | Culture signal (TTD) | PCR confirmation |
|---|---|---|
| VPTB57BNA | Negative | Negative |
| VPTB61BNA | Negative | Negative |
| VPTB75BNA | Negative | Negative |
| VPTB105ANA | Negative | Negative |
| BPTB 106ANA | Negative | Negative |
| VPTB 108BNA | Negative | Negative |
| - VPTB 109BNA | Positive (1 day) | Negative |
| VPTB 110ANA | Negative | Negative |
| VPTB111ANA | Positive ( 3 days) | Positive |
| VPTB111BNA | Negative | Negative |
| VPTB 112ANA | Negative | Negative |
| VPTB113ANA | Positive ( 5 days) | Negative |
| VPTB113BNA | Negative | Negative |
| VPTB114ANA | Negative | Negative |
| VPTB114BNA | Positive ( 28 days ) | Positive |
| VPTB 115ANA | Positive (5 days ) | Negative |
| VPTB 116ANA | Positive ( 12 days ) | Negative |
| VPTB117ANA | Negative | Negative |
| VPTB117BNA | Negative | Negative |

**BNA:Tissue samples cultured in absence of antibiotic supplement ,ANA: Faecal samples cultured in absence of antibiotic supplement**

In the previous experiment of samples cultured in the presence of the antibiotic brew, the faecal sample (VPTB105A) was positive on culture but PCR negative. However, PCR performed on the negative culture samples yielded 5 positive samples (VPTB 111A, VPTB113A, VPTB114A, VPTB115A and VPTB117A). From the samples cultured in the absence of the antibiotic brew, from the 6 culture positive sample, PCR confirmed 2 samples (VPTB111ANA and VPTB114BNA) as MAP. No isolation of MAP DNA was made from the culture negative antibiotics absent cultured samples. Four of the six signals obtained in this study were the samples from which MAP DNA was extracted from their culture negative samples. Six of the previously negative faecal

samples cultured in the presence of the antibiotic supplement were still negative when cultured excluding the antibiotic supplement. One faecal sample (VPTB116A) was previously negative but was subsequently positive when the antibiotic supplement was included and this sample was confirmed negative of MAP by PCR. Seven previously negative tissue samples were still negative on culture and PCR. One tissue sample (VPTB114B) was previously negative but now positive when cultured in the medium excluding the antibiotic supplement and it was confirmed MAP by PCR. One sample (VPTB109B) was positive in the previous experiment and when cultured in the presence of the antibiotic supplement. The same sample was confirmed as MAP previously but PCR was negative on this sample cultured in the absence of the antibiotic supplement.

### *5.3.2. Effect of the antibiotic supplement on the detection limit of the Versa Trek Liquid culture automated system*

#### 5.3.2.1. *Detection limit in individual faecal samples*

Table 19 lists side by side both the results for the ANQAP strain spiked faecal sample in both the presence and absence of the antibiotic supplement.

The lowest concentration detected for the ANQAP spiked faecal samples cultured in the presence of the antibiotic supplement is *1.01x10$^2$MAP* cells per ml. Though this was the minimum cell concentration detected, three sample of higher cell concentration (*1.01x10$^5$(2), 1.01x10$^4$(2) and1.01x10$^4$MAP cells /m*) did not give a signal. Detection was 100% in all the samples cultured in the absence of the antibiotic supplement.

**Table 19: Culture results of ANQAP strain spiked faecal samples in the presence and absence of the antibiotic supplement**

| VPTB PT1 | Growth signal with antibiotic | Growth signal without antibiotic |
|---|---|---|
| $1.01x10^{7}$ (2) | Positive TTD8 | Positive TTD2 |
| $1.01x10^{7}$ | Positive TTD6 | Positive TTD3 |
| $1.01x10^{6}$ (2) | Positive TTD8 | Positive TTD3 |
| $1.01x10^{6}$ | Positive TTD6 | Positive TTD3 |
| $1.01x10^{5}$ (2) | Negative | Positive TTD3 |
| $1.01x10^{5}$ | Positive TTD41 | Positive TTD3 |
| $1.01x10^{4}$(2) | Negative | Positive TTD1 |
| $1.01x10^{4}$ | Negative | Positive TTD3 |
| $1.01x10^{3}$ | PositiveTTD48 | Positive TTD1 |
| $1.01x10^{2}$ | Positive TTD60 | Positive TTD6 |
| $1.01x10^{1}$ | Negative | Positive TTD12 |

Faecal culture results of samples spiked with controlled SA MAP type concentrations are listed in table 20 side by side for those cultured in the presence and absence of the antibiotic supplement. The minimum cell concentration detected for samples spiked with SA MAP type cultured in the presence of the antibiotic supplement was *$1.08x10^{4}$(2)*. Detection was 100% in the samples cultured in the absence of the antibiotic supplement and the lowest cell concentration was *$1.08x10^{1}$*MAP cells /ml. The lowest TTD was obtained at cell concentration $1.08x10^{7}$*(2)* and *$1.08x10^{6}$(2)* while the highest TTD was obtained at cell concentration *$1.08x10^{7}$* for samples cultured in the absence of the antibiotic supplement.

**Table 20: Culture results of faecal samples spiked with controlled cell concentrations in the presence and absence of the antibiotic supplement**

| VPTB108A Subculture dilutions | Signal for culture with antibiotic supplement | Signal for culture without antibiotic supplement |
|---|---|---|
| $1.08x10^7$(2) | Positive TTD3 | Positive TTD2 |
| $1.08x10^7$ | Negative | Positive TTD 12 |
| $1.08x10^6$(2) | Positive TTD3 | Positive TTD2 |
| $1.08x10^6$ | Negative | Positive TTD4 |
| $1.08x10^5$(2) | Positive TTD3 | Positive TTD3 |
| $1.08x10^5$ | Negative | Positive TTD3 |
| $1.08x10^4$(2) | Positive TTD3 | Positive TTD3 |
| $1.08x10^4$ | Negative | PositiveTTD6 |
| $1.08x10^3$(2) | Negative | Positive TTD3 |
| $1.08x10^3$ | Negative | Positive TTD3 |
| $1.08x10^2$ | Negative | Positive TTD3 |
| 1.08x101 | Negative | Positive TTD3 |

#### 5.3.2.2. *Detection limit in pooled faecal samples*

Laboratory created pools at controlled SA MAP cell concentrations are listed in table 21. The results included in the table are a summary of the positive signals for faecal pools of 2, 5, 10, 15 and 20. Pools were created at lowest cell concentrations, intermediate cell concentration and at highest cell concentrations detected in individual samples. For comparative evaluation, culture results of those cultured in the presence of the antibiotic supplement and those cultured in the absence of the antibiotic supplement are listed alongside. Pools created at all the above stated cell concentrations and in the absence of the antibiotic supplement were all positive, while

those cultured in the presence of the antibiotic supplement, detection was up until the pool of ten for the intermediate cell concentration. At the lowest cell concentration, there was no detection in all the pools.

**Table 21: Detection limit at different concentrations in relaltion to pool size**

| Concentration | Culture signal for highest pool size in the presence of the antibiotic supplement | Culture signal for highest pool size in the absence of the antibiotic supplement |
|---|---|---|
| $1.08x10^{7}(2^{)}$pool | 100% detection (pool of 20) | 100% detection (pool of 20) |
| $1.08x10^{4}$(2)pool | Pool of 10 | 100% detection (pool of 20) |
| $1.08x10^{2}$pool | All negative | 100% detection (pool of 20) |

### *5.3.2.3. The effect of the antibiotic brew on the detection limit of the sub-cultures of MAP isolates*

To have an idea of the extent to which the decontamination method might be having on the SA MAP strain, direct sub-culture in the presence and absence of the antibiotic supplement was performed. Table 22 summarizes the culture signal results of sub-cultured samples for both the ANQAP and the SA MAP faecal isolate strain cultured in the presence and absence of the antibiotic supplement. For both the strains, detection was 100% in all the samples sub-cultured in the absence of the antibiotic supplement. As for the samples cultured in the presence of the antibiotic supplement, detection was until *1.01X10*$^{3}$ MAP cells /ml for the ANQAP strain and no detection for the SA strain sub-cultured samples at this range of cell concentrations.

The impact of taking only 5ml after sedimentation process during the decontamination process was evaluated against taking all the +-35ml present after sedimentation. There was no difference in the outcome of the culture signals from both methods.

**Table 22: Detection limit in sub-culture of serial diluted samples in the presence and absence of the antibiotic supplement**

| Strain | Highest dilution detected with antibiotic cultured (1.X107- 1X101 ) | Highest dilution detected without antibiotic cultured (1X107- 1X101 ) |
|---|---|---|
| ANQAP isolate | $1.01X10^3$ | $1.01X10^1$ |
| SA isolate | None | $1.08X10^1$ |

### *5.3.2.4 Sensitivity of culture and PCR of samples cultured in the absence and presence of the antibiotic supplement against histopathology.*

Only 15 samples retrieved for the antibiotic effect test experiment can be used for comparison against histopathology results (table 23). From these 15, eight are tissue samples and 7 are faecal samples. Of the 8 tissue samples, histopathology diagnosed 7/8 as positive and one was inconclusive. For the tissue samples cultured in the absence of the antibiotic supplement, from the 8 comparable samples, 7/7 (VPTB57B, VPTB110B, VPTB111B, VPTB112B VPTB113B, VPTB115B and VPTB117B) correlated with histopathology for MAP detection, the inconclusive sample on histopathology was tissue culture negative. For tissue samples cultured in the presence of the antibiotic supplement, only 4/7 (VPTB111B, VPTB113B, VPTB115B and VPTB117B) correlated with histopathology on positive MAP detection, the other 3 were negative and also the inconclusive sample (VPTB114B). As for the seven comparable faecal samples, histopathology diagnosed six as positive (VPTB110A, VPTB11A, VPTB112A, VPTB113A, VPTB115A, VPTB117A) and one (VPTB114A) was inconclusive as with the tissue sample. For the faecal samples cultured in the absence of the antibiotic supplement, 3/6 correlated with histopathology on positive MAP detection, also the inconclusive sample with histopathology was positive on faecal culture. On the other hand, samples cultured in the presence of the antibiotic supplement gave no culture signal 0/6 from faecal samples. When PCR was

performed on these samples, correlation between faecal culture and histopathology decreased from 3/6 to 2/6, the inconclusive sample was also confirmed as MAP species from the faecal culture sample. For tissue samples cultured in either the presence or absence of the antibiotic supplement, PCR confirmation did not results to a drop in the correlation of results with histopathology. For the inconclusive sample, PCR on the tissue sample was negative.

From the samples where the original sample was still available and was cultivated in the absence of the antibiotic supplement; only eleven are comparable with histopathology. Of this eleven, 5 are tissue samples and 6 are faecal samples. Of the 5 tissue samples, histopathology diagnosed 4 as positive and one as inconclusive. Tissue culture diagnosed 0/4, only the inconclusive sample was tissue culture and PCR positive. Lastly, for faecal samples comparable with histopathology. Histopathology diagnosed 5 as positive and one was inconclusive. Faecal culture correlated with 3/5 for MAP detection with histopathology. PCR confirmation on these samples dropped the correlation to just 1/5

Table 23. Histopathology results compared to culture of samples in the absence of the antibiotic supplement

| Identification number | Histopathology Results | Culture results in the absence of antibiotic | Culture results in the presence of the antibiotic |
|---|---|---|---|
| VPTB57B | Positive | Positive | Negative |
| VPTB110A | Positive | Negative | Negative |
| VPTB110B | positive | positive | Negative |
| VPTB111A | Positive | Negative | Negative |
| VPTB111B | positive | positive | positive |
| VPTB112A | Positive | Negative | Negative |
| VPTB112B | positive | positive | Negative |
| VPTB113A | Positive | Negative | Negative |
| VPTB113B | positive | positive | Positive |
| VPTB114A | Inconclusive | Positive | Negative |
| VPTB 114B | Inconclusive | Negative | Negative |
| VPTB115A | positive | positive | Negative |
| VPTB115B | positive | positive | positive |
| VPTB117A | positive | positive | Negative |
| VPTB117B | positive | positive | Positive |

## 5.4. Discussion

Throughout the study, an observation was made that the antibiotic supplement recommended as a contaminant suppressant might have a significant inhibitory effect on the successful culture and isolation of the South African MAP strain. For this reason, additional experiments were done to further test out the theory. Comparative evaluation was done between samples cultured in the presence and absence of the antibiotic supplement, respectively.

The control of Johne's disease relies greatly on culture results, as they demonstrate not only the presence of MAP in the sample but the state at which these MAP are in, live or dead. The state and the number of viable MAP organisms can help to determine the threat posed by the animal through transmission and spread of the disease (Behr & Collins, 2010) and this is more critical in faecal samples, as they are the key in the spread and transmission of MAP to other healthy animals. It is crucial that in the laboratory during the processing and incubation period, the opportunity of not only detecting MAP but also to determine if the bacterium is alive, dead or cultivable is not jeopardized. It is inevitable that during processing, some MAP organisms will be lost because of the unavoidable decontamination method used to cultivate MAP. However, these protocols are designed to have a minimum inhibitory effect on the targeted organisms (Bradner et al., 2013). To include an antibiotic during the cultivating process has proven to have negative consequences on the sensitivity of culture in this study. Even though the antibiotic is internationally used to control contaminants that come along with the sample (Whittington, 2009). The comparative evaluation results between the samples cultured in the presence and absence of the antibiotic supplement show that time to detection for the positive samples is prolonged if the

growth is not inhibited completely. In the absence of the antibiotic, the number of viable faecal culture isolates increased from 0 to 6, this means that 40% MAP infected excreting sheep were undetected when cultured in the presence of the antibiotic supplement. That gave a 0% correlation of results on positive detection between tissue and faecal culture. An overall correlation with regards to MAP detection between faecal and tissue culture was 3/9 (33.33%) of results for samples cultured in the absence of the antibiotic supplement. While it was 0/5 for samples culture in the presence of the antibiotic supplement. From the six faecal isolates detected in the absence of the antibiotic supplement, one correlated with tissue cultivated in the medium containing the antibiotic supplement.

Two animals (four samples) VPTB 112A/B and VPTB116A/B, faecal and tissue culture results of these samples cultivated in the medium supplemented with the antibiotic supplement did not correlate. However, there was a 100% correlation of culture results when we inoculated the samples in the culture broth that was not supplemented with the antibiotic supplement for the same samples. Lastly, the number of positive tissue culture results increased from 5 to 9 for the same samples cultivated excluding the antibiotic supplement. From these, four (VPTB111B, VPTB113B, VPTB116B, VPTB117B) correlated between those cultured in the presence and absence of the antibiotic supplement and one (VPTB115B) was later negative when cultured in the medium excluding the antibiotic supplement. Findings from this experiment do suggest that chances to isolate MAP from tissue and faecal samples can be increased when the recommended antibiotic supplement is excluded and this is more for the faecal samples.

Original samples cultured in the absence of the antibiotic supplement, results listed in table 23, two new faecal isolates (VPTB111A and VPTB 113A) and one tissue isolate

(VPTB114B) were detected and these were not detected in any of the previous experiments by culture and only by PCR. This experiment was conducted on the original samples received and unfortunately rarely were the samples enough for repetitive testing. As a result, proper comparative evaluation between antibiotic supplemented culture and non-antibiotic cultivated culture could not be made on this particular experiment. Even so, the positive culture signals cannot be overlooked, especially considering the distinct nature of MAP even in samples. These findings suggest that the SA strain might be partially or completely hindered by the antibiotic supplement. There are samples that were culture negative in the presence of the antibiotic supplement and PCR positive yet positive on culture in the absence of the antibiotic supplement. These results suggest viable MAP cells from the original samples at levels detectable by PCR. Because they did not grow, no metabolic processes and hence no growth signal was triggered. There is a possibility that a sample may have MAP cells below the PCR detection level, in the presence of the antibiotic supplement, these samples will go undetected.

Looking at results of the previous experiment of samples cultured in the presence of the antibiotic supplement in concurrent to findings obtained in this section, it is evident that time to detection for the samples for which we previously detected MAP was favorably changed. For one tissue sample that previously had a TTD of 79 days was decreased to just 6 days when the antibiotic supplement was excluded.

As much as the finding of more faecal culture positive isolates is giving hope, the possibility of increased false positive results is also expected. It is important that the signals are further investigated by a more specific method. As a result, from the 6 faecal samples were positive, only 4 were confirmed as true positive by PCR. It is

noteworthy that for each culture positive sample, time to PCR positivity varied. Some samples took longer to be PCR positive than the others. As a result, PCR confirmation results from samples cultured in a liquid medium should be interpreted with caution. One sample previously discussed (VPTB 57B) demonstrates this perfectly. The sample TTD was 79 days previously and PCR on this sample was negative and was therefore concluded as false positive. The reduced TTD was accompanied by PCR positive confirmation at the fourth week of incubation, though PCR done immediately after the signal was negative. Additionally, it is important to highlight that it is the SA faecal isolates that barely survived in the presence of the antibiotic supplement, while cultivation and isolation of the ANQAP strain was more successful. Detrimental effects by the antibiotic supplement are also reported in literature (Bradner et al., 2013; Gao et al., 2005). The observed less detrimental effect on the ANQAP strain might be because the strain is less susceptible to the antibiotic supplement.

The confirmed culture isolates cultured in the absence of the antibiotic supplement as MAP does not only open doors for further investigations on the types of antibiotics in the cocktail, but questions such as concentration dependent bactericidal effect. Sometimes antibiotics ability to kill an organism is greatly dependent on the concentration of the antibiotic (www.antimicrobe.org).

A 100% detection rate for MAP ($1.08X10^{7}$(2) MAP/ml – $1.08X10^{1}$ MAP/ml) when the antibiotic supplement was excluded concurrent with the 35ml supernatant centrifuge protocol as well as with the 5ml protocol in this study was made in samples spiked with defined MAP cell concentrations. While samples cultured in the presence antibiotic supplement, detection was only up to a concentration of $1.08X10^{4}$(2)/ml in

both protocols. There was no significant difference in the detection limit obtained following either the protocol in this study. A similar experiment was done; evaluating the two methods using35ml and 5ml protocol. The 35ml protocol yielded more culture MAP isolates compared to the 5ml protocol (Motiwala et al., 2004). When using either the 35ml or 5ml protocol, results are similar at higher concentrations and the results not the same at lower cell concentrations.

For the ANQAP strain, detection limit was $1.01x10^2$ MAP/ml for samples cultured in the presence of the antibiotic supplement and MAP was detected in all dilutions including the lowest concentration (*$1.01x10^1$)* for those cultured in the absence of the antibiotic supplement.

In pooled faecal samples, detection was 100 % (Pool of 2, 5, 10, 15 and 20) for samples cultured in the absence of the antibiotic supplement at all the concentrations. On the other hand of pools containing a concentration of *$1.08x10^7(2)$* MAP/ml cultured in the absence of the antibiotic supplement, detection was 100% in all the pools created. Pools created at the defined detection limit ($1.08X10^4$(2) MAP/ml in the individual samples cultured in the presence of the antibiotic supplement the cut off was at 10 animals per pool. At a lower concentration (*$1.08x10^2$*), MAP was not detected in any of the pools created. Therefore, the detection limit in individual samples cultured in the absence of the antibiotic brew is suggested to be lower than $1.08X10^1$ MAP/ml. Growth signal detection was successful in a pool of 20 faecal samples (when excluding the antibiotic supplement) at concentration $1.08X10^2$ MAP/ml. The number of MAP cells in the sample of 20 animals per pool containing one positive faecal sample was reduced to approximately 5 MAP cells per sample (see calculation below).

$$if\ 2g\ faecal\ sample = 100\ MAP\ cells, then\ 0.1g\ (\ one\ sample\ in\ a\ pool\ of\ 20)$$

$$= 5\ MAP\ cells$$

The sensitivity of faecal culture method in the absence of the antibiotic supplement when compared to histopathology increased from 0% (with antibiotic supplement) to 33%. The increased sensitivity when faecal samples are cultured in the absence of the antibiotic brew demonstrates that the sensitivity of the culture method is decreased by the presence of the antibiotic supplement. Shedding of MAP in faecal samples is key in the spread of the disease in a community of animals, hence, increasing the sensitivity of faecal culture method is key in the control and management of the disease and it has the advantage that it is ante-mortem. One animal (VPTB114A/B) was inconclusive on histopathology; however, faecal culture and PCR confirmation were positive for the faecal sample (VPTB114A) and for the tissue sample (VPTB114B) when cultured in the absence of the antibiotic supplement. These results suggest that even though histopathology is a standard reference method, diagnosis with culture is more direct and specific compared to histopathology. Culture is the gold standard in that it isolates the organism in its live form and this is direct a diagnosis for an infection. Additionally, excluding the antibiotic supplement allowed a more clear defined diagnosis compared to culture in the presence of the antibiotic brew and histopathology.

Sensitivity of tissue samples cultured in the absence of the antibiotic supplement compared to those cultured in the presence of the antibiotic supplement increased from 57.1% (without antibiotic supplement) to 100%. From this a conclusion can be made that tissue culture in the absence of the antibiotic supplement has the same sensitivity as histopathology the standard reference method. An added advantage to tissue culture is that it is more specific and able to differentiate infection from other

mycobacteria. However, the disadvantage to this is that the methods, tissue culture and histopathology are post-mortem.

Methods to selectively kill micro flora in samples and ways around to ensure maximum detection in samples differ between one laboratories to the other (Collins, 1996). Chemicals that are usually used during decontamination are the hexadecylpyridinium chloride, sodium hydroxide and the cetylpyridinium chloride. Antibiotics commonly used are the Vancomycin, amphotericin B and the nalidixic acid. The para-Jem (Versa trek liquid culture for MAP) is reported to contain these antibiotics in its antibiotic supplement cocktail (Bradner et al., 2013; Sattar et al., 2018). These antibiotics have in the past and in this study proven to be detrimental to the MAP organisms (Bradner et al., 2013; Pribylova et al., 2012). The chemical treatment followed by antibiotic supplement is suspected to have a compounded impact that might ultimately result in the MAP organism being susceptible to an antibiotic that they are normally resistant to (Bradner et al., 2013). While this may be true, the additional experiment performed in this study, where direct sub-culture there by bypassing the compounded effect during decontamination that involves the use of chemicals as suggested by Bradner et al (2013). Results obtained from this experiment (table25) point out the antibiotic supplement as the main and possibly the only culprit for the growth inhibition observed for the SA MAP organisms. The ratio of an antibiotic to the contaminants and the MAP organisms might have influenced the results of the above experiment. When sub-culturing, the isolate is considered clean from contaminants, hence the ratio antibiotic supplement to MAP organism is increased. The Versa Trek automated liquid culture system and medium was not specifically to isolate MAP but other pathogenic mycobacteria. The antibiotic supplement may have been chosen and work well to

promote their growth in the presence of contaminants. However, for the MAP strains isolated in this study, growth inhibitory of MAP by the antibiotic supplement was observed and as a result a different supplementation formulation may be needed. Antibiotic concentrations play a big role in inhibitory effects (Bradner et al., 2013; Pribylova et al., 2012).

Microorganism concentration in a sample play an important role in the sensitivity of the method (McKenna et al., 2018) and so does the antibiotic concentration (Bernier & Surette, 2013). The concentration and type of an antibiotic in a decontamination protocol should be based on the susceptibility of the non target bacteria to that antibiotic, minimum inhibitory concentration of the antibiotic required to kill the untargeted bacteria if it is susceptible to that antibiotic (Bernier & Surette, 2013). Hence there are a number of reasons why the sensitivity of the method increased (100%) when the antibiotic supplement was excluded, compared to when it was included ($1.08X10^4$(2) MAP/ml). The detection limit in the presence of the antibiotic supplement is unacceptably high because an infectious dose of only $10^3$ CFU per animal is enough to cause MAP infection and an infected animal can shed any levels above the infectious dosage and below the detection limit obtained in this study (Wu et al., 2007). There are levels at which bacteria of the same species and strain may be resistant/ susceptible to a certain antibiotic (Parrish et al., 2017), and only through tests such as antibiotic profiling on a type can the susceptibility/ resistance can be determined. There are two ways in which a bacterium may develop resistance, one is acquired and the other one is genetically gained (Normark & Normark, 2002). The former can be a reason why bacteria of the same species act differently under the same antibiotic concentration as it is depended on exposure. Hence the observed detection difference for the SA and ANQAP MAP types. Parish and colleagues noted

a very high and significant heterogeneity in minimum inhibitory concentrations between MAP types. The antibiotics they tested are rifaximin, rifampin, amikacin, clarithromycin, ethambutol, and gentamicin (Parrish et al., 2017). For this reason, validation of a diagnostic method for its intended use is important locally and it can help determine the above mentioned factor. The contaminants that come along with the samples cannot be avoided but the Versa Trek automated liquid culture system offers the advantage to determine the presence of contaminants that possibly survived decontamination. The machine allows continuous monitoring of samples and the generated graph characteristic of mycobacteria. By this, the antibiotic brew can be excluded at the beginning of the process and when the machine signals a possible contaminant that is outgrowing the targeted organism, only then the sample be removed and an antibiotic be added and re-incubated to eliminate or inhibit the growth of the contaminant.

## 5.5. Conclusion

There was an overall increased in the sensitivity of faecal and tissue culture method in the absence of the antibiotic supplement compared to histopathology. There was minimal decreased specificity for the faecal and tissue culture in the antibiotics exclusion method compared to histopathology. For the faecal and tissue samples used in this experiment, the antibiotic supplement did hinder the growth of some SA MAP isolates and this was more prominent for the SA faecal isolates than for the ANQAP faecal isolate. The detection limit of the SA MAP strain was above the infectious dose in the presence of the antibiotic but was reduced to $1.08X10^2$ MAP/ml when the antibiotics were excluded. We need to further evaluate this negative effect of the

antibiotic supplement on a larger sample size. Furthermore, antibiotic profiling is also recommended to be done on South African isolated strains before the method is fully adapted. Otherwise, there are substantially enough cases where the decontamination process alone proved to be enough without the need for the antibiotic supplement. This will also save costs for the test.

# *Chapter 6*

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APPENDIX A

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

# Animal Ethics Committee

| | |
|---|---|
| PROJECT TITLE | **Validation of *Mycobacterium avium* subspecies paraubercuIosis detection in ovine faecal samples (individual and pooled) under South African conditions using the Versa Trek liquid culture system and species verifying by PCR** |
| PROJECT NUMBER | **V142-16** |
| RESEARCHER/PRINCIPAL INVESTIGATOR | **N Masina** |

| | |
|---|---|
| STUDENT NUMBER (where applicable) | **UP_043 726 738** |
| DISSERTATION/THESIS SUBMITTED FOR | **MMedVet** |

| | | |
|---|---|---|
| ANIMAL ANIMALS | **No animals involved** | |
| NUMBER OF ANIMALS | **No animals involved** | |
| Approval period to use animals for research/testing purposes | | **November 2016-November 2017** |
| SUPERVISOR | **Prof. A Michel** | |

*KINDLY NOTE:*

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

| | |
|---|---|
| **APPROVED** | Date 28 November 2016 |
| CHAIRMAN: UP Animal Ethics Committee | Signature |

*S4285-15*

APPROVED

ARC • LNR

AEC13.16

**ONDERSTEPOORT VETERINARY INSTITUTE**
**ANIMAL ETHICS COMMITTEE**

## Application for clearance to use vertebrate animals (including their embryos and foetuses) for an experimental study or in a standard operating procedure for diagnostic purposes

**Where clearance is sought for a standard operating procedure (SOP), a separate clearance form must be completed per procedure**

**NOTE:**

- Please read the Animal Ethics Categories (AEC 01) form before you complete this application.
- This application must be typed.
- It must be signed by the Principal Investigator (the applicant) and other persons who are vouching for specialised aspects of the experimental design (i.e. statistician, safety officer, and persons responsible for supervising the use of scheduled medicinal substances).
- The application needs to be written simply but include all relevant detail.
- A score sheet must accompany all applications.
- If any animal during the experimental period gets sick/dies due to causes not related to the experimental work, a morbidity/mortality report must be submitted to the AEC accompanied by a full post mortem report.
- Once the experimental work starts a copy of the score sheet and a summary of the experimental work conducted must be visible at the experimental facilities in case of inspections by the AEC.
- **Submitting applications:** An electronic copy of the application should be emailed to SmitCH@arc.agric.za Ms Cecilia Smit, Secretary of the Onderstepoort Veterinary Institute – Animal Ethics Committee (OVI-AEC), (Tel: 012-5299378).
- **Deadline** for researchers to submit protocols will be the **1st of each month**. Late protocols go through to the next month.
- An AEC-reference no will be allocated to the submission and it will be distributed electronically to all AEC members for review and comments. Comments from AEC members will be submitted to Ms Smit by the 10th of the month.
- Ms Smit will collate all the comments and send them back to the AEC members, and applicants after the monthly AEC meeting.
- **Approved applications:** the original document with all the relevant signatures must be submitted to the secretary to arranged that it be Stamped and signed by the AEC-Chairman. The original document will be filed on AEC-Submission file and a copy be sent to the applicant.
- Revised applications must be submitted by the 20th of the month.
- AEC meetings will be held on the third Thursday of each month (except December) where all protocols and comments will be discussed, and final committee approval (or not) given, and communicated to applicants asap.
- Telephone enquiries on any animal ethics related matters may be directed to the Chairperson, Dr Paidamwoyo Mutowembwa (Tel: 012-529 9593, or MutowembwaP@arc.agric.za)
- **NB: No animals will be allowed at the ARC-OVI campus unless Section 20 approval is obtained from the Department of Agriculture, Forestry and Fisheries.**

APPLICATION FOR CLEARANCE AEC 2

APPROVED

ARC • LNR

| | | | | |
|---|---|---|---|---|
| Submission Date | | For Administrative Purposes | AEC REF | AEC 13 16 |
| AEC approval Date | 21 September 2016 | Signature (only on approval) | | 19 OCTOBER 2016 |

**A. PROJECT NO:**

**101664**

**B. PROJECT TITLE**

**Validation of *Mycobacterium avium subspecies paratuberculosis* detection in ovine faecal samples (individual and pooled) under South African conditions using the VersaTrek liquid culture system and species verification by PCR.**

**C. PROJECT LEADER/RESEARCHER (PRINCIPAL INVESTIGATOR)**

| Name | Contact Number | | e-mail address | Contact Address |
|---|---|---|---|---|
| Dr Tiny Hlokwe | 012 5299 149 | | HlokweT@arc.agric.za | Building 42 Bacteriology Section Onderstepoort Veterinary Institute |

| | | |
|---|---|---|
| **Division** | Zoonotic Diseases Section | |
| **Qualifications** | BSc; BSc Hons; MSc; PhD | |
| **Appropriate experience in animal research** | Twelve years' experience in paratuberculosis diagnostics | |
| **Details of involvement** | Co-supervision and training of the student | |

**D. RESEARCH TEAM MANAGER (RTM)**

| Name | Contact Number | e-mail address | Contact Address |
|---|---|---|---|
| Dr Claude Sabeta | 012 5299 439 | SabetaC@arc.agric.za | |

| | |
|---|---|
| **Division** | Food Feed and Veterinary Public Health |
| **Qualifications** | PhD ( Molecular Virology) |
| **Appropriate experience in animal research** | At least 20 years (small animals) |

Signature:.....Date..28.9.16.....

**D: Co-Workers** (involved directly with procedures on Animals

| Name | Contact Number | e-mail address | Contact Address |
|---|---|---|---|
| Not applicable | | | |

| | |
|---|---|
| **Division** | |
| **Qualifications** | |
| **Appropriate experience in animal research** | Not applicable |

E. OTHER Co-Workers

| Name | Contact Number | e-mail address | Contact Address |
|---|---|---|---|
| Ms Nomawethu Masina | 0721980139 | masinanomawethu@gmail.com | Building 42<br>Bacteriology Section<br>Onderstepoort<br>Veterinary Institute |
| **Division** | Zoonotic Diseases Section | | |
| **Qualifications** | BSc Hons (Microbiology) | | |
| **Appropriate experience in animal research** | 6 Months experience in paratuberculosis research | | |

| Name | Contact Number | e-mail address | Contact Address |
|---|---|---|---|
| **Prof Anita Michel** | 012 5298426 | Anita.Michel@up.ac.za | Faculty of Veterinary Sciences, University of Pretoria, Private Bag x4 Onderstepoort 0110 |
| **Division** | Department of Veterinary Tropical Diseases, Faculty of veterinary Science | | |
| **Qualifications** | Dr. med. Vet.; PhD | | |
| **Appropriate experience in animal research** | >20 years' experience in design and execution of research studies involving animals | | |

| Name | Contact Number | e-mail address | Contact Address |
|---|---|---|---|
| **Prof Gareth Bath** | 012 5298038 | Gareth.bath@up.ac.za | Faculty of Veterinary Sciences, University of Pretoria, Private Bag x4 Onderstepoort 0110 |
| **Division** | Small Stock Health and Production | | |
| **Qualifications** | BVSc | | |
| **Appropriate experience in animal research** | Experience in most aspects of sheep and goat health and production | | |

| Name | Contact Number | e-mail address | Contact Address |
|---|---|---|---|
| **Dr Sewellyn Davey** | 021 526 8600 | SewellynD@elsenburg.com | State Veterinarian Malmesbury |
| **Division** | State Veterinarian Malmesbury | | |
| **Qualifications** | BVSc | | |
| **Appropriate experience in animal research** | Specialist In Veterinary Surgeons | | |

## F. DECLARATION

1. Moral Philosophy

The ethical review of proposed animal experiments is predicated upon the acceptance by the OVI that vertebrate animals are organisms of moral concern and as such, must be accorded rights in their exploitation for the advancement of biological knowledge and for the promotion of the health and welfare of animals and man.

2. Animal Rights

In the research use of laboratory animals these comprise the right:

- Not to be used for research and/or to be killed for trivial, irrational, unjustified or inappropriate reasons
- To live, reproduce and grow under conditions that are comfortable and reasonably natural to their species
- To be kept free from disease, parasitism, injury and pain by prevention, rapid diagnosis and treatment
- To be able to express normal behaviour through providing sufficient space, proper facilities in which to live and in the company of the animal's own kind
- To be free from fear and distress by ensuring that the animal's living conditions, handling and treatment will either minimise or eliminate the infliction of these states upon the animal.

A. Humaneness

The principles of Humane Experimental Technique must be followed in the planning and conduct of animal experiments. These comprise:

- Replacement of animals with non-sentient research systems i.e. researchers should strive to avoid using laboratory animals if alternative methods can yield the data they need
- Reduction of the numbers of animals which are to be used to a minimum by design in order to achieve only sufficient statistical power to allow the objectives of the experiment to be achieved
- Refinement of the experimental methodology to be adopted by the implementation and if necessary improvisation of procedures which will have the least distressing or harmful effects on the animals and when this is not avoidable to counter those effects with the use of anaesthesia, analgesia and other effective strategies.

B. Relevance

The research must address an important question relevant to the OVI's objectives in advancing education, science and human and animal welfare through research.

C. Responsibility

Everyone using animals, whether for experimentation, testing, diagnosis, teaching or sourcing of tissues or body fluids is responsible in their personal capacity for assuring that the animals which they use are afforded the highest levels of welfare and protection from abuse, and violation of the rights accorded to them.

D. Declaration

6.1 I, Dr Tiny Hlokwe, as Principal Investigator in this application hereby declare that I am familiar with the precepts, policies, and responsibilities outlined under Section D and will personally undertake to see that these are upheld in the conduct of this study, should it be approved.

6.2 I also undertake not to deviate from the approved protocol without clearance for changes which may become desirable or necessary when the experiment is in progress without submitting a request for a deviation and having these changes cleared by the OVI-AEC.

6.3 Should the OVI-AEC request a report, at the conclusion of the study I undertake to report on its outcome to the OVI-AEC and if it has not been completed within six months of it being cleared by the Animal Ethics Committee, to submit progress reports at six monthly intervals until the study has been completed.

**Signature of Applicant:** ..... **Date:**..... 20/09/2016

| | |
|---|---|
| 6.4 | In my opinion, all persons named and working under my supervision have the appropriate training and skills needed to carry out their responsibilities for experimental procedures, care and handling of the species being used. |

Signature of Supervisor(When applicable) ..... Date 21.9.2016

**G. PEER REVIEW STATEMENT**

I N. Grebe (Reviewer's Name) declare that this research protocol has been peer reviewed by myself in my capacity as (tick answers):

| | |
|---|---|
| Director | |
| Programme Manager | |
| External review | |
| Other (specify) | Researcher with experience working with JD. |

Of the (Institute/Unit) on 21.9.2016 and has been judged by myself to be relevant, designed in accordance with accepted scientific practices and norms and is in my opinion likely to be successful in achieving its aims.

Signature (Reviewer) ..... Date 21.9.2016

**H. PROTOCOL**

**a. Nature of Project (tick applicable answer)**

A.

| New Project | Extension to Approved Project | Amendment to Approved project |
|---|---|---|
| X | | |

B.

| Research | Training | Animal products | SOP | Other (specify) |
|---|---|---|---|---|
| X | | | | |

C.

| Start Date | End Date |
|---|---|
| Unknown | Ongoing |

**1. Classification of project according to Categories A-E**

In order to comply with the Institutes requirements for animal experimentation it is essential that you read the attached list for the explanation of categories carefully before marking the relevant block with an **X**

| Category | |
|---|---|
| A | X |
| B | |
| C | |
| D | |

**2. Background Information**

Paratuberculosis poses a big threat to sheep and cattle farming and in particular to wool production and has a significant effect on the economy (Motiwala et al. 2004). Livestock diseases such as paratuberculosis have devastating outcomes on animal health and impact on national and international trade and remain endemic in many parts of the world (Gao et al. 2002). In addition, animals with unknown paratuberculosis status slaughtered at abattoirs pose a high risk of disseminating the organisms in the abattoir environment and may contaminate other meat being processed, posing a risk for human consumption (Gao et al. 2002). Paratuberculosis is a chronic enteritis of ruminants for which the main clinical signs are intermittent diarrhoea and weight loss. Usually animals are infected in their first year of life by ingesting food contaminated with faeces from their dams and other infected animals. Because the incubation period can vary from months to several years, the disease is not manifested clinically until the animal

becomes an adult but the majority may remain clinically in apparent carriers. There is no effective treatment for paratuberculosis and clinically affected animals ultimately die from the disease. The etiological agent of paratuberculosis is *M. avium* subspecies *paratuberculosis*, (MAP) which belongs to genus Mycobacteria (Thorel et al. 1990). Unlike bovine tuberculosis, which is another important mycobacterial disease that has been given much attention to the extent that a control scheme is available in South Africa and many developed countries, paratuberculosis on the other hand, remains an unsolved problem for the veterinary scientific community still incapable of reaching a consensus of a better way to deal with it.

**3. Aim/s of the Proposed Study/SOP**

Although a controlled disease in South Africa, paratuberculosis remains an unsolved and increasing problem for the farming and veterinary scientific community. MAP is currently only diagnosed serologically, but the downside of such tests is that they have low sensitivity and are costly. The causative agent can only be confirmed by culture. We aim to validate an internationally accepted, improved culture technique in South Africa, which is more affordable and will ensure better surveillance and control of the disease, hence higher productivity and economic returns. This information will also assist in identifying sources of infection and routes of disease transmission. Control and eradication of paratuberculosis is also in the best interest of public health.

**4. Potential Benefits of the Research Findings**
(For ethical cost/benefit assessment)

The validated method will be applied routinely as a diagnostic service offered by the Tuberculosis laboratory. The method offers a reduced turnaround time compared to previous culture methods, allowing more efficient diagnostic service. The validated and adopted method will offer cost effective diagnostic services by eliminating the need to use specialized media. Determining detection limit in pooled faecal samples will further offer reduced cost diagnostic services associated with individual sample processing. Additionally, the famers will be given an option of submitting recommended pooled faecal samples, which will be less costly. Results reported by the system will be more trusted as true negatives or as true positives as the method is more sensitive compared to the

currently used diagnostic method. This can help famers to avoid culling of non-infectious animals. In addition, farmers will be able to remove or isolate infected and infectious animal which do not present clinical symptoms.

**5. Hypothesis**

If a hypothesis is being tested (in exploratory research) give the postulate to aid the reviewers in following the rationale of the proposed study/SOP.

Not applicable

**6. Experimental Design and Procedure/s**

Describe briefly in layperson's terms in a sequence of short annotated sentences (a), (b), etc. all the steps to be performed in conducting the experiment including operative procedures, collection of samples (give blood volumes and routes of collection if applicable), other measurements to be done, duration of study, data processing and comparisons. Include a description of how the animals will be allocated to experimental and control groups and the treatment assigned to each group (where applicable).

Also see attached standard operating procedure (TBME007).

**Sample collection**

Abattoirs from the Eastern and Western Cape Provinces will be targeted for the collection of samples (500 and 200 from infected and negative flocks respectively) under the supervision of a state veterinarian or animal health technician. Faecal and tissue samples will be collected from animals originating from flocks with confirmed history of *M. avium subspecies paratuberculosis*, and will be cultured in the automated liquid culture system. The gold standard method for validation will be histopathological examination of a standard set of intestinal and lymph node samples of all sheep sampled. These test will be conducted at the Pathology department of the Faculty of Veterinary Science.

**Processing of samples**

**(a) Faecal samples**

The faecal samples will be collected and stored on ice. For these samples, the VersaTREK liquid culture system will be used for isolation of *M. avium subspecies paratuberculosis.* Pooled faecal samples (from more than 10 animals) will be used. Briefly, a double decontamination method with hexadecylpyridinium (HPC) will be

followed as described by Kim *et al.* 2004. One milliliter of the processed sample will be added to culture bottles that contain 11.5 ml of *para* JEM® broth, 1ml of paraJEM growth supplement, 1ml of paraJEM egg yolk supplement, 500 ml of paraJEM antibiotic supplement and 50 ml of paraJEM blue (TREK diagnostics systems Inc). The bottles will be incubated until a positive signal is detected by the culture instrument or for a maximum of 42 days.

**Identification of *M. avium subsp. paratuberculosis***

(a) Ziehl Neelsen staining and microscopy

About 1ml of sample from culture bottles indicating a positive signal will be centrifuged. Ziehl Neelsen staining will be performed using the bacterial culture pellet according to standard procedures. Slides will be viewed under the microscope for acid fast bacteria.

(b) Polymerase Chain Reaction (PCR) test)

Acid fast isolates will be subjected to Polymerase Chain Reaction using primers targeting the IS*900* sequences of the *M. avium* subspecies *paratuberculosis* (Paolicchi *et al.* 2012).

**Tissue samples**

Fresh tissue samples as well as those in 10% buffered formalin from the ileum, ileo-caecal valve region as well as one caecal and ileal lymph nodes will be collected from slaughter animals from infected flocks as described by Michel and Bastianello (Michel and Bastienello, 2000). Fresh tissue samples will be subjected to double incubation procedure and processed for culture following the procedure used for faecal samples.

**Histopathology**

Tissue samples in 10% buffered formalin will be subjected to histopathological examination according to standard procedure at the Pathology Department, Faculty of Veterinary Sciences, University of Pretoria.

**DNA extraction using the Maxwell-16 automated DNA extraction system**

DNA for PCR amplification will be extracted using the automated Maxwell-16 DNA extraction platform (Anatech instruments (Pty) Ltd) and the boiling method.

**VNTR Typing**

The amplification of specific tandem repeats at previously published specific loci within the *M. avium subspecies paratuberculosis* genome will be applied. The same DNA template used for species identification by IS*900* or the extracted genomic DNA will be used for VNTR typing according to parameters described by Möbius *et al.* 2008. The sizes of the PCR products will be estimated on a 2% agarose gel and results will be confirmed by sequencing of the amplicons. The resulting VNTR profiles will be analyzed using the Bionumerics software (Applied Maths, Belgium). The phylogenetic tree will be constructed to determine the genetic relationships of the isolates.

**Reproducibity testing**

Approximately 20 faecal samples including a mixture of known positive and negative *M. avium subsp. paratuberculosis* samples from a quality assurance scheme (Proficiency testing scheme) will be cultured to determine the VersaTrek liquid culture system's ability to detect *M. avium subsp. paratuberculosis.* In addition, interlaboratory testing (Molecular Diagnostic Service laboratory-KZN) will be conducted using DNA samples for identification of *M. avium* subspecies *paratuberculosis.*

**Determination of sensitivity**

Sensitivity (Se) is defined as the proportion of infected animals out of the total study number of diseased animals which were correctly identified as positive in the diagnostic assay in a known infected population. Five-hundred samples from known infected herds will be used to determine the sensitivity of the test.

Formula:

Sensitivity (Se) = Test positives (a)/known positive (a+c) X 100

**Determination of Specificity**

Specificity is defined as the proportion of non-diseased animals out of the total study number of non-diseased animals which were correctly identified as negative by a diagnostic test in a known uninfected population. Two-hundred samples from known uninfected herds will be used to determine the specificity of the test

Formula:

Specificity (Sp) = Test negatives (d)/ known negatives (b+d) X 100

**Determination of the detection limit of the liquid culture method in pooled and individual faecal samples**

Known confirmed positive for *Mycobacterium avium* subspecies *paratuberculosis* samples will first be classified as either high shedders of MAP, moderate shedders of MAP or low shedders. Detection limit will be determined in all three different patterns of shedding. Classification of shedders will be according to the time it took from the day of culture to the date of positive signal (Laurin et al 2015). In addition, detection limit in individual faecal samples will be conducted by quantifying a number of MAP present in a known confirmed positive sample. A method described by Treuer and Haydel (2012) for counting mycobacterial cells in liquid suspension will be followed Spiking of known confirmed negative samples with quantified known confirmed positive sample will follow and negative samples will be spiked in a decreasing order of number of MAP.

**7. Justify the use of selected species as an appropriate animal model**

Not applicable

| **8. Animal Requirements** | |
|---|---|
| Animal Species: | N/A |
| Strain / Gender / Bodymass / Age | N/A |
| Total Number Required: | N/A |
| Date Required: | N/A |
| Microbial Status: | N/A |
| Source of Animals: | N/A |
| Large farm animals:<br>Will Mr C. van Vuuren be able to supply the animals? | N/A<br><br>Signature: Mr C. van Vuuren DATE |
| Large farm animals:<br>Are there sufficient stables/pens to house the animals? | N/A<br><br>Signature: Mr C. van Vuuren DATE |
| Stable/pen number/name where animals will be housed | |

**9. Reduction of number of animals to a minimum to achieve scientific objective**

Not applicable

Not applicable

**10. Animal Caging and Care**

(Describe the provisions being made for the physical, psychological and behavioural wellbeing of the animals during the study and specify who will care for them on a daily basis).

Not applicable

**11. Restraint of the Animals**

What methods of physical restraint will be used on the animals and by whom?

Not applicable

**12. Invasiveness**

Describe the level of invasiveness of the procedure/s (nil, minor, moderate, major), the manipulation/s to be performed on the animals and the expected degrees of distress and pain which these may cause. State how these will be dealt with to ensure the welfare of the animals.

Not applicable

**13. Refinement**

Describe the specific steps that have been taken to refine the experimental procedures to make them as humane as possible.

Not applicable

**14. End Point for Experiments that Induce Illness in Animals**

Give the endpoints of data collection in experiments or procedures that may cause animals to become ill, lose weight, become distressed and justify these in terms of the needs of the experiment in order to attain its objectives.

Not applicable

**15. Euthanasia**

Will animals be euthanased at the end of the experiment? If yes, describe the procedure. If no, please state the fate of the animals. Will animals be euthanased should suffering become evident? If yes, describe the procedure and include dosages. If no, give reasons why not.

Not applicable

| | |
|---|---|
| YES | |
| NO | |

**Method of disposal of carcass**

Not applicable

**16. Administration of Scheduled Medicinal Substances (Medicines Control Act)**

Describe all substance administration to the animals and give routes of administration, dosages per body mass including anaesthetics, analgesics and agents used for euthanasia and state who is legally responsible for prescribing and administering the controlled Scheduled 3 – 6 Medicinal substances.

Not applicable

**Responsible Veterinarian (Print name) :**

**Qualification :**

**Acceptance Signature:**

**Date:**

**17. Statistical Analysis**

Describe briefly how the statistical analysis of data obtained from the study will be performed and who the statistician will be.

Analysis of data and other statistical combinations will be done using the Pivot tables (Microsoft Office Professional Plus 2010, Excel version 14.0.6129.5000-Pivot) and assistance in this regard will be provided by a statistician at the ARC-Central Office.

**18. Biohazard Statement**

Does the project pose any hazards to other animals and staff from the use of GMO's, infective agents, toxic or carcinogenic agents or ionising radiation. If it does, state safety procedures to be adopted to contain these hazards. Provide a brief approval statement below from the Institutional/Departmental Safety Officer to provide assurance of safety for this project with this person's signature of ratification.

No

**Safety Officer Name (Print)**

**Signature:** **Date:** 28/09/2016

**19. Facilities Requirements**

Are the proposed facilities where the animals will be housed in working condition and suitable for the housing of the animals for the duration of the entire experiment

Not applicable

**Facilities Manager – Name (Print)**

**Signature:** **Date:**

**20. Score sheet**

**Included**

| | |
|---|---|
| YES | |
| NO | x |

21. Has this application been submitted to any other Animal Ethics Committee?

| | |
|---|---|
| YES | |
| NO | x |

**If yes, include the name of Institution/Department responsible and the approval number.**

APPROVED

ARC • LNR

**Onderstepoort Veterinary Institute**

# Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

**TRAIL PERIOD**
**STARTING DATE: 01 October 2016 ENDING DATE: 31 December 2017**

| | | | | |
|---|---|---|---|---|
| **PROJECT NUMBER:** | 101664 | | | |
| **PROJECT TITLE:** | Validation of *Mycobacterium avium* subspecies *paratuberculosis* detection in ovine faecal samples (individual and pooled) under South African conditions using the VersaTrek liquid culture system and species verification by PCR.. | | | |
| **PROJECT LEADER:** | Dr Tiny Hlokwe | | | |
| **DIVISION:** | Zoonotic Diseases Section | | | |
| **CATEGORY:** | A | | | |
| **SPECIES OF ANIMAL:** | N/A | | | |
| **NUMBER OF ANIMALS:** | N/A | | | |

**RECOMMENDATIONS BY ANIMAL ETHICS COMMITTEE**

| Date of AEC meeting for consideration: | Action Taken: | SIGNATURE: AEC-Chairperson Dr P. Mutowembwa |
|---|---|---|
| 21/09/2016. | APPROVED | |

**PLEASE NOTE:** Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment