

MAKERERE



UNIVERSITY

**A CROSS-SECTIONAL STUDY OF PARATUBERCULOSIS IN
CATTLE FROM SELECTED DISTRICTS OF WESTERN UGANDA
AND ANALYSIS OF POTENTIAL VIRULENCE OF SELECTED
STRAINS**

BY

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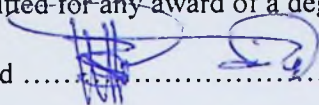
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**A THESIS SUBMITTED TO THE DIRECTORATE OF RESEARCH AND
GRADUATE TRAINING IN FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY
OF MAKERERE UNIVERSITY**

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DECLARATION

I, Judah Ssekitoleko, declare that this thesis hereby submitted for the award of the degree of Doctor of Philosophy of Makerere University is my original work and it has never been submitted for any award of a degree at any university or institution.

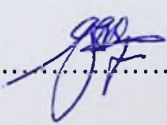
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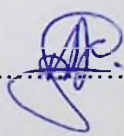
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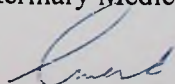
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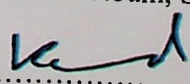
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DEDICATION

To my mother Rebecca Nabasirye and late father Matia Ssajjabbi,

my wife Dorothy and Children

To our Lord and Saviour Jesus Christ, be glory and honour, now and forever. Amen

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LIST OF ACRONYMS

μl	-	microlitre(s)
Av.	-	Average
CTAB	-	Cetyltrimethylammonium bromide
GDP	-	Gross Domestic Product
mg	-	milligram(s)
min	-	minute(s)
ml	-	millilitres(s)
NaCl	-	Sodium Chloride
nm	-	nanometers
PTB	-	Paratuberculosis
RT	-	Room Temperature
s	-	seconds
SDS	-	Sodium Dodecyl Sulphate
Se	-	Sensitivity
SNPs	-	Single nucleotide polymorphisms
Sp	-	Specificity

ABSTRACT

Paratuberculosis (Johne's Disease) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a devastating disease in many animals but most especially dairy cattle with serious economic losses in production. The disease occurs worldwide and is highly suspected to be zoonotic. In dairy cattle, paratuberculosis is characterised by chronic granulomatous enteritis marked by chronic or intermittent diarrhoea, leading to progressive weight loss, unthriftiness and cachexia which culminates into death. Losses associated with paratuberculosis include: reduced milk production, body wasting, increased veterinary costs, and early culling or eventual death of the affected animal. The occurrence of the disease and associated risk factors is not fully known in Uganda, and the virulence of the different local strains has not been documented yet these are important to design strategies for the management of paratuberculosis (PTB). To determine the prevalence of PTB, a survey was carried out in six districts of western Uganda in which 1,814 sera and faecal samples were collected from 93 herds of cattle. Serum was tested with ELISA, whereas, MAP DNA from faeces was detected by a molecular Recombinase Polymerase Amplification assay. A true cow-level sero- and molecular prevalence of 4.9% and 3% respectively and a within-herd sero-prevalence of $3.8 \pm 2.1\%$ were obtained. Herd-level prevalence of 43% and 41% were got based on ELISA and RPA respectively. The high herd-level prevalence is an indication of active PTB transmission among herds. Prolonged dry seasons were significantly associated with MAP occurrence which may be attributed to stress leading to increased susceptibility to infection. These results show the existence of PTB in the surveyed areas and the disease may become endemic in the country if no control measures are instituted. *Mycobacterium avium* subsp. *paratuberculosis* isolation and characterisation were also done to evaluate the potential virulence of the different MAP isolates using an *in vitro* infection model. Significant differences in terms of cytokine and virulence gene expression as well as the viability and quantity of MAP inside RAW

264.7 macrophages at different time points were observed among isolates, an indication of variable virulence. Phylogenetic analysis of the putative virulence genes of MAP exhibited differences when compared with the reference MAP K-10 strain for genes including; *umaA1*, *katG*, *kdpC*, *fabG2_2*, *papA2* and *impA*. These polymorphisms may explain the variable virulence observed in the *in vitro* infection study. The function of these genes and others requires further inquiry to establish their possible role in MAP pathogenesis in an *in vivo* model. This is the first study involving MAP strain virulence analysis in Uganda. The study is useful in addressing the plight of paratuberculosis as an emerging infection, especially in the design of strategies for its management. However, full data on MAP occurrence throughout the country and its possible involvement in human diseases are needed to inform policy on measures for PTB control and prevention.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

The livestock subsector contributed 16% of the agricultural Gross Domestic Product (GDP) and 3.9% of Uganda's GDP (UBOS 2021). The sector's contribution to the economy is still minimal due to the numerous challenges it faces. These constraints have hindered the realization of higher agricultural contribution to the GDP. Among the most important constraints are livestock diseases. Several diseases affect the livestock industry in the country among which include: tick-borne infections such as East Coast Fever, anaplasmosis, heartwater; and numerous viral as well as bacterial infections (Ocaido et al. 2005; Ocaido, Muwazi, and Opuda 2009; Byaruhanga et al., 2017). Presently, there are also several diseases reported as emerging or re-emerging infections affecting animals and almost 70% of these are zoonotic (Billinis 2013). One of such infections is paratuberculosis (PTB) which is an emerging and also neglected disease in Africa (Okuni et al. 2020). The disease is known to have devastating effects on the dairy industry which poses a threat to the income and food security for many people globally (Banda et al. 2021; Derbe et al. 2022). Previous studies have demonstrated occurrence of PTB in many European countries, in USA and other developed countries (Nielsen and Toft 2009; Whittington et al. 2019). In Africa on the other hand and Uganda in particular, not much is known especially on occurrence of the disease and the knowledge on virulence of the different strains (Okuni 2013). Currently, there are no control or prevention strategies for PTB in Uganda and if the situation remains the same, the disease may become endemic in the country.

Paratuberculosis (Johne's disease) is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in ruminants. It begins as a localized infection that may become systemic and often results into chronic granulomatous enteritis leading to chronic or intermittent diarrhoea (intractable in some species), weight loss and eventual death of the affected animal (Chacon, Bermudez, and Barletta 2004; Whittington et al. 2019). The organisms have also been isolated in humans with Crohn's disease and ulcerative colitis as well as in several other human diseases making it a potential zoonosis (Hermon-Taylor et al. 2000; Uzoigwe, Khaita, and Gibbs 2007; McNees et al. 2015; Elmagzoub et al. 2022). *Mycobacterium avium* subsp. *paratuberculosis* is a Gram-positive, aerobic, non-motile, non-spore-forming and acid-fast bacterium (Saviola and Bishai 2006). The disease was first described by Johne, H. A., & Frothingham (1895), as an atypical case of bovine tuberculosis

(Marino et al. 2017). It affects mainly large and small ruminants though several other species can be infected (Stevenson et al. 2009). Paratuberculosis is one of the most important single-aetiology diseases of the dairy industry world-wide causing considerable losses in production in terms of reduced milk yield, reduced weight gain, culling/death and increased cost of disease control (Olsen, Sigurgardottir, and Djonne 2002; Correa-Valencia, García-Tamayo, and Fernández-Silva 2018).

Currently, PTB has neither an effective treatment nor a commercial vaccine that can prevent infection. Control strategies are based on herd management practices that reduce the risk of infection of young animals, intra and inter-farm transmission as well as screening tests to identify and remove infected animals (Whittington et al. 2019; OIE 2022). Several knowledge gaps that hamper effective PTB control exist and these include: information on distribution of MAP genotypes which may vary in clinical presentations, comparison of MAP prevalence levels in the different regions and over time in the same region, development of reliable early-disease and on-farm diagnostics, knowledge on MAP transmission, survival and immune evasion mechanisms inside the host (Barkema et al. 2018).

Paratuberculosis has been extensively studied in Europe and in the United States of America where it causes an estimated annual loss of more than 200 million US dollars in the dairy industry (Losinger 2005). The losses are mainly due to reduction in milk production, culling and costs towards PTB control and prevention. In Canada, an annual loss of nearly CDN\$ 49 per cow was estimated (Tiwari et al. 2008). Annual losses of €234 and £27 per cow in infected herds were estimated in France and the United Kingdom, respectively (Dufour, Pouillot, and Durand 2004; Stott et al. 2005). Reports from Africa indicate that PTB is distributed in many countries though information on its prevalence and economic impact is still scarce (Okuni et al. 2020). Significant prevalence levels of PTB have been reported in some African countries such as Egypt where an animal-level prevalence of 13.6% was reported among dairy cattle with diarrhoea (Amin et al. 2015); Sudan where a cow-level seroprevalence of 6.3% was reported in Khartoum State (Elmagzoub et al. 2020); and in Uganda, where a true prevalence of 8.8% ($n = 943$) was obtained in the Central districts of the country (Okuni et al. 2011). Despite these findings, not much attention has been paid to this disease in terms of instituting control and preventive strategies to curb its spread in Uganda. Information on occurrence of the disease and the associated risk factors in other

parts of the country is missing yet this is necessary to plan strategies for control and prevention of the disease.

The present study aimed at determining the prevalence of PTB in selected districts in western Uganda as a step towards establishing full data on its occurrence in the country. Six districts were selected in this region, though the PTB situation is also not known in other regions of the country. Majority of the selected districts lie within the cattle corridor which are majorly livestock producing areas in the country. These districts are a major source of animals sold for both slaughter and breeding purposes to other areas of the country. A lot of milk and other related livestock products also originate from these districts to other parts of the country and beyond. Therefore, the PTB disease situation in these districts is of paramount importance as it gives a reflection of what may be happening in other regions of the country. The cattle farming systems in these regions tend to be similar with the neighbouring districts. Majority practice extensive cattle rearing system sometimes with fenced grazing units (paddocks) with limited intensive management. However, some farmers do practice communal grazing which exposes the animals to numerous diseases.

The survey employed both a molecular and serological diagnostic method for MAP. Serological methods coupled with PCR have been used in earlier studies for PTB surveillance. However, these available MAP diagnostics require complicated laboratory settings (Hansen et al. 2016); there is therefore, need to investigate into alternative fast and efficient diagnostic methods which can be applied for PTB surveillance. An antibody and a DNA test method were employed in the current study to diagnose MAP with the aim of applying a molecular assay that is fast and easy to use in limited-resource laboratory settings.

The occurrence of PTB in different countries has been associated with certain risk factors. According to some of the earlier studies, transmission of PTB was associated with calf feeding and housing system, breeding practices, attendance at calving, use of maternity pens and introduction of new stock from herds whose MAP infection status was unknown (Cashman et al. 2008; Vilar et al. 2015; Tiwari et al. 2009). Most of these factors are associated with contact of calves with infected faeces of the dam (Doré et al. 2012). Knowledge of risk factors is important in understanding the epidemiology of a disease and is important in planning proper disease management. These factors

may vary among regions thus the need to be investigated in the different areas. In the present study, risk factors associated with PTB occurrence have also been investigated.

Mycobacterium avium subsp. *paratuberculosis* is transmitted mainly through ingestion of contaminated pasture, water or milk/colostrum (Sweeney 2011). Environmental contamination with MAP is therefore an important aspect in the transmission of the disease (Smith et al., 2011). Infected animals shed MAP in faeces which finds its way on pasture for grazing and in water used by animals for drinking. Infected faeces may also contaminate milk or colostrum and other farm structures such as pens which act as a source of infection for calves and MAP is known to infect cattle mostly when young (Vilar et al. 2015; OIE 2022). The extent and focal areas of contamination of the environment with MAP in farms diagnosed with PTB are not documented in Uganda. The environmental study aimed to identify and assess the possible sources of MAP infection for animals.

Knowledge of MAP pathogenesis, strain diversity, and virulence mechanisms is scarce yet it is important in disease control and management, including vaccine and drug development (Stevenson 2015; Barkema et al. 2018). This information is also lacking in Uganda though an earlier study indicated existence of high MAP strain genetic diversity in Uganda which could contribute to variation in strain virulence (Okuni et al. 2012). Analysis of virulence of the different MAP strains as well as the possible genetic causes of variations in strain virulence has not been done in the country. The current study also aimed at analysing the virulence of different MAP strains using an *in vitro* RAW 264.7 murine macrophage infection model; as well as the phylogenetic analysis of the putative virulence genes of MAP to evaluate polymorphisms that could be responsible for variations in strain virulence. These approaches are intended to guide strategies for PTB prevention and control.

1.2 Problem statement

Mycobacterium avium subsp. *paratuberculosis* causes a serious and economically important disease in a wide range of animals most especially dairy cattle and is also implicated as a zoonotic pathogen. Although some studies in the central districts in Uganda showed that the disease had low to medium-level prevalence (Okuni et al. 2011; Erume and Mutebi 2012); no surveys have been carried out in other regions of the country yet the disease continues to affect the livestock enterprise. The extent of contamination of the farm environment with MAP and the risk factors associated with PTB infection are also not known. Studies in other countries indicate that there are several strains and genotypes of MAP which may lead to variations in the clinical and pathological presentation of the disease. No differences in strain virulence have been documented in Uganda to date. The variations in strain virulence could be associated with polymorphisms in different genes responsible for virulence within the MAP genome. There is little information concerning the molecular epidemiology and virulence of the different strains of MAP present in the country. This lack of an up-to-date systematic survey of the infection across the country and the scarce knowledge on virulence mechanisms of MAP strains is a hindrance to the control of this devastating infection in the cattle population and may lead to an endemic situation in the country.

1.3 Study aims

To contribute to the knowledge of the burden of *Mycobacterium avium* subspecies *paratuberculosis* in Ugandan cattle and the virulence of the local strains of this organism to guide on strategies for PTB control in the country.

1.4 Study objectives

1. To determine the prevalence and risk factors associated with PTB infection among cattle in Western Uganda.
2. To determine the extent of environmental contamination with MAP around infected farms
3. To determine the virulence of the different MAP strains in monocyte-derived macrophages
4. To determine the phylogenetic relationships of the different putative virulence genes of MAP among the different isolates from Uganda.

1.5 Research questions

1. What proportion of cattle is infected with MAP; and what are the risk factors for PTB infection in cattle in western Uganda?
2. What is the extent of contamination of the environment with MAP around infected farms?
3. How do the different strains of MAP differ in virulence in monocyte-derived macrophages?
4. What polymorphisms occur within the putative virulence genes of MAP that could be responsible for variations in virulence in the different strains?

1.6 Thesis structure and summary of each chapter

This thesis is presented according to the guidelines outlined in the graduate handbook of policies and procedures released by the Directorate of Research and Graduate Training of Makerere University (April 2022). The thesis is written in English (UK), and presented in manuscript form where each paper constitutes a chapter that is published or is to be published.

There are nine chapters in all, with chapter one being the general introduction to the whole study. It gives a brief background of the study, the problem being addressed, the study aims and objectives as well as the questions that the study attempts to address. Chapter two presents the general

literature review on MAP as an organism, its properties, and virulence attributes, and on PTB; the disease it causes in animals, and its zoonotic potential. Chapter three outlines the general methods used throughout the thesis to answer the questions pertaining to the research as well as a brief description of the site where the study was carried out and the methods for data analysis. Manuscripts are presented from chapter four to chapter eight; where chapter four has been published in the BMC Veterinary Research journal which is part of Springer Nature, chapter five will be published as a short communication, chapter six has been published in the Multi-disciplinary Digital Publishing Institute (MDPI) Journal under *microorganisms*, chapters seven has been submitted for publication while chapter eight is being prepared for publication.

Chapter four presents the molecular and serological survey of PTB in six districts selected from western Uganda. The study involved the collection of faecal and blood samples from cattle and testing them with RPA and ELISA respectively. All tests were done in the laboratory with the molecular RPA test modified to a suitcase laboratory setting. All faeces from MAP-positive cattle were cultured to harvest MAP organisms that were used in the *in vitro* macrophage infection experiments. Several risk factors associated with MAP occurrence were also investigated. This chapter highlights the plight of PTB in the country as an emerging cattle disease for which interventions are required to curtail its further spread and mitigate the negative impacts of the disease on the livestock industry.

Chapter five discusses the occurrence of MAP in the environment. Faeces from infected animals contaminate the environment including pastures, water and other farm structures. Cattle mainly get infected with MAP when they feed on contaminated pasture or milk/colostrum, especially as calves. It is therefore of great importance to understand the MAP infection situation in the environment as this is useful in designing strategies to manage the infection.

Chapter six presents a review of the virulence mechanisms of MAP which enable it to survive the macrophage intracellular environment. Some of these mechanisms include; the ability of MAP to interfere with lysosome maturation into a functional phagolysosome, inhibition of apoptosis, formation of granuloma and interference with cytokines production to favour its survival inside the host macrophage. These attributes are believed to be genetically mediated and some of the probable

MAP virulence genes are discussed. The genetic diversity of MAP strains, taxonomic classification and MAP pathogenesis are also discussed.

Chapter seven investigates the behaviour of MAP inside macrophages. Four MAP isolates obtained from this study were used to infect macrophages in an *in vitro* model and they exhibited different survival rates intracellularly. The isolates also showed variable expression profiles for the different virulence genes of MAP and cytokines. All these attributes were employed to elucidate the behaviour of MAP inside macrophages and could aid in understanding MAP strain virulence.

Chapter eight presents the phylogenetic analysis of the probable virulence factors of MAP among different isolates. Selected probable virulence factors of MAP identified in earlier studies were compared with similar gene sequences from the reference and other strains and with sequences from the current study isolates. The analysis was performed to investigate any differences arising as a result of polymorphisms within the MAP genome which could be responsible for the variable virulence among strains.

Chapter nine gives a general discussion of the thesis, highlighting the major research findings and the contributions of the study, future research areas as well as conclusions and recommendations drawn from the study.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy and Properties of MAP

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of paratuberculosis (Johne's disease) in animals. It is a gram-positive, aerobic, non-motile, non-spore-forming and acid-fast bacterium belonging to the genus *Mycobacterium* of the family *Mycobacteriaceae* and is a member of the *Mycobacterium avium* complex (MAC). Other members in the MAC complex include; *M. avium avium*, *M. avium silvaticum*, *M. colombiense*, *M. intracellulare* among others. (Frothingham and Wilson 1993; Saviola and Bishai 2006). *Mycobacterium avium* subsp. *paratuberculosis* is a facultative intracellular bacterium, rod-shaped (0.5 – 1.5 μm) with a thick and waxy cell wall made up of mycolate and peptidoglycan layers held together by arabinogalactan (Lea-Smith et al. 2007). They belong to the slow growing group of mycobacteria with a generation time of over 20 hours and takes between 6 – 16 weeks to produce visible colonies on solid media, though some strains may take up to 6 months. Media for culture of MAP include; Egg-based media like Löwenstein-Jensen medium, Herrold's egg-yolk medium and synthetic media such as Middlebrook 7H9, 7H10 and 7H11 (Elguezabal et al. 2011; Mayahi et al. 2013). *Mycobacterium avium* subsp. *paratuberculosis* is classified biochemically as being dependent on externally provided mycobactin; an iron chelating substance required in MAP metabolism, though some strains have been found to be mycobactin-independent (Cocito et al. 1994; Motiwala et al. 2004).

2.2 MAP survival in the environment

Mycobacterium avium subsp. *paratuberculosis* is a member of the non-tuberculous mycobacteria which are widely distributed in the environment, in healthy animals and humans and do not usually cause disease unless the host is debilitated or immune-compromised (Falkinham 2009). *Mycobacterium avium* subsp. *paratuberculosis* has also been found in other organisms such as the free-living amoeba (Samba-Louaka et al. 2018). This attribute has been exploited to investigate MAP behaviour in the intracellular environment as it mimics the natural host (Phillips et al. 2020). *Mycobacterium avium* subsp. *paratuberculosis* can survive in the soil or manure for more than a year under a dry shaded area but this period is reduced if the area is open (Whittington et al. 2004). The organism survives for almost similar time periods in water collection areas such as water dams (Whittington, Marsh, and Reddacliff 2005). It is more tolerant to temperature, chlorine treatment, pH variations and exposure to ultraviolet light than other pathogenic bacteria (Whittington et al.

2004). This makes it suitable for long-term survival in the environment which acts as a source of infection for MAP susceptible hosts. Viable MAP organisms have been recovered in pasteurized milk and this is a possible source of infection to humans (Grant, Ball, and Rowe 2002; Ellingson et al. 2005; Gerrard et al. 2018).

2.3 MAP strain diversity

Principally, three broad MAP strain groups are recognised depending on their growth characteristics, colony pigmentation and host associations; “Sheep type” or “Type S”, the “Cattle type” or “Type C” and the “Bison” or “B-type” (Stevenson 2015). *Mycobacterium avium* subsp. *paratuberculosis* colonies exist as pigmented and non-pigmented forms which is important in strain differentiation. Presently, MAP characterization depends mainly on whole genome sequence (WGS) analysis of genetic markers such as insertion elements, repetitive sequences and single nucleotide polymorphisms (SNP). Basing on these methods, MAP has been classified as Type I, II and III (Fawzy et al. 2018). Type I are slow growers (take more than 16 weeks to produce visible colonies on solid media) and are strongly associated with sheep; Type II grow more readily taking 6 to 16 weeks and are associated with cattle. It has been suggested that Type III is an intermediate between the two major types (Type S and Type C). Based on SNP analysis of the IS1311, MAP from bison have been classified as Type B strains from USA. With further molecular analysis, the USA bison-type were found to differ from the India bison and this gave rise to the Indian bison type (Sevilla et al. 2005). Type B are a subtype of the cattle type but are not restricted to the bison. Type III and Type I appear to be subgroups of Sheep type “S”(Bryant et al. 2016).

2.4 Host range

The natural hosts of MAP are mainly farmed and wild ruminants such as cattle, goats, sheep, deer, bison though other species such as alpacas, camels and rabbits have been found infected as well (Ayele, Machackova, and Pavlik 2001; Stevenson et al. 2009). Non-ruminant wildlife such as fox, weasel, rat, wood mouse, stoat, hare and badger have also been found to harbor the infection or may act as reservoirs (Beard et al. 2001; Stevenson et al. 2009). The disease has also been reported in non-human primates as well as in a dog (Roller et al. 2020; Miller et al. 2017).

2.5 MAP Pathogenesis

2.5.1 *Transmission*

In cattle, the fecal-oral mode of transmission is the most common and are infected mostly as young calves (Cousins et al. 2002; Sweeney 2011). Infected animals shed MAP in manure, colostrum and milk and young animals get MAP from milk and the contaminated environment (Manning and Collins 2001; OIE 2022). Once ingested, MAP enters the intestinal wall through the ileal mucosa via the microfold (M) cells residing in the Peyer's patches (Arsenault et al. 2014). It has however been demonstrated that MAP can cross the gut mucosa even in areas without Peyer's patches entering through enterocytes (Bermudez et al. 2010). Once inside the intestinal wall, they are phagocytosed by sub-epithelial macrophages. Infected macrophages migrate to local lymphatics and spread to regional lymph nodes where the organisms stimulate inflammatory and immunological responses (Coussens 2001; Tessema et al. 2001).

2.5.2 *Immune response*

Infected macrophages are known to produce a myriad of cytokines which play different roles in regulation of immune and inflammatory responses. Cytokines are also produced by other cells such as T-helper lymphocytes and when stimulated, result in formation of two T-helper cell sub-populations, T helper 1 (Th1) and T helper 2 (Th2) (Coussens 2004). In MAP infection, the Th1 cells produce pro-inflammatory cytokines such as interferon gamma (IFN γ), interleukin 2 (IL-2), IL-6, IL-12 and tumour necrosis factor alpha (TNF α) while Th2 cells produce anti-inflammatory cytokines such as; transforming growth factor β (TGF β), IL-4, IL-5, IL-10 and IL-13 (Coussens et al. 2004). These cytokines are believed to orchestrate cell-mediated and humoral immune responses that aim to contain the intracellular infection but end up augmenting the disease process. The early immune response to MAP infection consists of numerous infected macrophages with increased amounts of adhesion molecules which result in formation of granulomas in which the bacilli remain secluded for a long period of time, often years (Coussens 2004). The animal in this state presents no clinical symptoms and it can remain in this subclinical phase for a period of 2 - 5 years while the bacilli are contained in macrophages and microscopic granulomas (Stevenson 2005; Tanaka et al. 2005). Infected animals may not manifest clinical disease throughout their lifetime yet they

continue to contaminate the environment through shedding of MAP (Lombard 2011; Corbett et al. 2018). What determines the length of this duration both at host and pathogen levels is still unknown. On failure of macrophages to destroy MAP, the organisms begin to multiply intracellularly with production of immunoglobulin G1 (IgG1) antibodies characteristic of the late humoral response in MAP infection (Coussens 2004). Several studies have laboured to investigate the mechanisms of MAP survival in macrophages with limited explanation. In one of the earlier studies, it was observed that formation of persistently infected macrophages was associated with up-regulation of IL-10, transforming growth factor beta (TGF β) and reduction in IFN- γ (Khalifeh and Stabel 2004). MAP like other mycobacteria can disable maturation of the phagosome into a functional phagolysosome responsible for destruction of invading pathogens (Weiss et al. 2004). It has been demonstrated that MAP interferes with CD40 signaling responsible for the activation of macrophages by the T cells (Sommer et al. 2009). Failure of macrophage activation leads to a persistently infected macrophages which cannot destroy the pathogen. They instead act as tools for propagation and dispersal of the organism. (Simutis, Jones, and Hostetter 2007). Activated macrophages on the other hand are capable of killing MAP and control its growth (Bannantine and Stabel 2002; Marino et al. 2017).

2.5.3 MAP virulence

Virulence is the measure of the ability of a pathogen to infect, multiply and establish itself in the host cell. Virulence factors facilitate colonisation, adhesion and invasion of host cells by pathogenic bacteria. Mycobacterium however lacks the classical virulence factors such as toxins; it uses other virulence determinants which enable it to survive in the host intracellular environment (Forrellad et al. 2013). The ability of MAP to disable the activation mechanism of macrophages is one such virulence attribute. It is also suspected that there are differences in host-pathogen interactions attributed to the different strains (Stevenson 2015). O'Brien and co-workers showed that there were differences in MAP strain virulence in deer though the origin of the variations were not established (O'Brien et al. 2006). Several putative virulence factors that have been identified in MAP include; *impA* - probable inositol monophosphatase, *umaA1* - possible mycolic acid synthase, *gcpE* - isoprenoid biosynthesis, *kdpC* - probable potassium-transporting ATPase, conserved membrane transport proteins (*mmpl*) and the conserved polyketide synthases (*pks1*) among others (Shin et al.

2006). Most of these factors also occur in *Mycobacterium tuberculosis* (MTB) (Forrellad et al. 2013); hence they are envisaged to play similar roles in MAP. More virulence factors identified in MTB could as well be present in MAP or may have evolved into other virulence forms.

2.5.4 MAP survival in monocyte-derived macrophages

The different MAP survival attributes inside macrophages have been previously investigated using monocyte-derived macrophages (MDM) or RAW 264.7 murine macrophages in several *in vitro* studies (Gollnick et al. 2007; MacHugh et al. 2012; Lamont et al. 2014; Marino et al. 2017; Johansen et al. 2019). In these studies, different strains of MAP were used in the infection of MDM and attributes such as survival/multiplication of MAP cells, apoptosis, granuloma formation and cytokine production inside macrophages were monitored.

Enumeration of MAP to determine its survival or multiplication in MDM has been carried out targeting the F57 gene (254 bp) which is a single copy gene in the MAP genome using quantitative real-time PCR (Tasara and Stephan 2005). The commonly used target for MAP, the IS900 has several gene copies (14 - 20) and it may occur in other mycobacteria other than MAP; hence it would not give a good estimate of MAP cell quantities (Tasara, Hoelzle, and Stephan 2005). Another approach for quantifying MAP is by determining colony forming units (c.f.u/ml) to estimate the cell numbers in the culture media (Shin et al. 2007; Lamont et al. 2014), though it takes much longer time due to the slow growth rate of MAP.

Previous studies have shown that MAP inhibits apoptosis of MDM through upregulation of the Bcl2 gene which is known to have an inhibitory effect on apoptosis (Abendaño et al. 2014). The level of Bcl2 gene expressed by infected cells implies the extent of apoptosis of MDM and is measured by real-time qPCR of the Bcl2 gene expressed in mRNA extracted from infected MDM.

Macrophages infected with MAP produce pro- and anti-inflammatory cytokines responsible for cellular and humoral responses to counteract the diseases processes (Coussens et al. 2004; Stabel 2006; Cirone et al. 2020). A measure of cytokines elicited by the different MAP strains is used to infer virulence. Cytokine production by MAP-infected MDM is determined by means of quantitative real-time PCR (Abendaño, Juste, and Alonso-Hearn 2013).

2.5.5 Phylogenetic relationships among different putative virulence genes of MAP

Several putative MAP virulence genes have been identified previously some of which include; the conserved membrane transport proteins (*mmpI*), the conserved polyketide synthases (*pksI*), probable inositol monophosphatase (*impA*), possible mycolic acid synthase (*umaA1*), isoprenoid biosynthesis (*gcpE*) and probable potassium-transporting ATPase (Shin et al. 2006). To do a phylogenetic analysis, the genes of the study strains are compared with the sequences of reference strains from the Genbank to search for similarities or differences due to mutations or other forms of polymorphisms. Phylogenetic trees are drawn to further compare the virulence factors in the study strains to those of reference strains drawn from the GenBank. Different gene sequence analysis programs such as SnapGene Viewer, CLC Sequence Viewer, ClustalX/ClustalW, Basic Local Alignment Search Tool (BLAST), MEGA 11, and Geneious Prime, among others can be used in the analysis.

2.5.6 Symptoms of PTB

Clinical presentation of PTB in cattle includes chronic or intermittent watery diarrhoea, reduced milk production and chronic weight loss in spite of a good appetite (Clarke 1997). Infected cattle undergo different stages of disease presentation according to clinical signs, faecal shedding and immunological response. Firstly, during early infection, the animal shows no observable clinical sign. This is followed by low shedding of bacteria in faeces which can be detected, intermittent diarrhoea with loose to watery stool and detectable circulating antibodies. Finally, the disease progresses into profuse watery projectile diarrhoea, anaemia, submandibular oedema and emaciation which culminates into death (Clarke 1997; Whittington et al. 2019).

2.5.7 Pathology of paratuberculosis

Macroscopically, the lesions are primarily seen in the intestines and draining mesenteric lymph nodes. Intestinal lesions are either diffuse or segmental and are commonly found in the ileum though can be found in the whole length of the intestines (Whitlock and Buergelt 1996). The intestinal wall is thickened and oedematous and the mucosa has transverse folds or corrugations. The serosal and mesenteric lymph vessels are dilated and thickened. The mesenteric lymph nodes

are pale, swollen and oedematous. Grossly, in severe disease, the animal may become cachectic, alopecic and anaemic with oedematous and serous diffusions in the submandibular and different body cavities (González et al. 2005). Histopathological lesions are usually characterised by multifocal and/or diffuse granulomatous inflammations of the intestines and mesenteric lymph nodes (Clarke 1997). In sheep, the lesions can be categorized as multibacillary, lepromatous or paubacillary, tuberculoid depending on extent of cellular infiltrations and size of granulomata formation (Clarke and Little 1996).

2.6 Diagnosis for PTB

2.6.1 Clinical diagnosis

Paratuberculosis is clinically diagnosed from the typical symptoms of chronic diarrhoea and emaciation in spite of a good appetite (Clarke 1997). Other diseases, however, may also present with similar signs. Diagnosis of PTB in animals which have not yet shown clinical signs presents a challenge (Manning and Collins 2001). A definitive diagnosis therefore involves identification of the causative agent; through culture, DNA or antigen detection and/or detection of the immune response due to the disease.

2.6.2 Serological diagnosis – Detection of antibodies

Serological tests aim at detecting antibodies due to PTB infection. The production of antibodies in MAP infection does not have a significant protective value against PTB but are useful in detecting animals with fully developed lesions (Dieguez et al. 2009; Collins et al. 2005). Antibody detection in serum is based on three techniques; Enzyme-linked immunosorbent assay (ELISA), Complement fixation test (CFT) and Agar gel immunodiffusion test (AGID). ELISA is a fast and low-cost diagnostic tool and has been found to be more sensitive and specific compared to CFT and AGID test (Kalis et al. 2002; Kostoulas et al. 2006; Gumber, Eamens, and Whittington 2006). Since antibody levels in PTB rise late in the course of the infection, the sensitivity of ELISA is highest in animals of 2 years and above. Currently, with modification of the ELISA method to target certain biomarkers, the ability of the test to detect animals in latent PTB infection have improved (Li et al. 2017; Blanco Vazquez et al. 2020).

2.6.3 Microscopic examination

Using smear microscopy, mycobacterial bacilli can be demonstrated by their acid-fast nature when faecal or tissue sample is stained with Ziehl-Neelsen (ZN) staining technique (Dreier et al. 2006). This would be a cheap and fast way to demonstrate MAP though it has the disadvantage that other mycobacteria may also stain hence lowering the specificity coupled with a low sensitivity (Weber et al. 2009).

2.6.4 Culture

Culture and isolation of MAP from faecal and milk samples is the gold standard for the diagnosis of PTB to determine herd-level prevalence (Eamens et al. 2007). Culture can also be performed on tissues from intestines and mesenteric lymph nodes of affected cattle. Recent advances have made culture of MAP from blood samples possible (Bower, Begg, and Whittington 2011). Culture of MAP is traditionally done on Herrold's Egg Yolk medium with addition of mycobactin. MAP is known to depend on an external source of mycobactin for growth. Some studies have, however, revealed some strains that are mycobactin independent (Motiwala et al. 2004). This finding may be an important tool in strain differentiation. Other media used to culture MAP include; Middlebrook (7H9, 7H10, 7H11) and Lowenstein – Jensen media (Dimareli-Malli et al. 2013).

2.6.5 Molecular detection of MAP DNA

Mycobacterium avium subsp. *paratuberculosis* is commonly identified by the specific DNA insertion sequence 900 (IS900) using PCR methods. The gene is 1451 bp in length and there are several copies in the MAP genome (14 – 20) which increases sensitivity. The other target is the F57 gene which is a single copy gene in the MAP genome and offers a useful tool for MAP enumeration (Millar et al. 1996; Li et al. 2005; Tasara and Stephan 2005). PCR can be performed on faecal samples, blood, lymph node biopsies, intestinal tissues and rectal swabs (Alonso-Hearn et al. 2009). PCR can also be done on formalin-fixed and paraffin-embedded tissues hence making retrospective studies possible. This is also important in confirming PTB in cases where fresh tissue for MAP testing is not available (Whittington et al. 1999; Tripathi and Stevenson 2012). IS900 is very important in distinguishing between MAP and other members of MAC strains though some

members of the MAC have been found with some IS900-like elements (Motiwala et al. 2004). There are several other genome specific markers being validated such as the *hsp65* with potential use in MAP diagnostics (Motiwala et al. 2003). Modifications to the conventional PCR such as use of semi-nested, nested PCR, immunomagnetic PCR and real-time PCR coupled with sequencing have improved the sensitivity of PCR reactions (Djønne et al. 2003; Buergelt et al. 2006; Park, Allen, and Davis 2014).

2.6.6 Recombinase Polymerase Amplification for MAP DNA detection

This is an isothermal method in which the IS900 MAP specific gene sequence is amplified at constant temperature. This method has the advantage that when combined with MAP DNA speed extraction protocol, it can be performed at the point of need/care since it does not require very complex equipment and all reagents are stable at ambient temperature. The sensitivity and specificity of the RPA assay is 89.5% and 100% respectively (Hansen et al. 2016). In another study, the MAP serum or milk ELISA had sensitivities of 49% and 48% and specificities of 94% and 91% respectively during the late lactation stage (Angelidou, Kostoulas, and Leontides 2016).

2.7 References

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CHAPTER THREE

GENERAL METHODS

3.1 Introduction

In Uganda, the occurrence of PTB is not fully known in many parts of the country. Previous surveys have covered selected districts within central Uganda and these surveys have indicated the existence of the disease (Okuni et al. 2011; Erume and Mutebi 2012). In order to recommend a comprehensive control program, there is need to have full knowledge of the occurrence of PTB throughout the country. To partially achieve this purpose, this study has conducted PTB surveys in selected districts in the western part of the country, analysed risk factors associated with occurrence of the disease and the virulence of the different local MAP strains.

3.2 Study Area and Design

To investigate MAP prevalence in western Uganda, a cross-sectional study was done which covered six districts namely: Mbarara, Ntungamo, Ntoroko, Kiruhura, Bushenyi and Kabale (Figure 1). The study involved collection of fecal and blood samples from cattle as well as information regarding management practices and farm vegetation to assess risk factors associated with PTB occurrence. Currently, western Uganda comprises of 26 districts which lie within the southern and western highlands agro-ecological zones. Most of the districts covered by this study lie within the western cattle corridor which are major cattle producing areas of the country. These areas mostly experience a bimodal rainfall pattern and the vegetation is mainly savannah grassland (UBOS 2013). Livestock management system is mainly extensive grazing sometimes mixed with limited intensive management. The cattle population in Uganda stands at over 14 million and approximately 22% of these are kept in the western region. Majority of farmers keep mixed cattle breeds and these include the Friesians and their crosses as well the Ankole long-horned and other breeds.

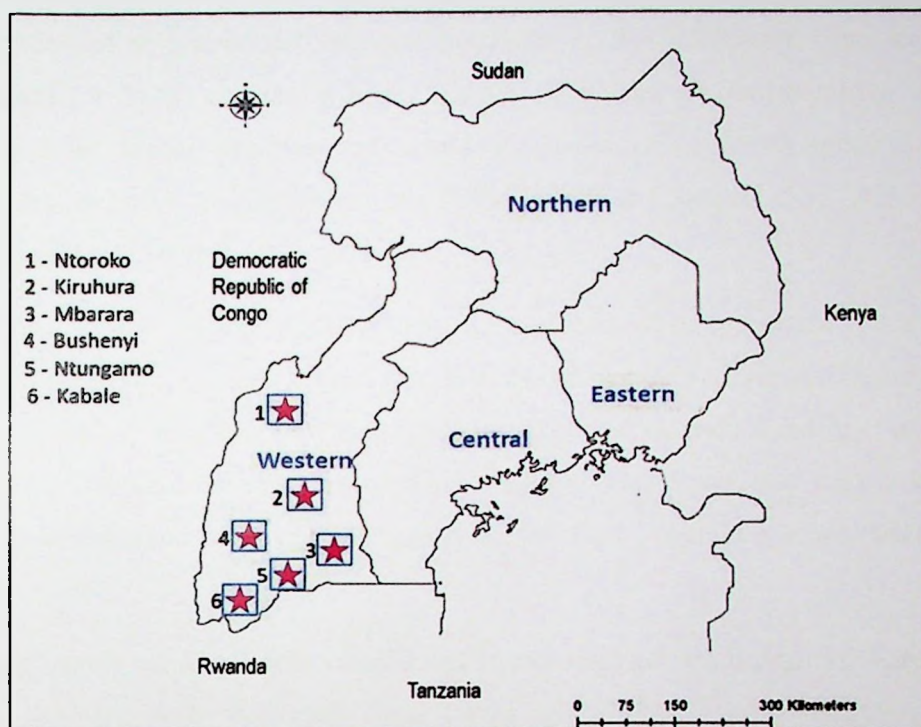


Figure 1: Map of Uganda showing districts where MAP survey was carried out; sampled districts are indicated by red stars. Source of map: Google

3.2.1 Sample size and Data collection

Samples were collected from cattle using a multistage sampling technique and the sample size was determined using the WINPEPI® programme (Version 3.18) assuming an expected MAP prevalence of 8% (Okuni et al., 2011), a design effect of two and an acceptable difference of 4% at 95% confidence level (Abramson 2011). The number of animals sampled from each district was calculated depending on the cattle population in a given district and the average size of each cluster/herd. Thus, the total number of samples taken from each district, n , was the sum of all the samples taken from each of the clusters. The first stage of selection was the district, the subcounty, the village and finally the herd. Within each herd, all cattle of two years and above were sampled. Thus, the total number (N) of samples in the study was calculated as follows:

$$N = n_1 + n_2 + n_3 + n_4 + n_5 + n_6, \text{ representing all the six districts.}$$

Samples collected from cattle including blood and faeces, were properly transported in a mobile fridge and ice-cooled boxes respectively and processed in the laboratory. Sera was harvested from blood and tested for MAP antibodies using ELISA (IDEXX® Paratuberculosis Screening). DNA was extracted from faecal samples and tested for presence of MAP specific genes using the Recombinase Polymerase Amplification test (RPA) method (Hansen et al. 2016) as described in details in chapter four (Section 4.2).

To determine the effects of various risk factors, questionnaires (Appendix I) were used to gather information from cattle owners about the history of Johne's disease related symptoms, calf management practices, breeding services, grazing practices, manure handling in addition to farm vegetation cover, rainfall patterns, as well as seasonal variations that may contribute to MAP introduction or persistence and eventual spread on the farm. Details are presented in chapter four, section 4.2.1.

Environmental survey of MAP was carried out in the districts of Bushenyi, Kiruhura and Kabale (Figure 1). Samples were collected from farms using boot swabs according to a procedure described by Eisenberg et al. (2013). A multi-stage cluster sampling technique was employed and sample size estimated using the WINPEPI® programme (Version 3.18) which takes into account the population of cattle in the district and the average herd/cluster size. One of the technical staff involved in the data collection wore a pair of boot swabs over clean rubber boots and walked through areas where cattle moved frequently such as the milking parlor, the resting area and the water drinking area. The boot swab was removed by inversion and together with the water samples, were transported to the laboratory in a cool box, processed and tested for MAP DNA using RPA molecular assay as is described in details in chapter five (Section 5.2).

To analyse virulence of the different MAP isolates, an *in vitro* RAW 264.7 macrophages infection model was performed. Faecal samples for all animals that tested positive with either ELISA or RPA from the prevalence study (Chapter 4.0) were cultured to isolate MAP organisms. Four pure MAP isolates were used in the *in vitro* macrophage infection to study the virulence attributes of MAP. The RAW 264.7 macrophages were grown into a monolayer and used in the infection experiment according to Abendaño, Juste, and Alonso-Hearn (2013). MAP survival inside macrophage, the expression of different inflammatory cytokines and virulence genes were determined by the specific

real-time qPCR at different time points (3 h, 24 h and 48 h). The number of MAP cells inside macrophages was determined by performing a real-time qPCR of the F57 gene of MAP which is a single copy gene and a reliable method to enumerate MAP cells. MAP enumeration was done on a standard curve analysis of the F57 gene. The viability of the MAP cells that had been phagocytosed was determined by use of AlamarBlue reagent (Resazurin) which has a property of getting reduced to highly fluorescent resorufin when it interacts with viable cells as used earlier (Carroll et al. 2009). The degree of reduction of AlamarBlue was measured using a spectrophotometer and percentage reduction calculated. The degree of reduction of AlamarBlue is the measure of viability. Selected pro- and anti-inflammatory cytokines expression profiles were also determined in MAP-infected macrophages as described in details in chapter seven (Section 7.2). The expression of the different probable virulence genes and cytokines gives an inference on the behaviour and effect of MAP on the host macrophages.

Phylogenetic analysis of the MAP isolates was performed to investigate any differences arising as a result of polymorphisms within the putative MAP virulence genes. The four MAP isolates were genotyped using whole genome sequence analysis (PacBio®, California, USA), putative virulence genes of the field study isolates were identified and comparison done with the virulence genes of MAP from Genbank to observe possible differences that may exist between the field strains and the MAP reference strain and other mycobacteria. This was done in an attempt to decipher polymorphisms that could be responsible for variations in MAP strains virulence. Putative virulence genes from the field isolates were aligned with similar genes from the reference MAP K-10 strain and other mycobacteria and analysed by means of phylogenetic trees to infer relationships among them as described in details in chapter eight; section 8.2.

3.2.2 Data analysis

Data collected from MAP survey and testing using ELISA and RPA was entered into spreadsheets (MS Excel), cleaned, coded and analysed. MAP prevalence data was analysed using MS Excel functions (mean and standard deviations). Risk factor data was also entered into MS Excel and analysed using STATA 15.1 statistical program (Texas USA) employing ordinary logistic regression model basing on the estimated intra-cluster correlation coefficient by Fleiss-Cuzick, discussed in details in chapter four. Data from environmental survey was entered into MS Excel for

subsequent analysis. Real-time PCR data from the virulence study was analysed by the Design and Analysis (version 2.6.0) program. Cytokine and virulence gene data was calculated as relative gene expression ($\Delta\Delta C_t$) using an appropriate formula (Taylor et al. 2007) and entered into spreadsheets for analysis. MAP DNA enumeration was calculated by means of a standard curve for the MAP F57 gene and also entered into MS Excel for subsequent analysis. MAP viability was calculated as percentage of AlamarBlue reduction using an appropriate formula (Carroll et al. 2009). All data was entered into spreadsheets and plotted as graphs for subsequent discussions. Details of data management and analysis are presented in the respective manuscripts from chapters four to eight.

3.3 Ethical consideration

Ethical approval for the studies was obtained from the Uganda National Council for Science and Technology (UNCST) under permit no. A35ES. Informed consent was obtained from cattle owners or their representatives for voluntary participation in the study and to provide the required information (Appendices III).

All the recommended animal handling practices were observed according to the World Organisation for Animal Health (OIE) regulations for use of animals in research and education (OIE 2012).

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CHAPTER FOUR

MOLECULAR AND SEROLOGICAL SURVEY OF PARATUBERCULOSIS IN CATTLE IN SELECTED DISTRICTS OF WESTERN UGANDA

Ssekitoleko, J., Ojok, L., Omala, S. K., Mukhtar, M. E., Eltom, K. H., Eltayeb, E. S., ... & Okuni, J. B. (2022). Molecular and serological survey of paratuberculosis in cattle in selected districts of Western Uganda. *BMC Veterinary Research*, 18(1), 438. <https://doi.org/10.1186/s12917-022-03535-7>

4.0 Abstract

Knowledge of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) herd infection status is important to plan appropriate control and prevention strategies for Paratuberculosis (PTB); however, in Uganda MAP infection status of most herds is unknown. This study aimed at determining the MAP infection status of cattle herds and the risk factors associated with occurrence of PTB in six western districts of Uganda. The survey covered a total of 93 herds where faecal and blood samples were collected from 1814 cattle. A Recombinase Polymerase Amplification (RPA) and an antibody-based (ELISA) assays were used to test for the presence of MAP DNA in faeces and MAP antibodies in serum, respectively. The apparent cow-level prevalence of MAP infection was 3.2% and 2.7% using ELISA and RPA respectively and the true cow-level prevalence using ELISA and RPA was 4.9% and 3% at 95% confidence level respectively. A herd-level prevalence of 43% (ELISA) and 40.8% (RPA) and a within-herd prevalence of $3.8 \pm 2.1\%$ based on ELISA were obtained. Among the risk factors investigated, long dry spells were significantly associated with high MAP infection ($p < 0.05$). These results indicate that MAP is actively present in most areas where the study was carried out. This poses a serious threat to the livestock industry and potentially to public health as MAP is highly suspected to play a role in the pathogenesis of several diseases in humans such as Crohn's disease, ulcerative colitis among others. Other areas of the country need to be surveyed as well in order to establish full data on MAP infection status to enable interventions for the control and prevention of the disease.

Key words: Paratuberculosis, Prevalence, Western Uganda, Recombinase Polymerase Amplification

4.1 Background

Paratuberculosis (PTB) or Johne's Disease (JD) is a disease of significant economic impact on livestock farming worldwide and affects farmed and wild ruminants such as cattle, sheep, goats, buffaloes, bison, alpacas, and deer (Stevenson et al. 2009). The disease has also been reported in other animals like stoats, badgers, camelids, and non-human primates (Ayele, Machackova, and Pavlik 2001; Roller et al. 2020); as well as in a dog (Miller et al. 2017). The causative agent,

Mycobacterium avium subsp. *paratuberculosis* (MAP), has been isolated severally from humans with Crohn's disease (CD) and ulcerative colitis as well as from many other diseases making it a potential zoonosis (Uzoigwe, Khaita, and Gibbs 2007; McNees et al. 2015; Elmagzoub et al. 2022).

The economic cost of PTB is due to deaths, reduced productivity, costs of treatments, reduced growth rate, and lack of market for replacement heifers; especially in countries where John's disease-free state is required (Hasonova and Pavlik 2006; Garcia and Shalloo 2015; Kirkeby et al. 2016). Dairy production is one of the sectors grossly affected by PTB (Losinger 2005; Tiwari et al. 2008).

Control of PTB is very difficult because there are no effective treatments, no vaccine that prevents the infection, lack of diagnostic methods that offer early detection; and measures to reduce infections are very expensive. Most diagnostic methods in use detect the infections only in animals with advanced clinical disease, when the animal is already spreading the mycobacteria and when no treatments can be effective (Woodbury, Chirino-Trejo, and Mihajlovic 2008; Mohana and Praveen Kumar P 2015; Hahn et al. 2017) and therefore, are of limited value in control and prevention. Nonetheless, diagnostic tests, play a key role in establishing an effective control program. Knowledge of the prevalence and risk factors of the disease in any locality is important in planning control measures that combine different approaches. The occurrence of PTB has been associated with certain risk factors though these may vary from one geographical region to another, hence the need for surveys in different regions. According to Doré and others (Doré et al. 2012), contact with faeces from the infected dam was the most important risk factor for MAP transmission to calves; other factors such as calf feeding and housing were all related to contamination with dam faeces. In a study from Ireland, the odds of a positive MAP test were increased with reduced attendance at time of calving (Cashman et al. 2008). A survey of possible risk factors for MAP in Canadian herds revealed that having more than one cow in the maternity pen, group housing of calves, recent purchase of heifers and herds with comorbidities, such as bovine viral diarrhoea virus were significantly associated with MAP occurrence (Tiwari et al. 2009). In another study from Brazil, (Vilar et al. 2015), failure to use maternity pens was the most significant risk factor associated to MAP transmission.

While knowledge about the prevalence and epidemiology of PTB and its causative agent are well documented in developed countries, in Africa, there are still large knowledge gaps around PTB (Okuni et al. 2020). In Uganda, the prevalence of PTB has been reported in selected districts of the central region (Okuni et al. 2011; Erume and Mutebi 2012). These studies have shown that MAP infection is established in the districts studied but hitherto, there has been no data from other parts of the country, especially in the major livestock producing regions, such as western Uganda. These areas are the major suppliers of Ugandan's milk and beef products. Additionally, little is known about the epidemiological factors that are associated with MAP infections and transmission in those districts.

The aforementioned studies relied on serology only. Use of both molecular and serological tests in the current study aimed at improving the ability of the diagnostic method to detect MAP in either faeces or blood of the sampled cattle. This paper describes the prevalence of PTB in selected districts of western Uganda and the risk factors associated with MAP infection. A combination of serologic and molecular methods was used for more accurate estimates of PTB prevalence.

4.2 Materials and methods

4.2.1 Study area and sample collection

To estimate the prevalence of MAP infection in cattle in western Uganda, a cross-sectional study covering six districts namely: Ntungamo, Kabale, Ntoroko, Mbarara, Kiruhura and Bushenyi (Figure 1) was carried out between October, 2019 and March 2021. These districts lie within the south and western highland agro-ecological zones. They experience a bimodal rainfall pattern and the vegetation mostly includes tropical woodland, bushland as well as savannah grassland (UBOS 2013). Farmers in this region mainly practice extensive grazing of cattle with fences to demarcate farming units and majorly graze on natural pastures with limited intensive production.

A multistage cluster sampling technique was employed and the sample size was estimated using the WINPEPI programme (Version 3.18) assuming an expected prevalence level of 8% (Okuni et al. 2011), design effect of two and an acceptable difference of 4% (Abramson 2011) at 95% level of confidence. The number of animals sampled from each district was calculated depending on the population size and the average size of each cluster/herd. The total number of samples taken from

each district, n , was the sum of all the samples taken from each of the herds. Within each cluster, all cattle of two years and above were sampled. Thus, the total number (N) of samples in the study was calculated as follows:

$$N = n_1 + n_2 + n_3 + n_4 + n_5 + n_6, \text{ representing all the six districts.}$$

To investigate the effects of various risk factors, questionnaires were used to collect information from the cattle owners or their representatives about history of Johne's disease-related symptoms, breeding practices, calf feeding practices, grazing practices and manure handling. Environmental factors were also investigated such as tree shade canopy on pastures, length of the dry season and water logging; that may contribute to MAP introduction, favour environmental persistence and spread in animals (See Appendix 1).

Blood and faecal samples were collected from all cattle after proper restraint following the recommended handling procedures. Approximately 4 ml of blood were collected from the jugular or coccygeal vein using a sterile vacutainer needle and a plain vacutainer tube (BD vacutainer®, Franklin Lakes, NJ, USA), labelled and stored in a mobile fridge (Dometic, Model CDF 26, Hong Kong, China) at 2 – 8 °C. Faeces was collected from the same animal directly from the rectum using clean arm-length or powder-free latex gloves, put in a 50 ml sample collection bottle (Sarstedt, Nümbrecht, Germany), labelled and stored in ice-cooled boxes for transportation to the laboratory. At the laboratory, serum was harvested into 1.5 ml cryovials, and stored at -20 °C for subsequent testing with ELISA. Faeces were also stored at -20 °C for subsequent MAP DNA extraction and detection using Recombinase Polymerase Amplification (RPA).

4.2.2 Detection of MAP antibodies

To test for the presence of MAP antibodies in sera, an Enzyme-Linked Immunosorbent Assay (ELISA) was performed using a commercial test kit (IDEXX® Paratuberculosis Screening, IDEXX Laboratories, Inc., Westbrook, USA), according to the manufacturer's instructions. Briefly, the serum and all test reagents were allowed to thaw at room temperature (RT) for about 30 min. The samples and controls sera (two positive and one negative) were diluted at 1:20 using dilution solution in the 96-well uncoated plates in the presence of *Mycobacterium phlei* and mixed by

agitating on a shaker and incubated for 15 min at room temperature (RT). One hundred microlitres of the pre-incubated samples and controls were dispensed into MAP antigen-coated wells in a 96-well microtiter plate, mixed gently by rocking for 5 seconds then covered with aluminium foil and incubated at RT for 45 min. The plate was then emptied by pouring off the contents in a fling and tapped on a blotting paper to remove excess fluid. The plate was washed thrice with approximately 300 µl of diluted wash buffer (1:20) for each well and blotted again. One hundred microliters of diluted conjugate (1:100) were added into each well, homogenised, covered and incubated at RT for 30 min. A second washing step followed and 100 µl of Tetramethyl Benzidine (TMB) solution were added to each well. The plate was covered with foil and incubated in the dark for 10 min. To stop the reaction, 100 µl of stop solution were added to each well. The optical density (OD) was read using a spectrophotometer reader (SPECTROstar Nano, S/N 601-1258 - BMG LABTECH, Ortenberg, Germany) at a spectrum absorbance of 450 nm. The OD of the samples and controls were entered into Microsoft Excel spreadsheets for analysis. The sample to positive ratios (S/P) were calculated from the mean OD of the positive control ($\bar{x}$ PC) and the OD of the negative control (x NC) as;

$$S/P = (OD \text{ of sample} - xNC) / (\bar{x}PC - xNC) * 100.$$

The manufacturer set the S/P cut-off point for a sample to be considered positive at $\geq 55\%$.

4.2.3 Detection of MAP DNA from faeces

4.2.3.1 DNA extraction

Mycobacterium avium subsp. *paratuberculosis* DNA was extracted from faecal samples using the SwiftX kit (Xpedite, Munich, Germany) in a mobile suitcase laboratory setting according to Hansen and colleagues (Hansen et al. 2019). Briefly, 100 mg of faeces were added to 500 µl of lysis buffer in a Precelly's SK 38 (Bertin Corp., Rockville, MD, USA) tube. and vortexed for 1 min. A negative extraction control was included for each set of samples extracted. Sixty microlitres of magnetic beads were added to the sample and mixed well for 10 s by vortexing or inverting. The tubes were then incubated in a heat block at 95 °C for 15 min; removed from the heat block every 2 min and vortexed. The tubes were removed from the heat block, either shaken down or tapped on a bench to remove condensate from the lid, transferred to a magnetic stand with loosened caps, and incubated

at RT for 2 min. Then the sample tubes were opened carefully and 10 µl of clear solution were transferred to a 1.5 ml tube containing 40 µl of water (molecular biology grade) to obtain a dilution of 1:5. The diluted sample containing the extracted DNA was directly used in the detection step.

4.2.3.2 MAP DNA detection using Recombinase Polymerase Amplification

To test for presence of MAP in faeces, the RPA test protocol was performed as previously described by Hansen et al. (2016). In brief, the dried oligos mix (primer and probe) and positive control were reconstituted by addition of 520 µl and 300 µl of molecular biology grade water respectively; the total reaction volume was 50 µl and the protocol was performed as follows: to RPA exo kit, 29.5 µl of rehydration buffer, 13 µl of oligos mix and 2.5 µl of magnesium acetate (280mM) were dispensed into the lid of each of the tubes, then 5 µl of the extracted DNA were added to the mix in the lid before closing the tubes. The negative control from the extraction and positive control comprising the molecular standard were included in each test run. The tubes were then centrifuged, vortexed and again centrifuged in this order and placed into a T8 – ISO tube scanner (Axxin, Fairfield, Australia). The T8 machine was pre-set to start automatically once the lid was closed. The test could be monitored on the screen as it progresses in a graphical form. After 4 min, the lid opened automatically; the strip was removed shortly, vortexed, centrifuged and placed back into the machine in the same order. The machine detects fluorescence resulting from the degrading probe. The detection process is completed after 15 min and the machine indicates ‘test completion’. Test results were viewed directly on the screen and downloaded into MS spreadsheets for subsequent analysis.

4.2.4 Data analysis

ELISA and RPA results were analysed using Microsoft spreadsheets (MS Excel). The true prevalence for the ELISA and RPA tests was calculated using EPITOOLS programme (Sergeant 2018; Reiczigel, Foldi, and Ozsvári 2010) at 95% confidence level taking into account the sensitivities and specificities of the two tests and the respective confidence intervals (CI). The ELISA kit (IDEXX Paratuberculosis screening, IDEXX Laboratories, Inc., Westbrook, USA), had an estimated sensitivity (Se) of 51.4% and specificity (Sp) of 99.3% (McCormick et al. 2010); whereas the Se and Sp of the RPA test were taken as 89.5% and 100% respectively as reported by

Hansen and co-workers (Hansen et al. 2016). Data for risk factor analysis was also entered into MS Excel and statistically analysed for association with occurrence of MAP on the farm using the Stata 15.1 statistical programme (Texas, USA).

4.3 Results

A total of 1814 heads of cattle comprising 93 herds from six districts were tested for MAP infection using ELISA and RPA. The cattle population comprised mixed cattle breeds common in Uganda and these included; Friesians and their crosses which were the majority in the survey, Ankole long-horned and others that were not classified to a particular breed (Table 1).

Table 1: Breeds of cattle sampled and tested for PTB in the six districts of western Uganda

Breed	Number of cattle tested	Proportion of cattle tested (%)	Number positive with ELISA	Number positive with RPA
Friesians	838	46	28	23
Friesian crosses	534	29.4	18	14
Ankole long-horned	213	12.6	6	8
Others	229	12	6	4
Total	1814		58	49

Of the 1814 heads of cattle, 58 were ELISA positive giving an apparent cow-level prevalence of 3.2% and 40 of the 93 herds had at least one MAP-positive animal resulting in a herd-level prevalence of 43% (Table 2). The average district seroprevalence was 3.36($\pm$ 1.25)%, being lowest in Kiruhura with 2.0% and highest in Ntoroko with 5.0% and a with-in herd prevalence of 3.8($\pm$ 2.12)%.

Table 2: Seroprevalence of paratuberculosis in six districts of western Uganda based on ELISA test results

District	Number of cattle tested (n)	Number positive	District apparent prevalence (%)	District true prevalence (%)	No. of herds tested	Positive herds	Herd Preva- lence (%)	Within- herd Prevalence (%)
Mbarara	242	7	2.9	4.3	13	7	53.8	2.1
Ntoroko	180	9	5.0	8.5	9	7	77.8	7.3
Ntungamo	335	16	4.8	8	14	8	57.1	3.4
Kiruhura	402	8	2.0	2.5	15	5	33.3	1.6
Kabale	315	10	3.2	4.9	18	8	44.4	5.2
Bushenyi	341	8	2.3	3.3	24	5	20.8	3.2
Total	1814	58	Av. 3.36	Av. 5.3	93	40	42.8	3.8

On the other hand, an apparent cow-level prevalence of 2.7% and a herd-level prevalence of 40.86% for herds which had at least one cow testing positive were obtained using RPA (Table 3). Similar to ELISA results, the prevalence based on MAP DNA detection was lowest in Kiruhura at 1.24% and highest in Ntoroko at 5.6% (Figure 2), with a district level prevalence of $3(\pm 1.5)\%$. A true cow-level prevalence of 4.9% (95% CI: 3.5 - 6.7) and 3% (95% CI: 2.29 - 3.97) were obtained based on ELISA and RPA test methods, respectively. The two tests were very close despite being based on different analytes. In three districts, ELISA showed higher prevalence estimates, while in two, RPA showed higher estimates but in one, the two were almost at parity (Figure 2). Comparing positivity estimates based on the ELISA and RPA tests, a total of 107 samples were positive on either test. However, of these, only six tested positive for both tests, implying that there was an overall increment in positivity for both tests.

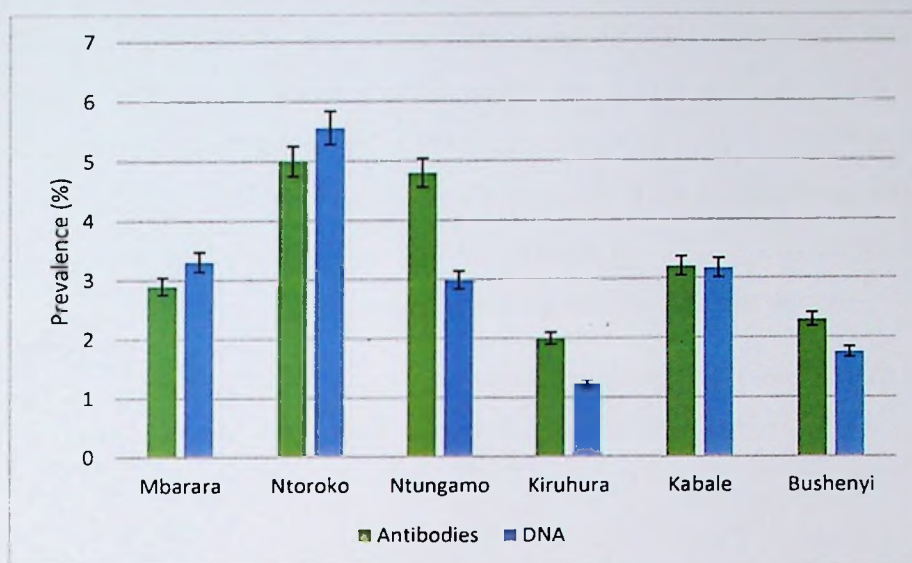


Figure 2: Comparison of the prevalence estimates of MAP infection in the six districts using ELISA and RPA tests

Legend: The estimates by the two tests were almost at parity especially for samples from Kabale though ELISA resulted in higher estimates in three of the six districts

Table 3: Prevalence of MAP DNA in faeces of cattle in six districts of western Uganda

District	Number of cattle tested (n)	Number positive	District apparent prevalence (%)	District true prevalence (%)	No. of herds tested	Positive herds	Herd Prevalence (%)
Mbarara	242	8	3.31	3.7	13	7	53.85
Ntoroko	180	10	5.56	6.2	9	6	66.67
Ntungamo	335	10	2.99	3.3	14	6	42.86
Kiruhura	402	5	1.24	1.4	15	4	26.67
Kabale	315	10	3.17	3.6	18	9	50
Bushenyi	341	6	1.76	2	24	6	25
Total (N)	1814	49	Av. 3.0	Av. 3.4	93	38	40.86

For risk factor analysis, the intra-cluster (herd) correlation coefficient was used as a measure of contribution of herd variations to the overall variations in MAP infection. The estimated intra-cluster correlation coefficient by Fleiss-Cuzick kappa was 0.013 (95% CI: 0 - 0.125) meaning that the contribution of herd-based variations in MAP infection was only 1.3% of the total variations in the infection. This implied that ordinary logistic regression model other than mixed-effects model would suffice to predict the associations. Indeed, variance of random effects for the herd intercept was zero based on exploratory mixed-effects logistic model (results not shown).

The analysis with the binary logistic regression model revealed only the length of the dry season exhibited significant association with MAP occurrence ($p < 0.05$) (Table 4).

Table 4: Binary logistic regression estimation of risk factors associated with prevalence of PTB in six districts of Uganda

Dependent variable: MAP infection status					
Covariates	Odds ratio	Std. Err.	Z	P -value	95% CI
Breeding service					
Bull used only on farm	1.000				
Bull is shared	1.577	0.703	1.02	0.306	0.659, 3.778
Artificial Insemination	2.797	2.232	1.29	0.198	0.659, 3.778
Artificial Insemination & Bull	1.383	0.622	0.72	0.471	0.573, 3.41
Manure handling					
Taken to plantation	1.000				
Used in plantation or sold	0.496	0.245	-1.420	0.156	0.188, 1.307
Left on the farm/pasture	1.027	0.269	0.100	0.918	0.615, 1.716
New stock introduced					
No	1.000				
Yes	1.172	0.307	0.600	0.546	0.700, 1.960
Length of dry season					
No or short dry spell	1.000				
Long dry spell	1.604	0.373	2.030	0.042	1.017, 2.531

Tree shade density

Heavy canopy	1.000				
Intermediate	1.492	1.134	0.530	0.599	0.336, 6.619
Sparse	1.893	1.422	0.850	0.395	0.434, 8.252

Water logging

No	1.000				
Yes	1.027	0.268	0.100	0.918	0.616, 1.714

Legend: The predicted probability of getting infected with MAP is 0.06 during the season of a long dry spell compared to 0.04 for a short or no dry spell. Other factors such as the breeding method, manure handling, or introduction of new stock did not exhibit significant association with PTB infection.

4.4 Discussion

This study aimed at determining the PTB infection status in selected districts in western Uganda and the associated risk factors. The obtained results indicate that MAP is actively present in this region. These results do not differ significantly from those obtained in earlier studies in other parts of the country (Okuni et al. 2011; Erume and Mutebi 2012). This situation is also not different from other African countries such as Egypt which reported 12.6% (n=500) MAP ELISA positive cattle (Abdellrazeq et al. 2014), and Sudan where a recently report has shown an apparent seroprevalence of 6.3% (n=175) in cattle in Khartoum State (Elmagzoub et al. 2020). Countries without a national control program such as India have reported high individual animal seroprevalence rates of MAP ranging between 21.4% - 29.8% among the cattle population (Singh et al. 2008; Singh et al. 2010). The trend in India indicates an increase in MAP prevalence in domestic livestock from 11.4 to 44.2% from 1985 to March 2017 (Chaubey et al. 2017). A similar trend can be envisaged in most African countries since no control strategies have been put in place to curb the spread of MAP infection. However, the scenario is different in countries, such as Denmark and Japan, that earlier had high prevalence but instituted control measures that brought the prevalence down (Verdugo, Toft, and Nielsen 2015; Yamamoto et al. 2018; Momotani 2012). Although the individual animal PTB prevalence is still low in Ugandan cattle, there is a cause to worry as the prevalence at the herd level is very high. Based on ELISA, the herd-level prevalence was close to 45%, whereas, with the

combined ELISA and RPA results, herd-level prevalence was 64%; this is an indication that there is high transmission between herds and the disease spread remains uncontrolled, as there are presently no control or prevention measures in place which may exacerbate the situation. Currently, PTB is not among the diseases routinely examined at inspection points before the issuance of animal movement permits. This implies that the spread by animal movement could be one of the most important avenues for the spread of the disease among herds.

There were variations in the level of MAP detection between the serologic and molecular diagnostic tests. In some districts, the nucleic acid detection method identified more MAP-positive animals compared to the antibody method. However, overall, the ELISA had more positive results compared to the RPA test. These differences could be attributed to some animals that are shedding MAP in faeces but have not mounted a detectable immunological response due to the delay of the humoral immune response in PTB infection and others may have variations in shedding patterns or are intermittent shedders (Olsen, Sigurgardottir, and Djonne 2002; Mitchell et al. 2015). From previous studies, the nucleic acid-based method detected more MAP-positive animals compared to the antibody method (Clark et al. 2008; Elsohaby et al. 2021) whereas, in another study, more positive animals were detected with serology as compared to the molecular method (Wells et al. 2006). Generally, using a combination of both molecular and serologic test methods to diagnose for PTB improves ability to detect positive animals; and this was true for the current study.

The RPA diagnostic technique is fast and simple as it involves few pipetting steps. The test is minimally affected by the presence of inhibitors hence the extracted DNA can be used directly in the detection step with no further purification. All RPA reagents are stable at ambient temperature and the test involves few equipment requirements which can be adopted in a mobile laboratory setting at the point-of-care or in the field. However, during MAP DNA extraction, heating and bead beating is essential to ensure optimal yield of DNA from the faecal sample. It is also important to minimize debris during the extraction to avoid excess background. The extracted DNA need to be used immediately in the detection step to avoid possible degradation during storage. This is the first time the RPA test has been applied in a PTB study in Uganda since its validation (Hansen et al. 2016) and the test has been effectively applied to screen animals for MAP in this study. The portability of the suitcase lab as well as the speed of molecular test (40 min including the extraction

protocol) have enabled the screening of large numbers of samples in a very short time and eliminate the storage problem.

Among the risk factors investigated, farms that experienced long dry spells were at higher odds of getting MAP infection compared to those that had shorter or no dry spells. The effect of prolonged drought could be attributed to nutritional stress as it is accompanied by lack of adequate feed and water, in addition to excessive heat. These could reduce the ability of animals to mount effective defence mechanisms against diseases, making them susceptible to MAP infection. Additionally, during dry seasons, animals from different herds and of different age groups congregate at water drinking points; this could facilitate disease transmission including PTB, which is mainly transmitted through the faecal-oral mode (Sweeney 2011). There were no significant differences in MAP infection among the different breeds of cattle in this study. On the other hand, factors such as manure handling, calf feeding, and housing methods were found to be associated with MAP infection in other studies. However, we were unable to establish such association because the majority of the farms visited used extensive grazing practices, sometimes on rangeland, which minimizes the contact of animals with the infective faecal material from other animals as is the case in intensive management systems.

4.5 Conclusions and Recommendations

This study has shown that PTB exists in all six districts of western Uganda, suggesting that the disease may be widespread across the region. The high herd-level prevalence and within-herd prevalence in some farms also indicate that MAP has been spreading between and within herds for many years. However, there is need to establish data on the prevalence of the disease in all parts of the country as this is necessary to lay strategies for MAP control and prevention. If no control measures are taken soon, PTB will continue to spread unchecked and is likely to become endemic in the country. Since MAP is suspected to be involved in the pathogenesis of Crohn's disease and associated with many others in humans, there is need to carry out a human study to determine the level of human exposure to MAP and the occurrence of CD and Ulcerative Colitis in Uganda. The RPA as a diagnostic technique, can be adopted for wider use to improve disease detection and early reporting.

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CHAPTER FIVE

SURVEY OF THE FARM ENVIRONMENT FOR CONTAMINATION WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN THREE DISTRICTS OF WESTERN UGANDA

(Manuscript)

5.0 Abstract

Cattle are majorly infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) the cause of paratuberculosis (PTB), when they ingest feed that is contaminated with the bacterium. The contamination is a result of infected cattle shedding MAP onto farm structures, pastures, and water sources. Knowledge of the level of contamination of the environment with MAP is therefore important in the management of PTB. This information as well as the distribution of the organism in the different locations on the farm, is however not known in Uganda. This study aimed at identifying MAP in presumably contaminated farm locations such as the milking areas, resting areas, water drinking points and in water used for drinking. Environmental samples, including manure (boot swabs) and water, were collected from three districts of western Uganda. The farms were selected taking into consideration the cattle population in the district and the average herd size. A total of 55 manure and water samples were collected and tested using a molecular recombinase polymerase amplification (RPA) assay which detects MAP DNA. None of the 55 manure and water samples tested positive for MAP. However, some of the farms sampled (36%) had MAP-positive cattle according to an earlier survey that tested animals using both ELISA and RPA tests. This implies that some of the farms were contaminated with MAP as a result of shedding from the infected cattle. These farms could not be detected for contamination possibly due to the low MAP prevalence within herds as well as the extensive management system employed in majority of farms whereby the MAP quantities within the environmental samples were lower than the detection limit of the test method used. For future studies, it is recommended to perform multiple sampling of the farm over time to achieve a representative sample, to perform culture and determine the colony forming unit per gram (c.f.u/g) of the manure sample. Additional areas from the farm such as manure collection areas and water sediment areas need to be sampled as well.

Key words: Environment, *Mycobacterium avium* subsp. *paratuberculosis*, contamination, paratuberculosis

5.1 Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes a devastating disease especially in ruminants though it affects many other animal species and is implicated in the pathogenesis of different diseases in humans (Cousins et al. 2002; McNees et al. 2015). It is a member of the *Mycobacterium avium* complex which is part of the non-tuberculous mycobacteria. These are widely distributed in the environment, in animals and humans (Falkinham 2009). Other organisms such as free-living amoeba have been found to harbour MAP (Samba-Louaka et al. 2018). *Mycobacterium avium* subsp. *paratuberculosis* can survive in soil or manure for more than a year under a dry shaded area but this period is reduced if the area is open (Whittington et al. 2004). The bacterium survives for almost similar time periods in water collection areas such as water dams (Whittington, Marsh, and Reddacliff 2005). *Mycobacterium avium* subsp. *paratuberculosis* is resistant to disinfectants, this makes it suitable for long-term survival in the environment. The latter then acts as an infection source from where MAP can find its way to a susceptible host (Whittington et al. 2012; Donat et al. 2015).

Infected animals shed MAP in faeces which contaminates the pasture and the water used for drinking; *Mycobacterium avium* subsp. *paratuberculosis* transmission is largely known to occur through ingestion of contaminated pasture, milk, colostrum or water (Sweeney 2011). Cattle normally get infected when young, though most animals do not manifest clinical disease until two or more years of age yet they continue to shed MAP in the environment thus spreading the infection (Lombard 2011). The extent of contamination of pasture, water, and other farm structures such as calf and maternity pens is not known in Uganda. The pattern of distribution of MAP contamination on the farm environment is also not known. This study aimed at determining the extent of contamination of the farm environment with MAP due to shedding from infected cattle. This information is important in designing strategies for prevention and control of paratuberculosis since the environment is known to be an important source of MAP infection to animals and possibly to humans.

5.2 Materials and Methods

5.2.1 Study area

This study was carried out in three districts in western Uganda: Kabale, Bushenyi and Kiruhura (Figure 3). These districts lie within the southern highlands and southern drylands agroecological zones. Cattle are reared on relatively extensive areas which are demarcated into paddocks or grazing units. Environmental samples were collected from the farms selected within the three districts. A multi-stage cluster sampling technique was employed using the WINPEPI® programme (Version 3.18) which takes into account the population of cattle in the district and the average herd/cluster size. MAP prevalence was estimated at 8% (Okuni et al. 2011) with an acceptable difference of 4% and a design effect of 2 (Abramson 2011) at a 95% confidence level. The total number of farms sampled (N), was the sum of the farms selected from each district (n); Hence, $N = n_1 + n_2 + n_3$.

5.2.2 Sample collection and processing

Environmental (manure) and water samples were collected from 55 farms according to a procedure used earlier by Eisenberg et al., (2013). One staff wore boot swabs (polyethylene bag) over a clean pair of rubber boots and walked through the milking area, the resting/sleeping area, and the water drinking point in a transect taking approximately 15 steps through each of these areas. These sites were targeted because they are high traffic areas for cattle where manure accumulates. The bag was then removed from the feet by inversion and properly closed taking care not to lose any manure stuck on the bag. In addition, a manure sample was directly picked from the ground and processed together with the boot swab sample. A water sample was likewise collected from the cattle drinking point in a 50 ml tube (Sarstedt®, Numbrecht, Germany). Both the water and manure samples were properly labelled, put in a cool box and transported to the laboratory for subsequent processing and testing.



Figure 3: Map of Uganda showing districts where environmental samples were collected; indicated by red stars. Source of map: Google

5.2.3 DNA extraction

The manure sample was retrieved from the boot swab by addition of 5 ml of distilled water and concentrated by centrifugation at 3,000g for 5 min. The supernatant was discarded and the sample was directly used in the DNA extraction step.

DNA extraction from water or manure sample was done using the SwiftX kit (Xpedite, Munich, Germany) in a mobile suitcase laboratory setting as previously described (Hansen et al. 2016). Briefly, approximately 0.5 ml of homogenised sample was added to 500 µl of lysis buffer in a Precellys SK 38 (Bertin Corp., Rockville, MD, USA) tube and vortexed for one min. A negative extraction control was included. Sixty microlitres of magnetic beads were added and mixed well for 10 seconds by vortexing or inversion. This was followed by incubation of the tubes in a heat block at 95 °C for 15 min, removing the tubes from the heat block every 2 min and vortexed. After

incubation, the tubes were removed from the heat block and either shaken down or tapped on a bench to remove condensate from the lid and transferred to a magnetic stand with loosened caps and incubated at RT for 2 min. The sample tubes were then opened carefully and 10 μ l of clear solution were transferred to a 1.5 ml tube containing 40 μ l of molecular biology grade water to obtain a dilution of 1:5. The diluted sample containing the extracted DNA was directly used in the detection step.

5.2.4 Recombinase Polymerase Amplification assay for detection of MAP DNA

DNA sample from water or manure was tested for presence of MAP using the RPA assay as earlier described by Hansen et al. (2016). Briefly, the dried oligos mix (primer and probe) and positive control were reconstituted by addition of 520 μ l and 300 μ l of water (molecular biology grade) respectively. To the RPA exo kit (TwistAmp® United Kingdom), 29.5 μ l of rehydration buffer, 13 μ l of oligos mix and 2.5 μ l of magnesium acetate (280mM) were added into the lid of each of the tubes followed by 5 μ l of the extracted DNA and the tubes were then closed. Negative and positive controls were included in each run. The tubes were centrifuged, vortexed and again centrifuged in this order and placed into a T8 – ISO tube scanner (Axxin, Fairfield, Australia). The T8 machine was pre-set to start automatically once the lid is closed. The test could be monitored on a screen as it progresses in a graphical form. The lid was set to open automatically after 4 min, the strip was removed shortly, vortexed, centrifuged and put back in the T8 machine in the same order. The machine detects fluorescence resulting from the degrading probe as amplification progresses. The detection process was completed after 15 min, and the machine indicates “test completion”. Test results were viewed directly on the screen and downloaded in MS excel for subsequent analysis.

5.3 Results

A total of 55 manure and water samples collected from 55 farms and tested for MAP DNA using RPA assay are shown in table 5: None of the samples (water and boot swab) tested positive for MAP in all 55 farms tested.

Table 5: Environmental samples from selected farms tested for MAP DNA using RPA in three districts of western Uganda

District	MAP DNA (Boot Swab)	MAP DNA (Water)
Kabale	17	17
Kiruhura	15	15
Bushenyi	23	23
Total	55	55

5.4 Discussion

The aim of this study was to assess the extent of environmental contamination with MAP in selected farms as a result of shedding from infected cattle. The result showed that none of the 55 tested samples was positive for MAP DNA. However, some of the herds (36%), from which samples were picked had animals testing positive for MAP with either ELISA or RPA tests in an earlier survey (Appendix II). In the same study, an average within-herd prevalence of 3.8% was obtained based on ELISA (see chapter four). The positive animals identified in some of the herds were assumed to contaminate the environment with MAP. However, some of the following circumstances could have led to the RPA negative results. Most of the farms surveyed were managed on an extensive system and some were on range management. This implies that there was limited manure accumulation and hence contact of animals with infective material was also limited. Earlier studies have indicated that it is sometimes difficult to detect MAP in environmental samples in low prevalence herds (Smith et al. 2011). In one of the studies, it was shown that MAP quantities of >30 c.f.u/g in the sample material were found to directly correlate with positivity of the environmental sample (Smith et al. 2011). On the other hand, MAP quantities that fell below 10 c.f.u/g could not be detected by the culture method (Raizman et al. 2004). The detection limit for the molecular assay (RPA) used in the current study was earlier determined as 16 MAP DNA molecules (Hansen et al. 2016). This implies that the samples and the sampling strategy employed did not provide sufficient MAP quantities that could be detected by the test method employed. Another reason for failure to detect MAP is that there is variation in shedding and load of MAP by infected animals (Mitchell et al. 2015). Some animals are intermittent shedders leading to low or no

MAP organisms being shed. Given the extensive grazing system of most farms surveyed, it is probable that the MAP quantities within the samples obtained were below the detection limit of the molecular diagnostic test. In future studies, it will be necessary to take multiple samples from the farm at different time intervals. Other areas of the farm should be included for sampling such as; water run-off from the farm, manure heaps or slurry. Culture is reported to be superior to molecular detection, therefore it is recommended that similar studies in future do culture all samples and determine the colony forming units (c.f.u/g) of the manure sample to establish the detection limit of the molecular assay and the level of contamination in the environment.

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CHAPTER SIX

***MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* VIRULENCE: A REVIEW**

Ssekitoleko, J., Ojok, L., Abd El Wahed, A., Erume, J., Amanzada, A., Eltayeb, E., ... & Okuni, J. B. (2021). *Mycobacterium avium* subsp. *paratuberculosis* Virulence: A Review. *Microorganisms*, 9(12), 2623. <https://doi.org/10.3390/microorganisms9122623>

6.0 Abstract

To propose a solution for control of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections in animals as well as in humans, and develop effective prevention, diagnostic and treatment strategies, it is essential to understand the molecular mechanisms of MAP pathogenesis. In the present review, we discuss the mechanisms utilised by MAP to overcome the host defense system to achieve the virulence status. Putative MAP virulence genes are mentioned and their probable roles in view of other mycobacteria are discussed. This review provides information on MAP strain diversity, putative MAP virulence factors and highlights the knowledge gaps regarding MAP virulence mechanisms that may be important in the control and prevention of paratuberculosis.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*; Johne's disease; virulence; pathogenesis

6.1 Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a weakly Gram-positive acid-fast bacterium which causes Johne's disease or paratuberculosis in animals especially ruminants (Cousins et al. 2002; Olsen, Sigurgardottir, and Djonne 2002). It is also implicated in the cause of Crohn's disease (inflammatory bowel disease) in humans (Hermon-Taylor et al. 2000). Paratuberculosis is one of the most serious infectious diseases of dairy cattle worldwide, causing considerable losses in production in terms of reduced milk yield, reduced weight gain, culling/deaths and increased cost of disease control (Correa-Valencia, García-Tamayo, and Fernández-Silva 2018).

Among the attributes for a potentially pathogenic bacterium are its ability to gain access to a susceptible host, establish itself through evasion of the host defense mechanisms and multiply in the host, leading to disease or tissue damage and eventual dissemination to other hosts (Webb and Kahler 2008). Genetic factors that enable the organism to achieve these pathogenic attributes are considered to be virulence factors. A lot still remains to be understood regarding the pathogenesis of MAP and its virulence in its numerous hosts. There are reports of variations in virulence among

the various strains as well as differences in host susceptibilities (Verna et al. 2007). This review is an attempt to elucidate the basis and mechanisms of differential virulence of MAP strains and isolates as reported to date.

6.2 Taxonomic Classification of MAP

Mycobacterium avium subsp. *paratuberculosis* is a Gram-positive bacillus, aerobic, non-motile, non-spore-forming and acid-fast bacterium belonging to the genus *Mycobacterium* of the family Mycobacteriaceae and is a member of the *Mycobacterium avium* complex (MAC) (Saviola and Bishai 2006). *Mycobacterium avium* complex are widely distributed in the environment, in healthy animals and humans and do not usually cause disease unless the host is debilitated or immunocompromised. Other members in the MAC complex include: *M. avium avium*, *M. avium silvaticum*, *M. colombiense*, *M. intracellulare* among others. They belong to the slow-growing group of mycobacteria, taking between 8–16 weeks to produce visible colonies on solid media like Egg-based media such as Löwenstein–Jensen medium, Herrold's egg-yolk medium and in synthetic media such as Middlebrook 7H9, 7H10 and 7H11, though some strains may take up to 6 months (Olsen, Sigurgardottir, and Djonne 2002; Verna et al. 2007). *Mycobacterium avium* subsp. *paratuberculosis* is classified biochemically as being dependent on externally provided mycobactin, though some strains have been found to be mycobactin independent (Motiwala et al. 2004).

6.3 MAP Strain Diversity

Principally, three broad MAP strain groups are recognised depending on their growth characteristics, colony pigmentation and host associations: Sheep type or “Type S”, the Cattle type or “Type C” and the Bison or “B-type” (Stevenson 2015). Some of the molecular techniques used in MAP strain typing for identification of genetic diversity include: IS900 restriction fragment length polymorphism (RFLP), IS1311 RFLP (Conde et al. 2021), IS1311 polymerase chain reaction–restriction enzyme analysis (Hodgeman et al. 2021), pulsed-field gel electrophoresis (Dimareli-Malli et al. 2009); short sequence repeat (Sevilla et al. 2008), mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis (Bryant et al. 2016) and single-nucleotide polymorphism (SNP) typing (Leao et al. 2016). These typing techniques have divided MAP into Type I, II and III (Figure 4). Type I is predominantly isolated from sheep (Type S) and

Type II is for isolates from cattle and other animals as well as humans. Type III is a subgroup of Type S. Based on SNP analysis of the IS1311, MAP first isolated from bison from Montana, USA, was classified as Type B (Whittington, Marsh, and Whitlock 2001). With further molecular analysis, the bison type was found to differ from a similar type from India giving rise to the Indian bison type (Sevilla et al. 2005). Type B strains are a subtype of Type C but are not restricted to the bison. Type III and Type I appear to be subgroups of Sheep type (Bryant et al. 2016). Recent studies recommend that MAP characterisation should be based on genome sequence analysis of genetic markers such as insertion elements, repetitive sequences and single nucleotide polymorphisms (Bryant et al. 2016; Lim et al. 2021) for better resolution, although this would be very expensive for small epidemiological studies.

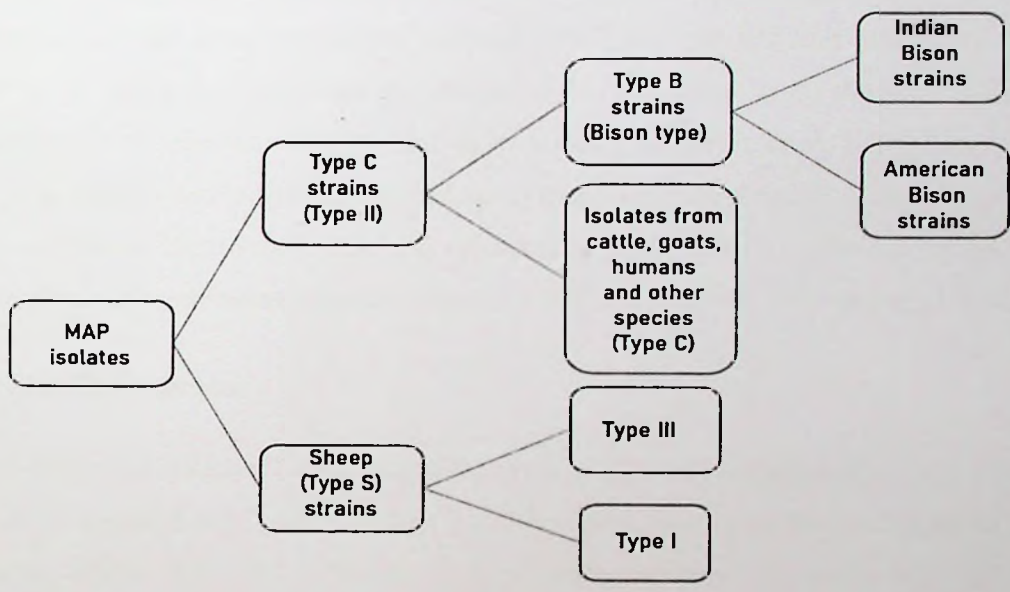


Figure 4: An illustration of MAP strain differentiation based on whole genome SNP-based phylogenetic analysis (Bryant et al. 2016).

6.4 MAP Pathogenesis

6.4.1 Transmission

MAP is transmitted in cattle mainly through the fecal–oral mode and infection occurs mostly in young calves (Cousins et al. 2002; Sweeney 2011). Risk factors for MAP transmission include: importation or introduction of new animals whose MAP infection status is unknown, pooled milk feeding of calves and lack of or failure to use maternity pens on the farm (Cashman et al. 2008; Vilar et al. 2015). Infected animals shed MAP in manure, colostrum and milk and young animals become infected through ingestion of colostrum and milk from the infected animals, contaminated water and pasture (Manning and Collins 2001; OIE 2012). Upon ingestion, MAP enters the intestinal wall through the ileal mucosa via the microfold (M) cells residing in the Peyer's patches (Arsenault et al. 2014). It has also been demonstrated that MAP can cross the gut mucosa even in areas without Peyer's patches entering through enterocytes (Bermudez et al. 2010). Here, they resist intracellular degradation and eventually get phagocytosed by subepithelial macrophages. Infected macrophages migrate to local lymphatics and spread to regional lymph nodes where the organisms stimulate inflammatory and immunological responses (Coussens 2001; Tessema et al. 2001).

6.4.2 Immune Responses

Infected macrophages stimulate T-helper lymphocytes and clonal expansion of two T-helper cell subpopulations: T helper 1 (Th1) and T helper 2 (Th2), which secrete different cytokines (Coussens 2001; Coussens 2004). The Th1 cells produce pro-inflammatory cytokines such as interferon gamma (IFN γ), interleukin 2 (IL-2), IL-12, IL-6 and tumour necrosis factor alpha (TNF- α) while Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 (Coussens et al. 2004; Coussens et al. 2005). These cytokines are believed to orchestrate cell-mediated and humoral immune responses that aim to contain the intracellular infection but unfortunately end up augmenting the disease process. The early immune response to MAP infection consists of numerous infected macrophages with increased amounts of adhesion molecules, which result in formation of granulomas in which the bacilli remain secluded for a long period of time (Coussens 2004). The animal in this state presents no clinical symptoms and it can remain in this subclinical phase for a period of 2–5 years while the bacilli are contained in macrophages and microscopic granulomas

(Stevenson 2005; Tanaka et al. 2005). Infected animals may not manifest clinical disease throughout their lifetime, yet they continue to contaminate the environment through shedding of MAP (Lombard 2011; Wolf et al. 2014; Corbett et al. 2018). What determines the length of this duration both at host and pathogen levels is still unknown. With the failure of macrophages to destroy MAP, the organisms begin to multiply intracellularly with production of immunoglobulin G1 (IgG1) antibodies characteristic of the late humoral response in MAP infection (Coussens et al. 2004).

6.5 Fate of MAP inside Macrophages

Mononuclear phagocytes, principally macrophages, serve as the intracellular niche for MAP survival and multiplication (Tooker, Burton, and Coussens 2002). Macrophages possess several receptors involved in mycobacterial uptake which include complement receptors (CR1, CR3 and CR4), mannose receptors, immunoglobulin receptors and the scavenger receptors (Stokes and Doxsee 1999; Cirillo 2001; Underhill and Ozinsky 2002). Reports suggest that complement opsonisation is important for the uptake of MAP by bovine mononuclear phagocytes and that these phagocytes can synthesise and secrete complement proteins to opsonise particles for phagocytosis (Woo and Czubrynski 2008).

The innate immune system also employs germline-encoded pattern-recognition receptors for the initial detection of microbes. There are several classes, some of which include the Toll-like receptors (TLRs) and Nucleotide-binding oligomerisation domain-like receptors (NLRs) as reported by Kawasaki and Kawai (2014). Antigen-presenting cells such as macrophages express these TLRs, which initiate immune responses mediated by different cytokines. Reports have indicated that TLRs and NLRs are involved in the recognition of MAP by the innate immune system (Ferwerda et al. 2007).

Mechanisms of virulence responsible for MAP colonisation, entry and persistence in macrophages and eventual disease development remain elusive. Several mycobacterial factors are believed to interplay and interact with host factors to determine the virulence and pathogenesis of MAP within the animal host (Figure 5). Once inside macrophages, MAP survives and proliferates in the phagosome using means which are not fully understood. One such means is the ability of MAP to disable the reactive oxygen anion intermediates (ROI) such as superoxide anions, hydrogen

peroxide and hydroxyl radicals (Werling et al. 2004), which have a mycobactericidal effect. *Mycobacterium avium* subsp. *paratuberculosis* secretes superoxide dismutase, which is a possible counter mechanism for protection of the bacilli in macrophages (Liu et al. 2001). Macrophages also produce reactive nitrogen intermediates such as nitric oxide (NO) when stimulated with IFN- γ and TNF- α which are also known to have a mycobactericidal effect (MacMicking, Xie, and Nathan 1997). Macrophages infected with *M. tuberculosis* (MTB) have been shown to inhibit recruitment of inducible nitric oxide synthase (iNOS) in phagosomes containing mycobacterium as a measure to counteract NO production (Miller et al. 2004). A similar mechanism is believed to work in macrophages infected with MAP, though this requires further investigation since Hostetter and colleagues found little evidence of iNOS activity in Johne's disease lesions (Hostetter et al. 2005).

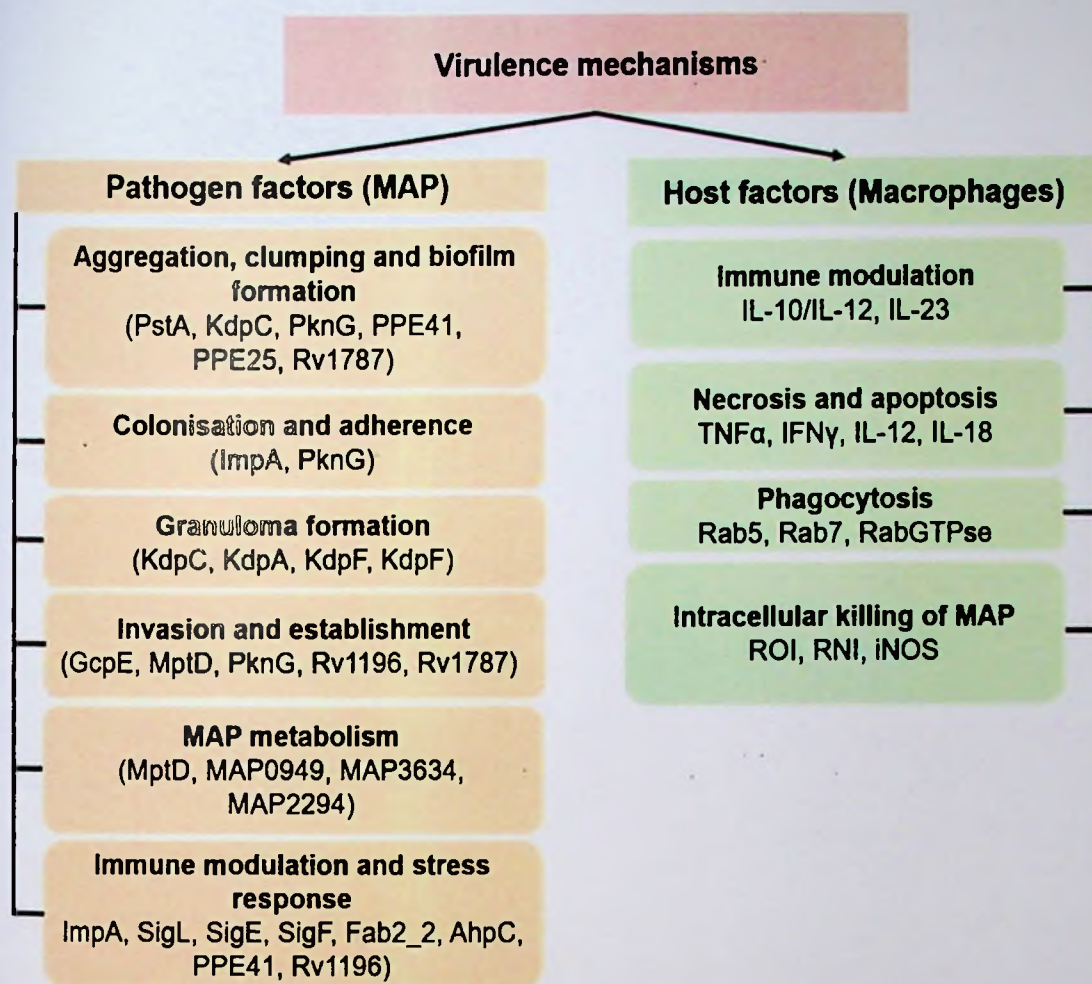


Figure 5: Schematic representation of putative MAP virulence mechanisms and associated host factors.

Legend: The virulence mechanisms enable MAP to gain entry, survive, multiply and cause disease in the host by interacting and sometimes disabling the host defense mechanism and metabolism. Since there is limited information concerning the expression and actions of virulence factors in MAP, most of the current understanding of MAP virulence is imputed from the functions of such factors in related mycobacteria.

Another microbicidal mechanism by phagocytes is the phagosome–lysosome fusion to form a functional phagolysosome in which the lysosomal vacuoles containing hydrolytic enzymes kill and degrade invading microbes (Bainton 1981; Vergne et al. 2004). Live MAP has been shown to

perforate this vacuole by secreting lipids that create pores in the membrane surrounding the vacuole. Mycobacteria are also known to interfere with phagosomal maturation through inhibition of the ATP-dependent proton pump (H^+ -ATPase) which maintains an acidic environment necessary for optimal activity of the hydrolytic enzymes (Sturgill-Koszycki et al. 1994). MAP cells have been shown to inhibit phagosome–lysosome fusion and phagosomal acidification in murine cells (Kuehnel et al. 2001; Woo et al. 2007). This is through blocking of Rab5 activity, therefore preventing the fusion of early endosomes. Activation of MAP-infected murine macrophages with IFN- γ and lipopolysaccharide (LPS) resulted in enhanced phagosome–lysosome fusion and increased killing of MAP cells intracellularly as compared to the unstimulated (Hostetter et al. 2002). *Mycobacterium avium* subsp. *paratuberculosis* cells have been shown to stimulate production of IL-10, an anti-inflammatory cytokine, which counteracts the effects of IFN- γ in macrophage infection and aids MAP survival (Buza et al. 2004). Consequently, downregulation of IL-10 gene resulted in reduced survival of MAP but also increased production of INF- γ in bovine peripheral blood mononuclear cells (Buza et al. 2004; Weiss et al. 2005). Similar reports showed that infection of bovine macrophages with MAP resulted in reduced apoptosis of macrophages (Woo et al. 2007; Weiss et al. 2004) which enhances MAP survival.

Mycobacteria, unlike other pathogenic bacteria, lack the classical virulence factors such as toxins; they use other virulence mechanisms which enable them to survive in the macrophage intracellular environment (Forrellad et al. 2013). The mechanisms utilised by MAP to enter and survive in the intracellular host environment include resistance to intracellular degradation by macrophages and inhibition of apoptosis and interference with cytokines production. Cytokines control different processes in the cell through protein regulation to overcome the infection. MAP, however, modifies the response of macrophages to infection so that it is able to survive inside the macrophage (Tessema et al. 2001).

Although the mechanisms for the intracellular survival of MAP within macrophages are not entirely understood, several reports suggest that these mechanisms have a genetic basis (Stevenson 2015; Ghosh, Steinberg, and Talaat 2014). Khalifeh and Stabel reported that formation of persistently infected macrophages is associated with upregulation of IL-10, transforming growth factor beta (TGF β) and reduction in IFN γ (Khalifeh and Stabel 2004). It has been shown that MAP interferes

with CD40 signaling, which is mediated by IL-12P40 and inducible Nitric oxide synthase (iNOS) in monocytes-derived macrophages (Sommer et al. 2009). The CD40 pathway is responsible for the activation of macrophages by the T cells. Failure of T cell activation and consequently macrophage activation leads to persistently infected macrophages incapable of destroying the pathogen that instead act as a vehicle for propagation and dispersal of the organism leading to infection (Coussens 2001; Simutis, Jones, and Hostetter 2007). Activated macrophages are capable of killing MAP and control their growth; however, at high multiplicity of infection, MAP is cytotoxic to macrophages and also inhibits apoptosis (Bannantine and Stabel 2002; Marino et al. 2017).

6.6 MAP Virulence Factors

Virulence factors facilitate adhesion, colonisation and invasion of host cells by pathogenic bacteria; they include enzymes of several lipid pathways, cell surface protein regulators and signal transduction systems molecules (Figure 5). Bacterial virulence genes may be located on transmissible genetic elements such as transposons, bacteriophages, plasmids or within the bacterial genome organised in a contiguous region known as the pathogenicity island (Hacker et al. 1997). A gene will probably contribute to virulence if it is present in a pathogen but absent in a closely related nonpathogenic organism or inactivation of the gene results in attenuation of the virulence phenotype and replacement with an intact copy of the inactivated gene restores virulence (Falkow 2004).

6.6.1 Factors That Facilitate Adhesion, Colonisation, Entry and Persistence of MAP

From studies so far conducted, it has been demonstrated that certain genetic elements within the pathogen genome are responsible for virulence. Once removed or disrupted, the organism displays an attenuation phenotype. The disruption of genes can be achieved experimentally by means of insertion or deletion mutagenesis and construction of transposon mutant libraries. Using a transposable element Tn5367 introduced into the MAP genome by means of a conditionally replicating mycobacteriophage phAE94, Harris and colleagues generated a library of 5620 MAP insertion mutants and also demonstrated that the transposon insertion sites are distributed relatively randomly throughout the MAP genome (Harris et al. 1999). In a related study, Shin and coworkers constructed a library of transposon mutants whose genes were phenotypically involved in

membrane transport protein, iron, tryptophan, or mycolic acid metabolic pathways (Shin et al. 2006). In the aforementioned study, the transposable element Tn5367 was used to generate a library of MAP mutants. A total of 5060 mutants were generated, of which 1150 mutants were characterised by sequencing and bioinformatics analysis. Out of these, using a mouse model of PTB, seven novel virulence determinants were identified. Bacteriological and histological analyses employed in the study were able to categorise the putative colonisation virulence factors into two classes: those that exhibited impaired organ colonisation and low inflammatory scores; and those that showed low colonisation rates but at a later stage of infection, implying a role for persistence in macrophages. These genetic factors are involved in *M. tuberculosis* (MTB) virulence and might have significance in MAP virulence as well.

In Table 6, we present a list of virulence factors of MAP and related mycobacteria which are believed to play a role in MAP virulence and pathogenesis. The gene *gcpE* encodes a protein involved in isoprenoid biosynthesis via the mevalonate-independent 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Sasseti, Boyd, and Rubin 2001). It is considered an essential gene in *Escherichia coli* (Altincicek et al. 2001). In a study using a calf model in which MAP strains were deposited in the ileum, mutants of the *gcpE* gene were unable to traverse the intestinal barrier to the mesenteric lymph nodes, unlike the wild-type strain. This indicated that the mutant was less invasive, throwing more light on the process of MAP pathogenesis and virulence mechanism (Wu, Livesey, et al. 2007). However, more information is required on the probable use of the *gcpE* gene as a potential target for vaccine development in PTB control.

The nonribosomal peptide synthetase gene (*pstA*) encodes an enzyme involved in glycopeptidolipid biosynthesis which is associated with biofilm formation (Wu et al. 2009). It has been described in mycobacteria such as MTB, *M. smegmatis* and *M. avium* (Ojha et al. 2008; Recht and Kolter 2001; Yamazaki et al. 2006). Glycopeptidolipids are species-specific mycobacterial lipids and have been demonstrated to be major constituents of cell envelopes of several nontuberculous mycobacteria, both pathogenic and nonpathogenic (Billman- Jacobe et al. 1999). Biofilm formation is believed to contribute to bacterial virulence by inducing a persistent source of infection and may contribute to antibiotic resistance (Falkinham III 2007). According to Shin and coworkers, *pstA* mutants showed significant reduction in tissue colonisation in a mouse model of PTB (Shin et al. 2006). In a later

study, *pstA* mutants were shown to be deficient in the ability to form biofilm when compared with the intact MAP strain (Wu et al. 2009), an indication that *pstA* could contribute to biofilm formation in MAP. In the same study, it was observed that *pstA* mutants also exhibited significant reduction in bacilli length compared to the parent strain, which raises the question of whether cell elongation could contribute to biofilm formation. The exact role of *PstA* in MAP pathogenesis requires further scrutiny.

Table 6: Factors believed to influence virulence in MAP and other mycobacteria

Virulence Factor	Name/Function	Probable Role in MAP Virulence	References
GcpE	Protein involved in Isoprenoid Biosynthesis	Involved in the MEP pathway. Important in tissue invasion during early MAP infection	(Wu, Livesey, et al. 2007)
PstA	Non-ribosomal peptide Synthetase	Glycopeptidolipid biosynthesis and associated with biofilm formation	(Wu et al. 2009)
KdpC	Probable potassium-transporting ATPase C chain	An inducible high-affinity potassium uptake system. In MAP it has been associated with organ colonisation and granuloma formation	(Shin et al. 2006; Gaßel and Altendorf 2001)
PapA2	Conserved polyketide synthase-associated protein	Virulence-enhancing lipids of MTB. In MAP it is associated with tissue colonisation	(Shin et al. 2006; Onwueme et al. 2004)
ImpA	Inositol monophosphatase Protein	Involved in cell wall permeability and persistence in macrophages	(Shin et al. 2006; Nigou and Besra 2002)
FabG2_2	Putative oxidoreductase	Involved in colonisation and persistence in macrophages during	(Shin et al. 2006; Li et al. 2005)

MAP infection				
UmaA1	Mycolic acid methyltransferase	Involved in cell wall biosynthesis and tissue/organ colonisation	(Li et al. 2005; Settles, Kink, and Talaat 2014)	
MptD (MAP3733c)	Putative ATP binding cassette transporter	Important in MAP adaptation during early infection through lipid metabolism	(Meissner et al. 2014)	
PknG (MAP3893c)	Serine/threonine protein kinase G	Contributes to biofilm and granuloma formation in MTB. In MAP it induces production of IFN γ leading to macrophage phosphorylation	(Khan et al. 2017; Bach et al. 2018)	
MAP0949	Probable diguanylate cyclase	Involved in bacterial cell surface adhesions. Important in adaptation and evasion of the host immune system.	(Purcell and Tamayo 2016; Hall and Lee 2018)	
MAP2291	Haemoglobin-like oxygen carrier—glbO	Protects MAP against oxidative stress	(Phillips et al. 2020)	
MAP3634	Hypothetical protein of mycobacterial L,D-transpeptidases	Involved in bacterial cell wall synthesis through polymerisation of peptidoglycans	(Phillips et al. 2020)	

The *kdpC* gene is a probable potassium-transporting ATPase C chain which encodes an inducible high-affinity potassium uptake system of *E. coli*. It is part of the Kdp complex, which are membrane bound subunits comprising the KdpC, KdpF, KdpB and KdpA (Gaßel and Altendorf 2001). The role of the *kdpC* gene in mycobacterial pathogenesis is not yet clear. From the study by Shin and others (2006), mutants for the *kdpC* gene were deficient in the ability to colonise mouse organs (liver and intestines) and this could possibly be due to a defect in the potassium-shuttling mechanism which resulted in the attenuation phenotype exhibited by the mutants. The *kdpC*-deficient mutants showed limited granuloma formation compared to the wild types. The *papA2*

gene is a member of the conserved polyketide synthase-associated protein (Pap) family which encodes virulence-enhancing lipids of MTB (Onwueme et al. 2004). Disruption of the *papA2* gene in MAP resulted into attenuation in a murine model of PTB (Shin et al. 2006).

The *impA* gene encodes an inositol monophosphatase protein which earlier studies showed that it played a role in cell wall permeability in *M. smegmatis* and in the synthesis of phosphatidylinositol dimannoside (Nigou and Besra 2002; Parish et al. 1997). On the other hand, *fabG2_2* gene encodes a putative oxidoreductase activity in MAP (Parish et al. 1997). Both mutants of *impA* and *fabG2_2* genes exhibited low colonisation levels late in the infection experiment, which implied that they could possibly play a role in bacterial defense in the persistence stage of MAP infection (Shin et al. 2006).

The *umaA1* gene, which encodes a mycolic acid methyltransferase, is involved in cell wall biosynthesis (Li et al. 2005). Disruption of *umaA1* resulted in reduced colonisation levels of mutants in mice (Shin et al. 2006). This is, however, in contrast to a similar model for MTB in which *umaA1* mutants exhibited a hypervirulence phenotype (McAdam et al. 2002) and this may suggest a difference in role played by the gene in MTB from that of MAP. MAP *umaA1* together with *fabG2_2* gene mutants have been tested for their vaccine potential in mice and were found to induce a MAP specific IFN- γ important for eliciting cell-mediated immunity. The *umaA1* mutants further induced production of IL-17a, a cytokine important for mycobacterial protective immunity. Indeed, mice vaccinated with the *umaA1* and *fabG2_2* gene mutants exhibited significant reduction in organ colonisation and low histological scores compared to control animals when challenged with a virulent MAP strain (Settles, Kink, and Talaat 2014).

Though the above-described virulence genes have been identified, there is little understanding of the pathways through which they act and whether the same effects observed *in vitro* and in mouse models can be observed in ruminant models of PTB. Moreover, similar genes in closely related species may play different roles in pathogenesis due to differences in gene organisation on the genomic island, as was observed in MAP and *Mycobacterium avium* subsp. *avium* (Wu et al. 2006; Rathnaiah et al. 2017). How virulence factors affect host-pathogen interactions is still only left to speculation. These probable virulence determinants could represent novel functional classes

necessary for mycobacterial survival during infection and could provide suitable targets for vaccine and drug development.

6.6.2 Factors Which Affect Metabolism of MAP

Different pathogenic bacteria have been demonstrated to undergo adaptations that enable them utilise the host metabolites that would otherwise have been toxic. An example is *M. tuberculosis*, which has the ability to break down the host fatty acids, detoxify the products and utilise the resulting molecules to acquire carbon during intracellular infection. The ability of pathogens to acquire nutrients within the host environment is part of the crucial processes contributing to bacterial virulence (Lee et al. 2013).

Mycobacterium avium subsp. *paratuberculosis* has been shown to undergo significant genetic modifications during host– pathogen interaction within macrophages. One such modification is iron limitation, which triggers nitric oxide build-up linked to nitric oxide synthase production, this prompts MAP to enter into an iron sequestration program. This kind of pathway is likely to contribute to MAP virulence by aiding establishment and long-term survival of the organisms within host macrophages (Ghosh, Wu, and Talaat 2013). However, it is not known how MAP influences the host metabolic state for its benefit and whether the genetic factors contributing to its virulence are also involved in these processes. To date, only a couple of genes have been shown to modulate MAP virulence within macrophages (Figure 5).

Meissner et al. (2014) studied the role of MAP *mptD* gene (*map3733c*) to deduce its potential role in the host during MAP infection. The *mptD* gene whose operon is predicted to encode a putative ATP-binding cassette transporter belongs to a small group of functionally uncharacterised genes. Macrophage infection experiments were performed in which the gene was observed to have a significant role in MAP adaptation during early infection. Metabolic profiling revealed profound disorders in lipid metabolism. This pointed to the probable importance of *mptD* gene in metabolic adaptation required for MAP persistence in the host.

In a recent study, Philips and others used an *Acanthamoeba castellanii* (amoeba) model to predict virulence mechanisms of MAP (Phillips et al. 2020). A library of gene knockout mutants generated by Mycomart7 (Mmt7) transposon was used to identify MAP clones that can either enhance or

inhibit the amoeba metabolic activity, and this mirrored the pattern of MAP survival or attenuation in macrophages. They observed that MAP mutants that induced high amoeba metabolic activity were defective in the intracellular growth inside macrophages. From bioinformatic analysis of mutant sequences, some genes were identified that could possibly be responsible for the altered ability for survival of MAP mutants in the RAW 264.7 macrophages (Phillips et al. 2020).

The *map3893c* gene encodes the serine/threonine-protein kinase G (PknG) which is a well-characterised virulence factor in MTB where it is known to contribute to biofilm development and granuloma formation (Khan et al. 2017; Wolff et al. 2015). It is also known to block the recruitment of active Rab711-GTP to phagosomes containing the pathogen, thereby inhibiting the phagolysosome fusion (Walburger et al. 2004; Pradhan, Shrivastva, and Mukhopadhyay 2018). It appears that the role of PknG in MAP pathogenesis contributes to macrophage phosphorylation signaling and other adaptor molecules by inducing an immune response through production of IFN- γ (Bach et al. 2018). The exact role of PknG in MAP is not fully understood, but because it shares high homology with that of the MTB complex, it is anticipated to perform similar roles. This, however, needs further investigation.

Another macrophage-related putative virulence factor is MAP0949, a probable diguanylate cyclase whose absence results in severe attenuation of MAP during macrophage infection. Its wild phenotype is, however, restored once the disrupted gene is complemented by the functional gene (Phillips et al. 2020). In other bacteria, it has been postulated that it is involved in stimulating degradation of second messenger cyclic di-GMP (c-di-GMP) involved in bacterial cell surface adhesions (Purcell and Tamayo 2016). The c-di-GMP and c-AMP second messenger signaling molecules control expression of a variety of environmental and quorum sensing signals as well as regulating several key virulence mechanisms required for bacterial adaptation and evasion of the host immune system (Hall and Lee 2018; Johnson and McDonough 2018; Römling, Galperin, and Gomelsky 2013).

Inducible nitroxide synthase (iNOS) is an important enzyme responsible for the activation of reactive nitrogen oxide species that kill intracellular mycobacteria. Virulence of mycobacteria is probably mediated by the *map2291* gene, a haemoglobin-like oxygen carrier (*glbO*) which encodes the globin protein occurring across the *Mycobacterium avium* complex (MAC). It is 86% homologous to MTB oxygen-binding *glbO* which has a function related to oxygen affinity and

reactivity (Bustamante et al. 2016). MTB-truncated haemoglobin O (trHbO) displays moderate NO-scavenging activity which signifies involvement in both NO detoxification and aerobic respiration (Pawaria et al. 2008). Since *map2291* consists of MTB *glbO*-like domain, it can be hypothesised that the *map2291* gene prevents intracellular killing of MAP by protecting the pathogen from microaerophilic conditions and oxidative stress (Phillips et al. 2020). Moreover, one study also showed evidence of limited iNOS activity in MAP granulomas (Hostetter et al. 2005).

Virulence of MAP may also be determined by the *map3634* gene, which encodes a hypothetical protein that contains IgD-like repeat domain of mycobacterial L,D-transpeptidases and is responsible for the final polymerisation steps involved in the formation of glycan strands and cross-linking peptide stems of the peptidoglycan cell wall in most bacteria (Mainardi et al. 2008). Inactivation of these transpeptidases has been demonstrated to be detrimental to MTB and *M. abscessus* (Sabe et al. 2019; Kumar et al. 2017), signifying its importance as a virulence factor in these organisms. MAP mutants lacking *map3634* gene exhibit reduced survival rate in macrophages, presumably due to defective bacterial cell wall synthesis (Phillips et al. 2020).

6.6.3 Factors That Affect Aggregation and Clumping

The PE/PPE proteins are among the probable factors contributing to MAP virulence. The PE/PPE proteins are large families of proteins with each member sharing a conserved N-terminal domain with the characteristic motifs Proline–Glutamate (Pro–Glu or PE) or Proline–Proline–glutamate (PPE) that are an important domain in mycobacteria and have been for long believed to play a role in virulence (Cole et al. 1998). They have been well characterised in MTB, though they also occur in other mycobacteria such as *M. leprae*, *M. avium* and *M. bovis* (Cole 2002). They are thought to be virulence factors responsible for evasion of host immune responses and a potential source of antigenic variation in MTB (Akhter et al. 2012). It is reported that some PE/PPE families are deleted from avirulent *Mycobacterium tuberculosis* (Garcia-Pelayo et al. 2004). Zheng and colleagues also showed that a number of PE/PPE genes found in virulent strains of MTB contain single nucleotide variations (Zheng et al. 2008). Differential expression of PE/PPE genes in *M. tuberculosis* and *M. bovis* seems to suggest host specificity. At least one *M. tuberculosis* PE protein is known to cause cell death while another causes apoptosis of host macrophages (Basu et al. 2007).

There is some evidence that inactivation of the PE-polymorphic GC-rich repetitive sequence (PE-PGRS) gene in *M. bovis* BCG strain will lead to loss of cell aggregation (clumping), dispersed growth and reduced infection of macrophages (Brennan et al. 2001). The iron-dependent regulator (IdeR) of MTB has been reported to control several PE/PPE proteins such as Rv0279, Rv0285 and Rv2123, and the evidence that iron is necessary for virulence points to the PE/PPE proteins being virulence factors (Prakash et al. 2005; Mohareer, Tundup, and Hasnain 2011). In MAP the PE/PPE family comprises 1% of the genome and lacks the intact PE-PGRS subfamily present in MTB and other mycobacteria such as *M. bovis* and *M. marinum* [(Li et al. 2005).

The exact role of PE/PPE proteins in MAP virulence has, however, not yet been elucidated. PE-PGRS family proteins Rv0834c, Rv3097c, Rv097 are expressed within 24 h post infection in macrophages and are believed to aid the establishment of the infection in macrophages (Akhter et al. 2012). It is suggested that PE-PGRS Rv1759 binds to the cytoskeletal protein, fibronectin thereby facilitating entry of MAP into the host through opsonisation (Bannantine and Bermudez 2013). Another PE-PGRS protein, Rv1787 (PPE25) has been shown to be important for growth of *M. smegmatis* within macrophages, while Rv1196 (PPE18) and PPE44 trigger responses that favour a switch from Th1 to Th2 by shifting IL-10/IL-12 balance and down regulating IL-12/TNF α . Because of the high degree of polymorphisms among the PE-PGRS genes observed even among the different strains of mycobacteria (Fleischmann et al. 2002), they are potential determinants of differential virulence in mycobacterial strains. As has been recommended for other mycobacteria, the macrophage infection model of MAP can be used to answer questions about activation of various PE/PPE genes, their regulators and mRNA stability (Mohareer, Tundup, and Hasnain 2011). Another aspect of MAP pathogenesis and virulence is to understand how this organism modulates IL-10/IL-12 response.

6.6.4 Global Gene Regulators and Stress Induced Genes

Global gene regulators (GGR) are involved in the control of several other genes such as the sigma factors which have been shown to be important in regulating MAP virulence. In one of the studies (Ghosh, Wu, and Talaat 2013), of the 19 alternative sigma factors encoded in MAP (Li et al. 2005), only sigL was induced at an early stage when subjected to stressors to mimic the host

microenvironment such as oxidative and cell wall stressors. It has been suggested that *sigL* regulates the synthesis of cell envelope lipids and is responsible for modification of secreted proteins in MTB (Hahn et al. 2005). The importance of *sigL* in MAP survival in macrophages during early adaptation and the ability of mutants to produce protective immune response against PTB (Ghosh, Steinberg, and Talaat 2014) further highlighted the importance of GGR in possible development of effective live attenuated vaccines.

Wu and colleagues studied the gene expression profiles of MAP under different stress conditions such as heat shock, acidity and oxidative stress. Several sigma factors such as *SigH*, *SigE* were differentially coregulated with a large number of genes. Deletion mutagenesis revealed attenuation of gene mutants that included; *lipN*, *lpqP*, *aceAB*, and *prpA* in a murine model of PTB which could participate in tissue colonisation indicating their role in MAP pathogenesis (Wu, Schmoller, et al. 2007). Pribylova and coworkers also reported that *SigE*, *SigL*, *SigF*, alkyl hydroperoxide reductase (AhpC) and the major membrane protein (MMP) are over-expressed during heat stress in MAP (Pribylova et al. 2011). Further analysis of the role of stress-induced genes in MAP pathogenesis and virulence is needed.

6.6.5 The Role of Polymorphism in the MAP Genome on its Virulence

Microbial genetic variations may be a result of spontaneous mutations due to the unstable nature of the purine and pyrimidine bases, resulting from errors that may occur during replication or be induced by exposure to certain environmental factors such as UV light (Stevenson 2015). Whole genome sequence analysis of the MAP genome has revealed occurrence of large sequence polymorphisms (LSPs) emanating from deletions, insertions, inversions, duplications or dislocations. These events can cause DNA rearrangements resulting in major phenotypic changes such as acquisition of virulence genes through insertions. Other sources of genetic variations include: insertion sequences, repeat sequences and single nucleotide polymorphisms (Stevenson 2015). The MAP genome comprises 19 different insertion sequences which are useful in mycobacterial species and subspecies differentiation such as IS1311 and IS900, which is MAP-specific (Bannantine et al. 2012). Repeat sequences are also important in genomic typing for strain differentiation and molecular diagnosis such as the mycobacterial interspersed repetitive units--

variable-number tandem repeats (MIRU-VNTRs) and short-sequence repeats (Thibault et al. 2008). Single nucleotide polymorphisms are the substitutions of a single nucleotide with another or a deletion or insertion of one nucleotide. Single-nucleotide substitutions in protein synthesis can be synonymous - resulting in no amino acid change - or nonsynonymous, which results in amino acid changes. SNPs can be utilised in intra-strain differentiation and offer the greatest microbial genomic variation and may contribute to variations in pathogenicity among MAP strains (Stevenson 2015; Leao et al. 2016; Bannantine et al. 2012). Genetic variations, including single nucleotide polymorphisms in PE/PPE-PGRS genes, have been found to occur more frequently in virulent strains of *M. tuberculosis* compared to the avirulent ones. All these different polymorphisms could play important roles in differential virulence in MAP but further study is required to determine if this is true.

6.7 Conclusions

Differences in MAP strain virulence have been reported by several studies, but the slow growth of MAP and its long incubation period have made it very difficult to understand the mechanisms for its pathogenesis and virulence in its natural hosts. Most of the current understanding is based on inference from other mycobacteria, with a few studies attempting to replicate these studies using MAP itself. The key highlights of MAP virulence focus on genes such as the PE/PPE - PGRS family, mycobacterial protein kinases (*PknG*) and the modulation of IL-12/IL-10 switch during infection. There are still many knowledge gaps in our understanding of how differential expression, stability and secretion of these gene products affect MAP uptake, persistence, frustration of phagocytosis and toxicity, which are the hallmarks of mycobacterial virulence in its hosts. Future studies need to focus on elucidating the different protein pathways involved during mycobacterial pathogenesis.

6.8 References

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CHAPTER SEVEN

EVALUATION OF THE POTENTIAL VIRULENCE OF SELECTED ISOLATES OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* FROM CATTLE IN UGANDA

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7.0 Abstract

Understanding the pathogenesis of paratuberculosis is essential in the management of this economically important disease. *Mycobacterium avium* subsp. *paratuberculosis* strains are known to differ genotypically and this could contribute to variations in strain virulence and disease outcome. The behaviour of MAP inside the host is not adequately known and the mechanisms utilized by these organisms to overcome the host defence system are not entirely understood. This study aimed at evaluating the potential virulence of the different MAP isolates from cattle in Uganda. This was achieved by analysing the cytokine expression profiles of RAW 264.7 macrophages when infected with four different MAP isolates. Specific real-time PCR was used to determine the expression of different cytokines, probable MAP virulence genes as well MAP quantities inside the macrophages at different time points (3 h, 24 h and 48 h). Significant differences in the relative cytokine and virulence gene expression profiles were observed during the infection among the different isolates. In particular, there was an up-regulatory trend for pro-inflammatory cytokines (IFN- γ , TNF- α , IL-12p40) and down regulation of the anti-inflammatory cytokines (IL-10, TGF- β) in MAP2 isolate. The anti-apoptotic gene on the other hand was generally down-regulated during infection with all isolates except in MAP2 where it was up-regulated after the 24 h time point. The regulation of the probable virulence genes including: *kdpC*, *katG*, *papA2*, *impA* and *umaA1* varied considerably among isolates with a relative up-regulation in MAP4 and a down-regulation in MAP2 later in the infection. There were significant variations among isolates in MAP cell replication inside the macrophages as well as its viability. MAP4 isolate exhibited least ability for survival inside macrophages whereas isolate MAP1 on the contrary exhibited higher virulent behaviour in regard to relative cytokine expression and replication intracellularly compared to the other isolates. This shows that MAP strains differ in behaviour inside macrophages which is an indication of the differential virulence. This knowledge is important in designing strategies for PTB management.

Key words: Virulence, Cattle, Uganda, *Mycobacterium avium* subsp. *Paratuberculosis*, Strains

7.1 Introduction

Paratuberculosis (Johne's disease) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a serious and economically important disease in a wide range of animal species especially ruminants worldwide. The infection leads to a chronic granulomatous enteritis which sometimes presents itself only after several years in form of severe wasting diarrhoea, resulting in cachexia and complete loss of productivity of the affected animal. In recent years, the suspicion that MAP is a zoonotic pathogen has been strengthened (Patel et al. 2006; Waddell et al. 2015).

The disease is known to occur worldwide though the exact number of infections is only estimated and is probably higher than assumed. Early diagnosis of the disease is difficult due to the long incubation period (often years), where the animal does not present with clinical signs yet it continues to spread the infection. The organism grows extremely slowly and this complicates studies on its behaviour in host cells. Hence, prophylaxis for the disease is not normally successful due to the limited knowledge of the interaction of the organism with the host at the cellular level. Studies have shown that there are several strains and genotypes of MAP distinguished based on growth characteristics and host associations and these include: the "cattle type" or "Type C", the "sheep type" or "Type S" and the "Bison" or "B type" (Stevenson 2015). *Mycobacterium avium* subsp. *paratuberculosis* has also been classified into type I, II and III based on whole genome sequence analysis of genetic markers (Fawzy et al., 2018). These different strains and genotypes may contribute to variations in the clinical and pathologic presentation of the disease (Hodgeman et al. 2021). However, differential virulence in MAP strains has not yet been extensively investigated.

Microbial virulence is the ability of the pathogenic bacteria to colonise, enter and persist in the host cell through regulation of different protein secretion systems, lipid pathways and other cell surface signal variations leading to infection (Hacker and Heesemann 2002). Inside the host cell, MAP is known to have an effect on intracellular processes such as apoptosis and regulation of the production of different cytokines. Infected macrophages produce a plethora of cytokines which play multiple roles in modulation of immune and inflammatory responses. Different cytokines may have multiple actions on multiple target cells, implying that they may not be specific in function (Thomson and Lotze 2003). Cytokines may overlap in action in response to an infection. Early MAP infection is associated with a pro-inflammatory cytokine response involving production of

cytokines such as; interferon gamma (IFN γ), interleukin 2 (IL-2), IL-12 and tumour necrosis factor alpha (TNF α) whereas the late infection is associated with production of anti-inflammatory cytokines such as; transforming growth factor - beta (TGF- β), IL-4, IL-10 and IL-13 (Coussens et al. 2004). MAP interferes with these cellular processes to enable its survival in the intracellular environment and overcome phagocytosis (Woo and Czuprynski 2008). It has been demonstrated that MAP infection is associated with changes in expression profiles for genes involved in host immune responses that enable its survival and multiplication inside the host macrophages (Marino et al. 2017). Mycobacterial genes that are believed to play a role in the entry, survival and establishment of MAP in the host macrophages include: the non-ribosomal peptide synthetase gene (*pstA*), the conserved polyketide synthase-associated protein (*papA2*), serine/threonine protein kinase G (*pknG*), putative oxidoreductase (*fabG2_2*), probable inositol monophosphatase (*impA*), possible mycolic acid synthase (*umaA1*), isoprenoid biosynthesis (*gcpE*), catalase-peroxidase (*katG*), probable potassium-transporting ATPase (*kdpC*), among others (Shin et al., 2006; Wu et al. 2007; Granger et al. 2004).

In vitro infection experiments have been performed previously using primary macrophages - monocytes-derived macrophages (MDM) or special cell lines such as RAW 264.7, J774 among others to investigate MAP virulence attributes such as viability (survival and multiplication), effect on apoptosis, cytokine production and different gene expression profiles to infer the fate of MAP inside macrophages (Gollnick et al. 2007; MacHugh et al. 2012; Lamont et al. 2014). *Mycobacterium avium* subsp. *paratuberculosis* is known to interfere with these processes which enables it survive the body defense mechanism and establish an infection.

Variations in virulence of the different strains could be associated with polymorphisms due to mutations within the MAP genome. Phylogenetic analysis of the different putative MAP virulence genes is necessary to infer the source of these variations. Unfortunately, there is a paucity of information concerning virulence of the different MAP strains. The general data situation on paratuberculosis is particularly poor in many African countries (Okuni et al. 2020). Reasons for this are mainly the lack of general awareness of the occurrence of this disease in domestic and wild animal populations as well as the infrequent reporting of cases. Finally, it should be noted that PTB is considered a purely animal disease and therefore does not have the highest priority in control or

research programs as, for example, malaria or tuberculosis. This means that sufficient knowledge on MAP is lacking and the pathogen can continue to spread unhindered without assessment of the consequences for both humans and animals.

In this study, questions concerning the behavior of MAP during infection were addressed. The expression of virulence genes by four field MAP isolates from Uganda during an infection period of 3 to 48 h as well as the respective cellular cytokine responses to MAP infection were examined. The behaviour of MAP isolates within these time periods was observed in respect to its replication and viability at the intracellular level. Knowledge of MAP behaviour and virulence mechanisms is important in the design of control and prevention measures for paratuberculosis.

7.2 Materials and methods

7.2.1 MAP cultivation and preparation for infection

Fecal and blood samples were collected from cattle in a MAP survey recently carried out in Uganda (details discussed in chapter four). In this study, faeces and serum was tested for presence of MAP DNA and antibodies using RPA assay and ELISA respectively. All fecal samples from animals that were positive on either the molecular or serologic test were cultured on Herrold's Egg Yolk agar slants (HEYM) containing Vancomycin, Amphotericin B and Nalidixic acid with mycobactin J (Becton, Dickinson and Co., Sparks MD 21152, USA) at 37 °C for a period between 6 weeks to 6 months. All slants that developed colonies typical of MAP were confirmed using both RPA and real-time PCR. *Mycobacterium avium* subsp. *paratuberculosis* positive colonies were sub-cultured on HEYM to achieve pure colonies. A single colony was then inoculated in approximately 5 ml of BBL™ Middlebrook 7H9 broth produced by Becton, Dickinson and Co. (Sparks MD 21152, USA) with glycerol and supplemented with Mycobactin J (IDvet, rue Louis Pasteur – Grabels - France) and addition of OADC enrichment (BD BBL™ Middlebrook, Becton, Dickinson and Co., Sparks MD 21152 USA) in a 15 ml centrifuge tube (Sarstedt®, Numbrecht, Germany) and incubated at 37 °C for 3 weeks to achieve optimal mycobacterial growth. Four successfully cultivated MAP isolates; (MAP1, MAP2, MAP3 and MAP4) and confirmed as MAP using real-time PCR of the IS900 gene, were used in the infection experiments.

For the experiments, approximately 10 ml of bacterial cell suspension was centrifuged for 10 min at 3,000g and a single resuspension of the resulting MAP pellet in PBS was performed. Brief homogenization by vortexing was followed by adjustment of the bacterial density by turbidity comparison to the McFarland Standard No. 1 (3×10^8 CFU/ml), (Lenexa, Kansas, United Kingdom) by slow addition of PBS.

7.2.2 Macrophage preparation

MAP-susceptible RAW 264.7 murine macrophages (CLS Cell Lines Service GmbH, Eppelheim, Germany) were selected for these infection studies. Cultivation was performed under standard conditions of 37 °C and 5% CO₂ (ESCO CelCulture CO₂ Incubator; CCL-170A-8, Changi South Street, Singapore) in RPMI 1640 (Sigma- Aldrich, St. Louis, USA) media with the addition of 10% fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 1% non-essential amino acids, and 1% penicillin/streptomycin (Life Technologies, Grand Island, USA) in 25 cm² and 75 cm² tissue culture flasks (avantor™, Radnor, PA -- USA and TPP®, Trasadingen, Switzerland). The macrophages were grown for 24 hours to achieve confluence before infection.

7.2.3 Infection

Infection experiments were performed in duplicate in 24-well culture plates (Cellstar® Greiner bio-one, Kremsmünster, Austria) with a 24-hour RAW 264.7 monolayer of approximately 10^5 - 10^6 cells/ml. Fresh RPMI media was added and incubated with 100 µl each of the previously prepared MAP suspension (McFarland No. 1) – at a multiplicity of infection (30:1) - and at an atmosphere of 37 °C and 5% CO₂ for 3, 24, and 48 hours. After 3-hour incubation, complete removal of the cell culture medium was performed using a pipette followed by washing of the monolayer twice with fresh RPMI 1640 medium to remove extracellular MAP. Subsequently, 1000 µl of fresh RPMI medium was added to each well and incubation was continued at an atmosphere of 37 °C and 5% CO₂. For each time interval, one well of non-infected cells was included as a control.

7.2.4 Intracellular MAP Viability assay

After the respective time intervals, the cell culture medium was completely disposed and the monolayer was washed with PBS. Subsequently, cell lysis and release of intracellular MAP was performed by adding 0.25% Triton® X-100 (AppliChem, Darmstadt, Germany) at an exposure time of 1 min. The lysate was transferred to 1.5 ml tubes and centrifuged at 5000g for 2 min. After this step, the resulting pellet was resuspended in 150 µl of fresh Middlebrook 7H9 broth, vortexed and briefly centrifuged. The entire suspension was then transferred to one well of a 96-well plate.

To determine viability, 15 µl of AlamarBlue (Life Technologies, Eugene, USA) were added to the suspension per well. Darkened incubation for 24 hours (optimal time for AlamarBlue reduction) was followed by absorbance measurements at wavelengths of 570 and 600 nm using a spectrophotometer (SPECTROstar Nano, S/n 601-1258 – BMG LABTECH, Ortenberg, Germany). The calculation of the percentage AlamarBlue reduction was performed using a formula (Carroll et al. 2009).

7.2.5 Calculation for correction factor for media blank and media plus AlamarBlue

All reads were done in duplicate after 24 h incubation with AlamarBlue (AB) in 7H9 MiddleBrook media (Table 7).

Table 7: Calculation for correction factor

	Ab 570nm	Ab 600nm (Reference)
15 µl AB + 150 µl of media	0.340	0.468
150 µl media	0.056	0.059
Difference	0.284	0.409

Correction factor (R_o) = Absorbance at 570nm/Absorbance at 600nm

$$0.284/0.409 = 0.694$$

Formula for percentage reduction of AB

$$AR_{570} = A_{570} - (A_{600} \times R_o) \times 100$$

Where,

- AR – AlamarBlue reduction
- A570 - Absorbance at 570nm
- A600 - Absorbance at 600nm
- R_o - Correction factor

7.2.6 Expression of cytokines and virulence genes during infection

At the end of the respective time intervals, the medium was completely aspirated from the respective wells and the monolayer was washed with PBS. This was followed by the addition of lysis buffer (containing 2-mercaptoethanol, Sigma Aldrich, St. Louis, USA) and manual homogenization by pipetting to release intracellular MAP. Ribonucleic acid from 300 µl of cell lysate were extracted using the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's recommendations.

Extraction of RNA was followed by the performance of real-time RT-PCR for detection of cytokine and virulence gene expression using a SYBR Green RT-PCR kit (Sigma Aldrich, St. Louis, USA) on a 96-well, 0.2 ml, QuantStudio™ 5 PCR Cycler (ThermoFischer Scientific, Waltham, USA). The reactions total volume was 25 µl, containing 1 µl of each primer (10 µM), 0.025 µl Reference Dye, 0.125 µl reverse transcriptase (M-MLV RT) and 12.5 µl of SYBR Green Taq Ready-Mix. The temperature profile was a 2-minute initial activation at 94 °C, followed by 40 cycles of denaturation for 15 sec at 94 °C, annealing/extension for 1 min at 60 °C as recommended by the manufacturer. Reference (housekeeping) genes *Ig2* and Cancer susceptibility candidate 3A/metastatic lymph node 51 (*casc3A/mln51a*) for MAP virulence genes and RAW 264.7 cytokine normalization respectively were separately run. Primers for the specific cytokines, anti-apoptotic and virulence genes are given in Table 8.

Table 8: Primers used in the cytokine, virulence gene expression and MAP enumeration study

Gene name	Primer Sequence (5'– 3')	Primer Sequence (5'– 3')	Size	Source
	Forward	Reverse	(bp)	
MAP enumeration				
F57	GCCCATTTTCATCGATACCC	GTACCGAATGTTGTTGTCAC	254	Accession
Probe	6FAM– CAATTCTCAGCTGCAACTCGAA CACAC - BHQ1			no. X70277
Cytokines				
IL-12P40	TCAGACCAGAGCAGTGAGGT	GCAGGTGAAGTGTCAGAAT	243	(Smeed et al. 2007)

<i>IFNγ</i>	GGTCATTCAAAGGAGCATGGA	CTGCCATTCAAGAACTTCTGA	55	
<i>TNFα</i>	CGCATTGCAGTCTCCTACCA	GGGCTCTTGATGGCAGACA	238	
<i>TGFβ</i>	TGAGCCAGAGGCGGACTACT	TGCCGTATTCCACCATTAGCA	169	
<i>IL-10</i>	CTTGTCGGAAATGATCCAGTTTT	TCAGGCCCCGTGGTTCTCA	305	
<i>IL-6</i>	GCTGCTCCTGGTGATGACTTC	GGGTGGTGTCATTTTTGAAATCTT	236	
<i>BCL2-1</i>	CACCCAGGGACAGCATA	CGTCCCGGAAGAGTTCATTC		(Abendano et al. 2013)
Virulence genes				
<i>impA</i>	CTGACCTGGTTGCCGTTC	GCGGGATTTCGTTCTTGC	76	(Pribylova et al. 2011)
<i>kaiG</i>	CAACCAGGGCAAGTTCGTC	AAGCGGTCGTTGTTTCATCAC	66	
<i>kdpC</i>	CACCGTTCGTGAGCCTCT	ATCTGGCCGAGCGAATAGT	76	
<i>papA2</i>	GGCGTTCCACAGAATCC	CAGACACATCGCCCTGAC	73	
<i>umaA1</i>	TTGACCTACACCCAGAAGCAG	AACCGTAAATCGCTCATCG	66	
Housekeeping genes				
<i>Ig2</i>	AAACGATTTGAACAAGGTGCTC	CGAATAGGGCGCTGAATG	119	(Pribylova et al. 2011)
<i>Casc3A/mln-51a</i>	CTGCCTTGTCTTTTGCAGTA	TGCAAGTACAGGAGCAGAAT		(Taylor et al. 2007)

7.2.7 Intracellular MAP quantification

At the end of the respective time intervals (3, 24 and 48 h), the medium was completely aspirated from the respective wells and the monolayer was washed with PBS. This was followed by the addition of Buffer AL and Proteinase K for cell lysis and manual homogenization by pipetting to release intracellular MAP. Deoxyribonucleic acid from 560 μ L of cell lysate were extracted using the QIAamp® DNA Blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. This was followed by quantification on performing real-time qPCR with LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany) on a QuantStudio™ 5 PCR Cycler. The reactions total volume was 20 μ L, containing 1.0 μ L of each primer (10 μ M), 0.5 μ L (10 μ M) of probe, 10 μ L of Light Cycler 480 Probe Master and 5.0 μ L of template (MAP DNA standard). The temperature profile was initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 40 s as

recommended by the manufacturer, using primers that target the F57 gene (Table 8). To quantify MAP, a synthetic molecular MAP F57 DNA Standard was produced by Invitrogen and a standard curve drawn using serial dilution (10^7 - 10^1) to calculate the number of MAP copies.

7.2.8 Data analysis

Data for relative gene expression for both cytokines and virulence gene as well as viability was entered into MS Excel and analysed. Relative gene expression ($\Delta\Delta C_t$) was calculated as previously reported by Taylor et al. (2007);

$$\Delta\Delta C_t = (C_t \text{ gene of interest} - C_t \text{ reference gene}) \text{ infected sample} - (C_t \text{ gene of interest} - C_t \text{ reference gene}) \text{ control}$$

7.3 Results

7.3.1 Cytokines and virulence genes expression during infection

The four different MAP isolates showed significant differences in the relative expression of the different virulence genes within the 3 h, 24 h, and 48 h time intervals (Figure 6). In general, MAP1 showed an initial up-regulation of most of the virulence genes, followed by down-regulation of *papA2* and *umaA1* genes and a final up-regulation of the *kdpC* gene. MAP2 shows a down-regulation of all genes after the 24 h time point (TP). MAP3 on the other hand, showed pronounced down-regulation of the *katG* and *umaA1* genes at all time points. The rest of the genes showed variable levels in relative expression at different time points. Likewise, MAP4 exhibited pronounced down-regulation of *katG* and *umaA1* genes up to the 24 h time point. The other genes showed variable degrees of relative expression for the different time points (3 h, 24 h and 48 h); however, all the genes were up-regulated after the 24 h time point.

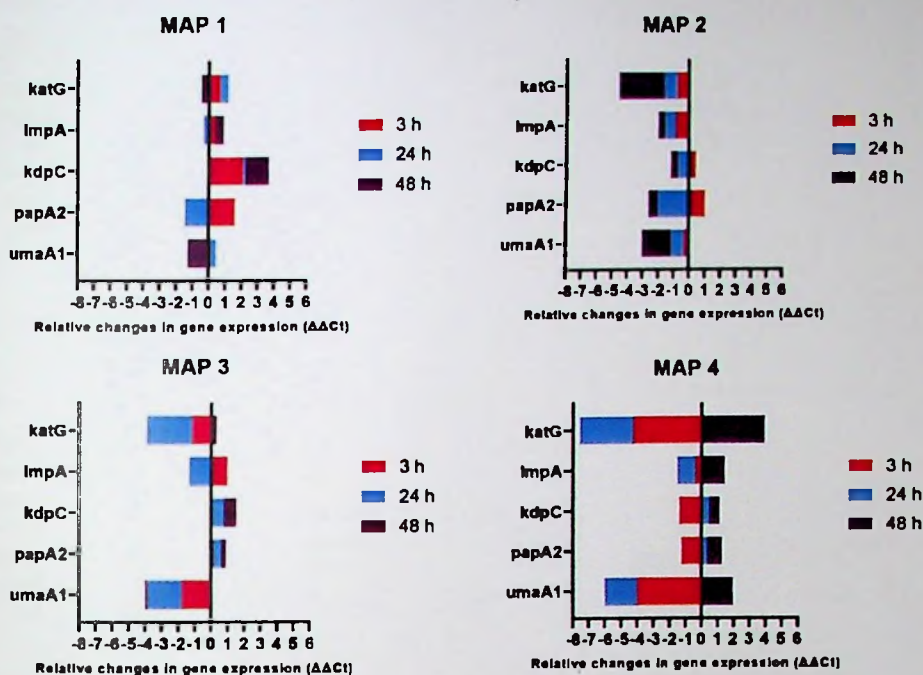


Figure 6: Relative changes in virulence gene expression profiles of MAP isolates after 3 h, 24 h and 48 h of infection

For cytokine expression, the very non-homogeneous picture with regard to the up- and down-regulation of the individual cytokines between the different isolates is striking (Figure 7). For MAP1, all cytokines were down-regulated between the 3 h and 24 h time points though there was a remarkable up-regulation of IL-6 at the 48 h time point. Macrophages infected with MAP2 showed relative up-regulation of the pro-inflammatory cytokines (IFN- γ , TNF- α , IL-12p40) and a down-regulation of the anti-inflammatory cytokines (IL-10, IL-6, TGF- β) after the 24 h TP. Isolate MAP3 exhibited a degree of variability in the relative cytokine expression with no clear-cut differences between the 3 h and 24 h TPs; though, after 24 hours, IL-12p40, IFN- γ and TGF- β were up-regulated. Isolate MAP4 on the other hand showed a clear down-regulation for all cytokines at the 3 h time point. Transforming growth factor- β was further down-regulated at the 24 h time point, though TGF- β and IL-10 were up-regulated after the 24 h time point. The anti-apoptotic (*Bcl2*) gene was down-regulated in MAP1, MAP3 and MAP4; it was only up-regulated in MAP2 after 24 h TP.

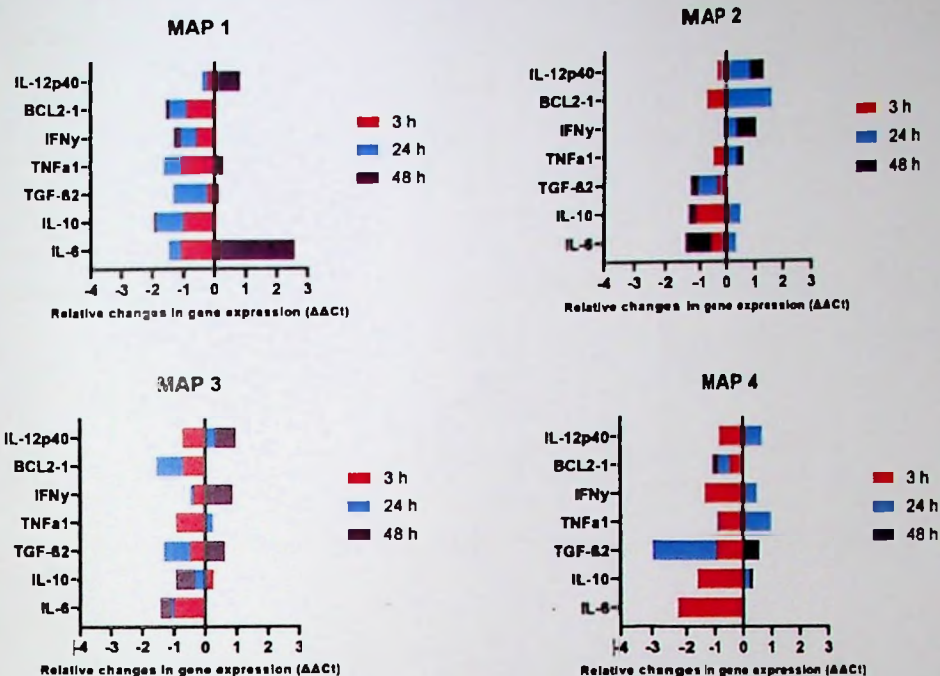


Figure 7: Relative changes in cytokine expression profiles of MAP isolates after 3 h, 24 h and 48 h of infection

7.3.2 Quantification of intracellular MAP

All MAP isolates replicated as evidenced by increase in intracellular DNA albeit at a low rate within 48 h (less than 0.5 log₁₀ levels). Only MAP2 showed a slight reduction of the intracellular DNA amount after 48 h (less than 0.5 log₁₀ levels). In general, the changes in intracellular MAP DNA at all time points and in all isolates can be considered less appreciable (Figure 8).

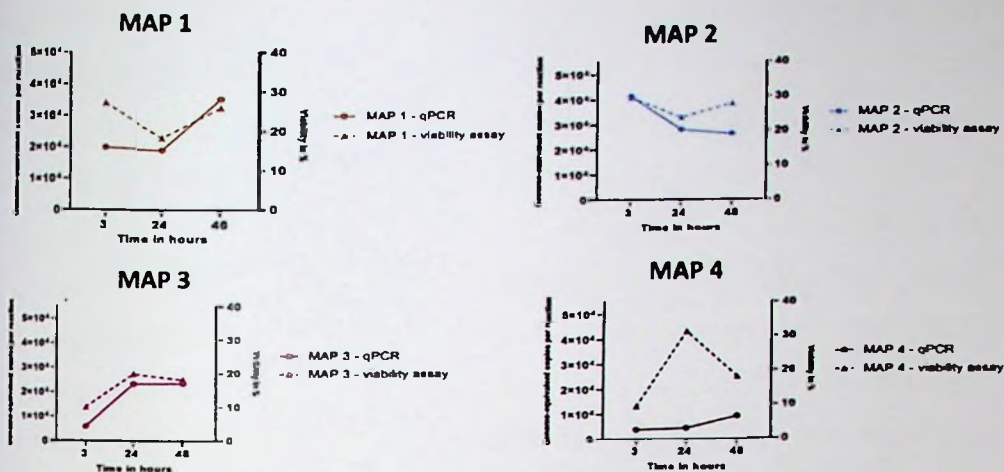


Figure 8: Intracellular MAP concentration and viability over time

7.3.3 Viability

The viability study revealed that the percent reduction of AlamarBlue and thus, cell activity followed that of MAP DNA quantities and this was true for MAP1, MAP2 and MAP3 for the 48 h infection period. MAP4 on the other hand, showed a reduction in viability against a slight increase in MAP quantity after the 24 h infection period. In addition, MAP4 was in low quantities intracellularly compared to the other isolates (Figure 8).

7.4 Discussion

For effective diagnosis and control of PTB, the behaviour of MAP inside the host cells and how it causes subsequent infection needs to be understood. In this study, we observed the pattern of regulation of some putative virulence genes of MAP, anti-apoptotic gene (*Bcl2*), MAP survival inside macrophages as well as the behaviour of some cytokines involved in inflammatory processes in RAW 264.7 macrophages infected with different MAP strains. Macrophages infected with MAP are known to produce different cytokines in response to the infection.

From the current experiment, macrophages infected with MAP1 isolate exhibited significant up-regulation of IL-6 after the 24 h infection period. Other cytokines including the tumour necrosis factor - alpha (TNF- α), interferon - gamma (IFN- γ) and interleukin -12p40 among the pro-inflammatory cytokines; IL-10 and transforming growth factor - beta (TGF- β) among the anti-

inflammatory cytokines (Coussens 2007; Hirata et al. 2008) varied considerably in regulation. The behaviour of MAP1 in regard to IL-6 response is suggestive of MAP survival strategy inside macrophages. For MAP2 on the other hand, the behaviour of macrophages during a typical infection was exhibited where the pro-inflammatory cytokines were up-regulated whereas the anti-inflammatory cytokines were down-regulated in response to the infection. This response is an indication of virulence attribute. In MAP3 and MAP4, the cytokine expression profile is not suggestive of a particular trend however, they both show an initial down-regulation of all cytokines and eventual up-regulation later in the infection. The anti-apoptotic gene (*Bcl2*) is generally down-regulated in all isolates except MAP2 which shows an up-regulation after 24 h TP. MAP is known to interfere with normal macrophage function by inhibiting apoptosis thereby promoting its survival in macrophages (Abendaño et al. 2014). MAP2 is demonstrating more ability to survive inside macrophages compared to the other isolates in this regard.

One of the previous studies showed an up-regulation of anti-inflammatory cytokines such as interleukin-6 (IL-6) and IL-10 which favours MAP survival intracellularly (Abendaño, Juste, and Alonso-Hearn 2013). A similar trend was exhibited by MAP1 in regard to IL-6. Different MAP strains have been shown to differ in their ability to survive in monocytes-derived macrophages (MDM). In an earlier study, it was observed that MAP strains of distinct genotype varied in their survival in MDM (Abendano et al. 2013). These MAP strains belonged to different hosts and from that study, it was concluded that MAP strains express differential levels of virulence due to host associations. In another study, MAP strains of bovine and human origin were used to infect MDM; an anti-inflammatory response was observed with up-regulation of IL-10 and down-regulation of the TNF- α (Janagama et al. 2006). The trends observed in the current study are suggestive of variable virulence among MAP isolates.

Some of the probable MAP virulence genes investigated in this study included: *kdpC*, *katG*, *papA2*, *impA* and *umaA1*. These genes are involved in cellular pathways that play different roles in enabling MAP to evade the host defence mechanisms and cause an infection in a susceptible host (Shin et al. 2006). In the current experiment with RAW 264.7 macrophages, there were remarkable differences in the relative expression of the virulence genes for all the four isolates at the different time points (TP). Particularly, MAP2 showed significant down-regulation of all virulence genes after the 24 h

TP. On the other hand, MAP4 exhibited marked down-regulation of all virulence genes in the initial 24 h, but later they were all up-regulated particularly *katG* and *umaA1* genes. A similar trend was observed in MAP3 though to a less extent with up-regulation of *kdpC* gene after 24 hours. The *umaA1* gene was reported to play a role in organ colonisation and cell biosynthesis (Settles, Kink, and Talaat 2014). The increased expression of the *katG* gene; a probable catalase/oxidase, is believed to be in response to the oxidative stress encountered by MAP within the macrophage environment as was reported earlier (Granger et al. 2004). In contrast, MAP1 did not show marked differences in gene expression between the up- and down-regulation profiles though *kdpC* gene was up-regulated throughout the experimental period as was the case with MAP3. The *kdpC* gene, a probable potassium-transporting ATPase C chain has been shown to be associated with organ colonisation and granuloma formation in MAP (Shin et al. 2006). These observations are suggestive of possible differences in virulence mechanisms of these strains.

MAP cell viability and concentration inside macrophages varied considerably among the isolates though with an overall increase after the 24 h TP and indication of successful replication inside macrophages. MAP1 in particular exhibited significant increase in number intracellularly after 24 hours of infection. MAP4 on the other hand, exhibited low levels of survival inside the macrophage at all time points. The viability dropped after the 24 h TP an indication of intracellular killing by macrophages unlike the other isolates which had significant amounts of MAP surviving inside macrophages up to the 48 h TP. All MAP isolates except MAP4 had significantly high numbers of copies (over 2×10^4 genome equivalents) after the 24 h TP; this is an indication of variable survival abilities of the different isolates, particularly MAP4 showed a lower survival ability. Generally, there was minimal appreciable increase in the amount of MAP cells inside macrophages for all strains at all time points implying a low multiplication rate. This is due to MAP having a long generation time of over 20 h (Elguezabal et al. 2011). Hence significant changes could not be observed during the 2-day incubation period of the experiment. We could not afford to go beyond 48 h of infection, as the RAW 264.7 macrophages were overgrown after this TP and were no longer confluent monolayer. For future studies, we recommend use of macrophages with a slow replication rate to avoid this problem and allow for longer experimental period if desired.

7.5 Conclusion

Overall, the different MAP isolates exhibited varying degrees of virulence in regard to cytokine and apoptotic gene responses. However, MAP1 exhibited more ability to survive and replicate inside macrophages, whereas MAP4 on the other hand exhibited least ability to survive compared to the other isolates. These assays present a way of understanding the behaviour of MAP inside macrophages and these isolates can be further assessed for their immunogenicity potential. Since PTB takes a long time to develop clinical disease, these *in vitro* models will aid the understanding of MAP pathogenesis. Virulence genes are proposed as possible targets to understand the behaviour of MAP inside the host macrophages. For future studies however, we recommend the use of models that mimic the *in vivo* environment of the host to better understand how MAP organisms interact with the host in a natural intercellular environment.

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CHAPTER EIGHT

PHYLOGENETIC ANALYSIS OF THE PUTATIVE VIRULENCE GENES OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* AMONG DIFFERENT ISOLATES FROM UGANDAN CATTLE

(Manuscript)

8.0 Abstract

Knowledge of the pathogenesis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the cause of paratuberculosis in animals is generally scanty yet it is important in the design of control and preventive approaches to manage the disease. In this study, we have analysed selected putative virulence genes of MAP for any polymorphisms resulting from insertions, deletions, or substitutions that may cause variations in virulence of the different MAP isolates. Phylogenetic analysis of the virulence genes of MAP from this study was done in comparison with those from the reference strain (MAP-K10) and other mycobacterial strains obtained for GenBank. Four MAP isolates (MAP1, MAP2, MAP3 and MAP4) from a recent study in Uganda (details in chapter seven) were sequenced and putative MAP virulence genes were identified, these included: the putative inositol monophosphatase (*impA*), S-adenosylmethionine-dependent methyltransferase (*umaA1*), potassium-transporting ATPase (*kdpC*), probable catalase/oxidase (*katG*), trehalose-2 sulfate acetyltransferase (*papA2*) and the putative oxidoreductase (*fabG2_2*). Phylogenetic analysis revealed that only the *impA* and *papA2* genes were conserved when compared with the reference MAP K-10 strain. Among the four isolates, all the virulence genes were similar except the *fabG2_2* gene of MAP4 isolate which deviated from the other isolates. Phylogenetic trees of the different virulence genes belonging to the four isolates revealed evolutionary deviations from the MAP-K10 and other mycobacteria. This is an indication that the MAP strains from Uganda have significant genomic differences and hence may be the source of the variations in strain virulence. More investigations are needed to understand how the genomic differences are expressed phenotypically in the pathogenesis of the disease in the host and whether there are other factors responsible for MAP virulence, and how these can be exploited in the design of strategies for the management of paratuberculosis.

Key words

Phylogenetic analysis, putative virulence genes, *Mycobacterium avium* subsp. *paratuberculosis*

8.1 Introduction

Paratuberculosis (Johne's disease) causes enormous economic losses in livestock production worldwide especially dairy cattle and is also highly suspected to be zoonotic (Garcia and Shalloo 2015; McNees et al. 2015). The disease characterised by chronic granulomatous enteritis is most common in ruminants though other animals may be affected (Stevenson et al. 2009). The causative agent, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been frequently isolated from patients with Crohn's disease, as well as from many other gastro-intestinal diseases, hence the suspicion that it could be zoonotic (Elmagzoub et al. 2022; Dow and Alvarez 2022). MAP is a member of the non-tuberculous mycobacteria and belongs to the *Mycobacterium avium* complex (MAC) group (Saviola and Bishai 2006).

There are several strains of MAP currently recognised based on growth and morphological characteristics of the bacterial colonies as well as on sequence typing techniques and they include: Cattle strain "Type C", Sheep strain "Type S" and the Bison "Type B" (Collins, Gabric, and de Lisle 1990; Whittington, Marsh, and Whitlock 2001). Sequence-based typing techniques for MAP genetic diversity include: the IS900 restriction fragment length polymorphism (RFLP), IS1311 RFLP, short sequence repeats (SSR); mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) method; single nucleotide polymorphism (SNP) typing (Conde et al. 2021; Dimareli-Malli et al. 2009; Sevilla et al. 2008; Bryant et al. 2016); among others. Based on these techniques, MAP strains have been grouped as Type I, these are predominantly S-Type, Type II are mostly Cattle-Type and also found in other animals and humans, and Type III appear to be a sub-group of the S-Type (Bryant et al. 2016). These strains are believed to vary in virulence in the different host species (Stevenson 2015). The variation in MAP strain virulence is believed to be genetically regulated involving certain genes. However, knowledge on pathogenesis of MAP and the virulence attributes of the different strains in different livestock species is scanty (Barkema et al. 2018; Okuni et al. 2020). Understanding MAP's behaviour and pathogenesis is important in disease management (Bryant et al. 2016).

Comparative genomics reveals genotypic variations between or within strains that could be responsible for differences in behaviour among strains (Hardison 2003; Eddy 2005). These differences may be observed in genes that confer virulence among pathogenic organisms on WGS

analysis (Wu et al. 2006). *Mycobacterium avium* subsp. *paratuberculosis* whole genome sequencing was first carried out by Li and colleagues (Li et al. 2005) on the reference strain, MAP-K10 though several WGS of the MAP genome have been done since then (Bannantine et al. 2014; Brauning et al. 2019). With the introduction of next generation sequencing (NGS) techniques, genomic analyses are now frequently based on WGS. A recent study has employed WGS analysis to distinguish among the different MAP strain types and predicted phenotypic features of MAP strain pathogenicity basing on genetic polymorphisms (Lim et al. 2021). WGS analysis has also been recently employed in a study to investigate virulence among two MAP isolates in comparison to the MAP-K10 reference strain and significant genomic differences were observed that could explain the variations in virulence (Colombatti Olivieri et al. 2022). MAP strains from Uganda were shown to have very high genetic diversity in an earlier study and this implied that there could be variations in strain virulence (Okuni et al. 2012). Several MAP putative virulence genes were identified by means of transposon mutagenesis, and these included: non-ribosomal peptide synthetase (*pstA*), the probable inositol monophosphatase (*impA*), possible mycolic acid synthase (*umaA1*), isoprenoid biosynthesis (*gcpE*), conserved polyketide synthase (*papA2*), putative oxidoreductase (*fabG*) and probable potassium-transporting ATPase (Shin et al. 2006; Wu et al. 2009). In the present study, these putative virulence genes were hypothesized to be responsible for the variable virulence observed in a murine infection model of four MAP isolates in one of our recent study (Chapter 7.0). These genes were selected to perform genomic analysis where the study isolates were compared with reference sequence (MAP-K10) to search for similarities or differences due to mutations or other forms of polymorphisms. Phylogenetic trees were drawn to further compare the virulence factors in the study strains to those of reference strains. The study strains were also compared with strains from other countries to determine their phylogenetic relationships. All these are important to elucidate MAP strain diversity and differential virulence which may provide useful tools for disease management.

8.2 Materials and methods

8.2.1 MAP cultivation

Four MAP isolates were obtained from a recent PTB study carried out in Uganda (discussed in chapter four). The method of cultivation of the isolates is as described earlier in chapter seven

(Section 7.2.1). Briefly, faecal and blood samples collected from cattle and tested positive for MAP DNA and MAP antibodies respectively were cultured on Herrold's Egg Yolk agar (HEYM) slants (Becton, Dickinson and Co., Sparks MD 21152, USA) for a period of more than four months. All MAP-positive cultures were sub-cultured into BBL™ Middlebrook 7H9 broth produced by Becton, Dickinson and Co. (Sparks MD 21152, USA) with glycerol and supplemented with Mycobactin J (IDvet, Rue Louis Pasteur – Grabels – France) in a 15 ml centrifuge tube (Sarstedt, Numbrecht, Germany) for three weeks.

8.2.2 MAP DNA extraction

The modified CTAB method was used (van Helden et al. 2001) for the extraction. Approximately 10 mL of cell suspension were heated at 80 °C for 5 min to kill the cells. This was followed by centrifugation at 3000g for 10 min to obtain a cell pellet. The pellet was then washed twice by resuspension into 1 ml of sterile 1×TE (pH 8.0) in a 2 ml Eppendorf tube (Hamburg, Germany). Brief homogenization by vortexing was followed by centrifugation at 5500 rpm (iFuge D12, India) for 5 min at room temperature (RT) and supernatant discarded. One hundred microlitres of sterile 1×TE were added and pellet was resuspended by vortexing. Then, 50 µl of freshly prepared 40mg/ml lysozyme was added, mixed gently and incubated overnight at 37 °C at 300rpm. This was followed by addition of 70 µl of freshly prepared 10% SDS and 5 µl of 20mg/ml proteinase K and mixed by inversion at least 10 times and incubated for 1 hour at 56 °C. The sample mixture was allowed to cool at RT for 5 min and then 100 µl of 5M NaCl and 100 µl of CTAB/NaCl solution (pre-warmed) added. The mixture was then inverted until it turned milky. This was then incubated at 65 °C for 1 hour. An equal volume (approximately 500 µl) of freshly prepared Chloroform:Isoamyl alcohol in the ratio 24:1 was added and inverted at least 10 times. The mixture was centrifuged at 5500 rpm (iFuge D12, India) for 15 min to form 3 phases. A 200 µl pipette was set at 150 µl and carefully transferred the upper aqueous phase in a sterile 1.5 ml microfuge tube; this was repeated once. An equal volume of analytical grade absolute isopropanol was added to the mixture and mixed gently by inverting at least 10 times. The mixture was then incubated at – 20 °C for 1 hour. This was then spun at 15,000 rpm (iFuge D12, India) for 10 min. The supernatant was pipetted off carefully without dislodging the pellet and discarded. One millilitre of 70% alcohol was added to the pellet and mixed gently by inverting the tube at least 10 times. The tube was spun at

15,000 rpm (iFuge D12, India) for 5 min and the supernatant pipetted off without dislodging the pellet. The tube was opened and left to air dry at RT for 20 min. One hundred microlitres of sterile 1×TE buffer (pH 8.0) or water (molecular biology grade) were added and the tube tapped at the bottom and incubated at 55 °C (for about 10 min) until the pellet was completely dissolved. The DNA was stored at – 80 °C for later use. The quality and quantity of the DNA was assessed using a nanodrop spectrophotometer and purity was determined at 260/280 nm absorbance.

8.2.3 Sequence data analysis

To obtain sequences for the putative MAP virulence genes, MAP DNA was sequenced by whole genome sequencing and analysis was performed to identify the virulence genes for each individual isolate. Phylogenetic analysis of the different virulence genes was done by comparing the respective virulence genes of the MAP isolates with related genes of the reference MAP K-10 strain and other mycobacteria. The Molecular Evolutionary Genetics Analysis tool (MEGA 11) was used to perform the multiple sequence alignments and to draw phylogenetic trees to infer relationships among the different isolates (Tamura, Stecher, and Kumar 2021). Phylogeny was inferred by Maximum likelihood and tested by the Bootstrap method using the Tamura-Nei model (Tamura and Nei 1993).

8.3 Results

Table 9 presents the selected putative MAP virulence genes identified in the four MAP genomes.

Table 9: Putative MAP virulence genes identified in the four genomes

Gene	Size (bp)	Name/probable role
<i>umaA1</i>	861	S-adenosylmethionine dependent methyltransferase
<i>kdpC</i>	876	Potassium-transporting ATPase subunit
<i>katG</i>	2247	Catalase/peroxidase
<i>papA2</i>	1518	Trehalose-2-sulfate Acetyltransferase
<i>impA</i>	801	Putative inositol 1-monophosphatase
<i>fabG2_2</i>	789	Putative oxidoreductase

8.3.1 Sequence alignments and phylogenetic trees

Alignment of the putative virulence genes from the study isolates with similar genes from the reference MAP K-10 strain revealed some deviations though some were conserved. Differences were also revealed on alignment with other mycobacteria. The alignment of the different MAP putative virulence genes with the reference strain and other mycobacteria as well as the corresponding phylogenetic trees are presented in the subsequent sections.

The probable inositol monophosphatase (*impA*) gene of the study isolates was conserved (Figure 9). However, some divergence was observed with other mycobacteria such as *M. lepraemurium* and *M. avium* subsp. *hominissuis* as shown by the phylogenetic tree (Figure 10).

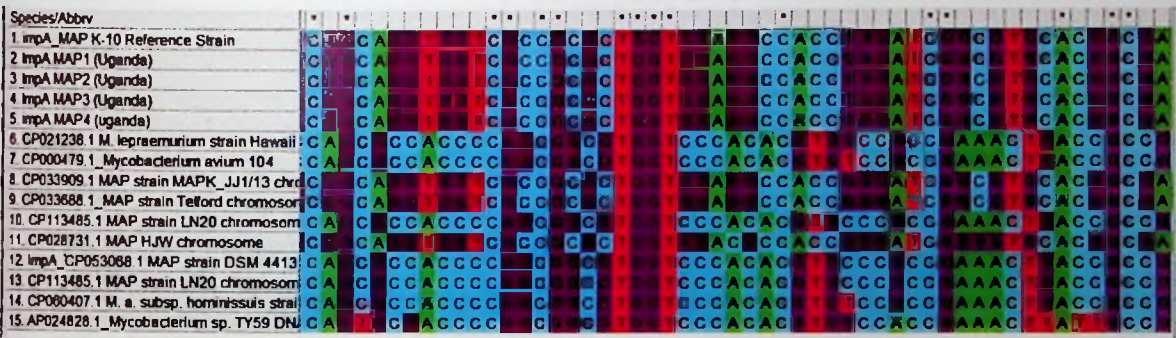


Figure 9: Alignment of the *impA* gene sequence of the four MAP isolates with reference MAP-K10 and other mycobacteria

Legend: Alignment of the *impA* gene with the reference MAP-K10 revealed that it was conserved. Divergence was however observed with other mycobacteria

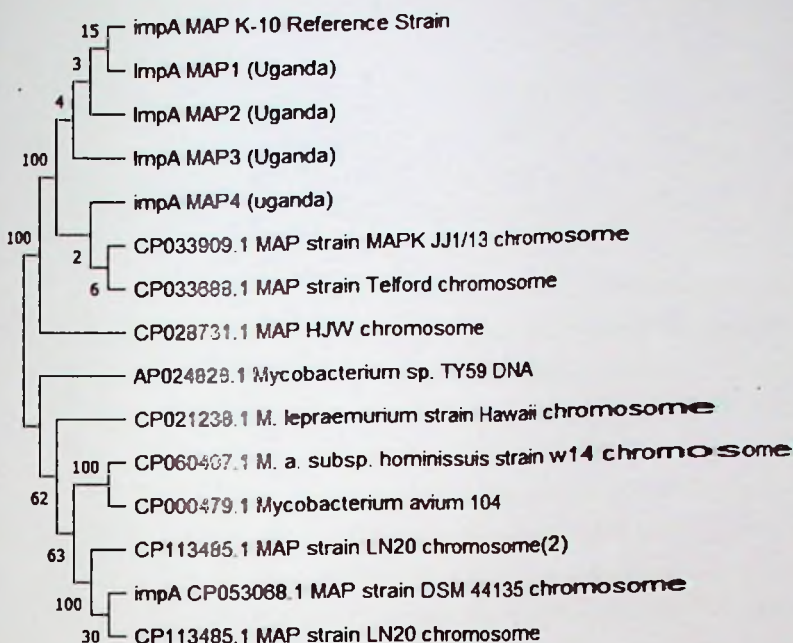


Figure 10: Phylogenetic tree of the *impA* gene aligned with mycobacterial strains

The trehalose-2-sulfate acetyltransferase (*papA2*) gene was also analyzed. The phylogenetic tree revealed two distinctive clusters of the *papA2* (Figure 12).

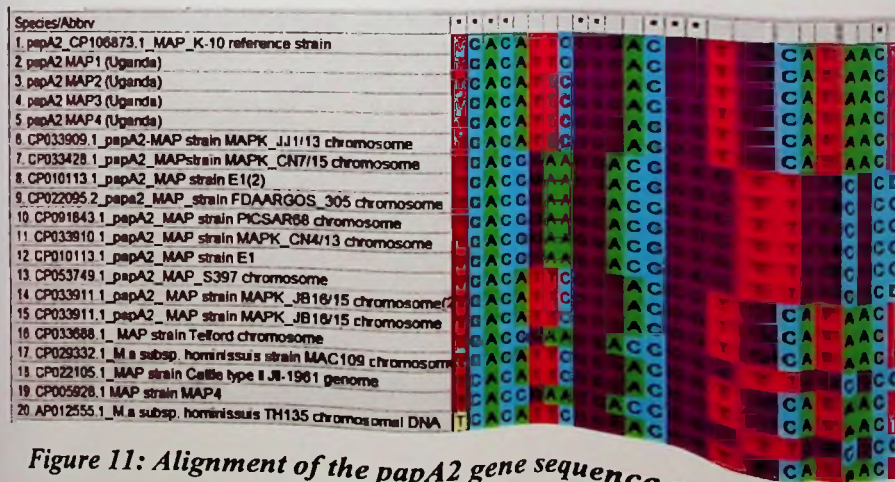


Figure 11: Alignment of the *papA2* gene sequence of the four *M.* and other mycobacterial strains

Legend: The papA2 gene aligned with the reference MAP-K10 revealed that it was conserved though divergencies were observed with other mycobacterial strains

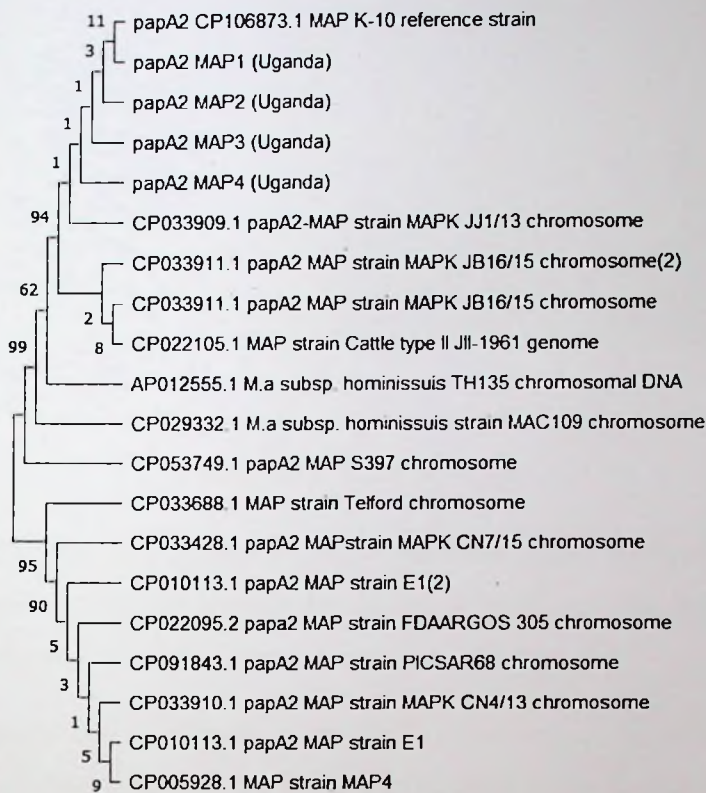


Figure 12: Phylogenetic tree of the papA2 gene aligned with the reference strain with other mycobacterial strains

Alignment of the study isolates with other mycobacteria and the reference strain revealed high variation in the putative oxidoreductase (*fabG2_2*) gene (Figure 13).

Species/Abbrev	
1 fabG2_2_CP106873.1_MAP_K-10 reference strain chromosome	
2 fabG2_2_MAP1 (Uganda)	
3 fabG2_2_MAP2 (Uganda)	
4 fabG2_2_MAP3 (Uganda)	
5 fabG2_2_MAP4 (Uganda)	
6 MTB_fabG2_2_CP054014.1_Mtb strain FDAARGOS_756 chromosome	
7 MTB_fabG2_2_CP047163.1_Mtb strain TDC7 chromosome	
8 CP021238.1_M. lepraemurium strain Hawaii chromosome fabG2_2	
9 CP000479.1 Mycobacterium avium 104_fabG2_2(2)	
10 CP000407.1 M.a subsp. hominissuis strain w14 chromosome fabG2_2	
11 CP089223.1_Mycobacterium avium subsp. avium strain fabG2_2	
12 AP020326.1_M.a subsp. hominissuis JP-H-1 DNA fabG2_2	
13 CP028731.1_M.a strain HJW chromosome fabG2_2	
14 CP089223.1_MAP strain FDAARGOS_1609 chromosome	
15 CP005926.1_Mycobacterium avium subsp. paratuberculosis MAP4_fabG2_2	
16 CP021238.1_M. lepraemurium strain Hawaii chromosome fabG2_2	
17 CP091843.1 MAP PICSA88 isolate 15-9556_3 chromosome fabG2_2	
18 CP000479.1 Mycobacterium avium 104_fabG2_2	
19 CP000407.1_M.a subsp. hominissuis strain w14 chromosome fabG2_2	
20 CP113485.1 MAP strain LN20 chromosome fabG2_2	
21 CP053068.1_MAP strain DSM 44135 chromosome fabG2_2	



Figure 13: Alignment of *fabG2_2* gene sequence from the four and other mycobacterial strain

Legend: Isolate MAP4 exhibited variations from the other alignment. The phylogenetic tree drawn for the *fabG2_2* gene among the different isolates.

There were three distinctive clusters formed by the different strains of the *fabG2_2* gene. Isolate MAP4 (Uganda) did not group with the other isolates (MAP1, MAP2, MAP3) and the reference strain (Figure 14).

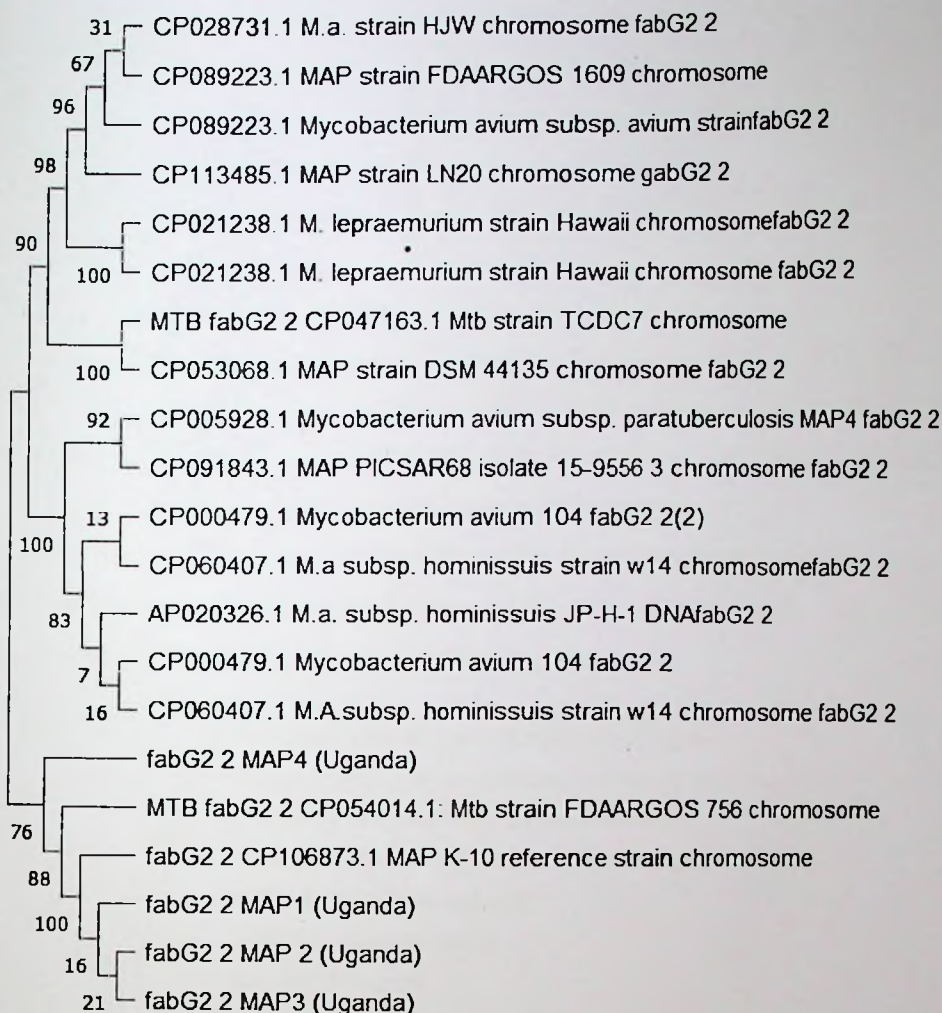
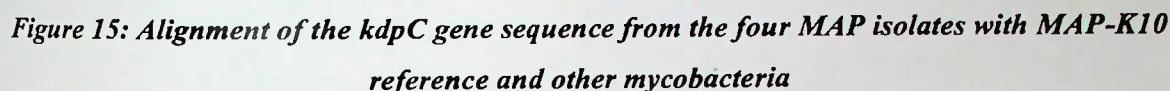


Figure 14: Phylogenetic tree based on the *fabG2_2* gene of the four MAP isolates aligned with the MAP K-10 reference strain and other mycobacteria

The potassium-transporting ATPase (*kdpC*) gene of the study isolates deviated from that of the reference strain (Figure 15). Phylogenetic tree produced different clusters for the different mycobacterial species (Figure 16).



Phylogenetic tree showing the relationship between various *kdpC* sequences. The tree is rooted on the left and branches to the right. Bootstrap values are indicated at the nodes. The sequences are listed on the right, with some color-coded: purple for KdpC MAP3 (Uganda), KdpC MAP4 (Uganda), KdpC MAP2 (Uganda), and KdpC MAP1 (Uganda); green for CP028731.1 kdpC M.a strain HJW chromosome, CP085978.1 kdpC M.A strain FDAARGOS 1808 chromosome, CP023147.1 kdpC M. marseillense strain FLAC0026 chromosome, and kdpC AP024247.1 M. intracellulare M007 DNA; and black for the remaining sequences.

Sequences and their corresponding bootstrap values (from top to bottom):

- CP053068.1 kdpC MAP strain DSM 44135 chromosome (7)
- CP091843.1 kdpC MAP strain PICSAR88 isolate 15-9556 3 chromosome (1)
- CP033688.1 KDPc MAP strain Telford chromosome (0)
- CP022095.2 kdpC MAP FDAARGOS 305 chromosome (0)
- CP010114.1 kdpC MAP strain E93 (0)
- CP005928.1 kdpC MAP MAP4 (3)
- KdpC MAP3 (Uganda) (2)
- KdpC MAP4 (Uganda) (8)
- KdpC MAP2 (Uganda) (97)
- KdpC MAP1 (Uganda) (97)
- CP028731.1 kdpC M.a strain HJW chromosome (97)
- CP085978.1 kdpC M.A strain FDAARGOS 1808 chromosome (27)
- CP023147.1 kdpC M. marseillense strain FLAC0026 chromosome (27)
- kdpC AP024247.1 M. intracellulare M007 DNA (100)
- CP035744.1 kdpC M.A subsp. hominissuis strain MAH11 chromosome (99)
- kdpC CP106873.1 MAP strain K-10 Reference (93)
- CP022105.1 kdpC MAP strain JIL-1961 genome (27)
- CP113485.1 kdpC MAP strain LN20 chromosome (27)

Figure 16: Phylogenetic tree based on the *kdpC* gene of the four MAP isolates aligned with the reference MAP strain and other mycobacteria

The s-adenosylmethionine-dependent methyltransferase (umaA1) study isolates though there were some differences observed (UmaA1 19). The *umaA1* gene is not conserved hence the reference strains (Figure 20).

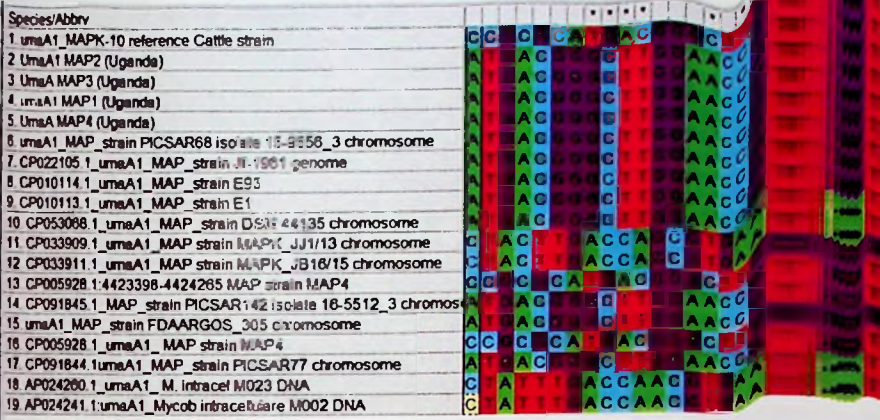


Figure 19: Alignment of the *umaA1* gene sequence from reference strain and other mycobacteria.

Legend: The *umaA1* gene was not conserved as observed from the phylogenetic tree revealed clustering of the Uganda isolates with other closely related countries.

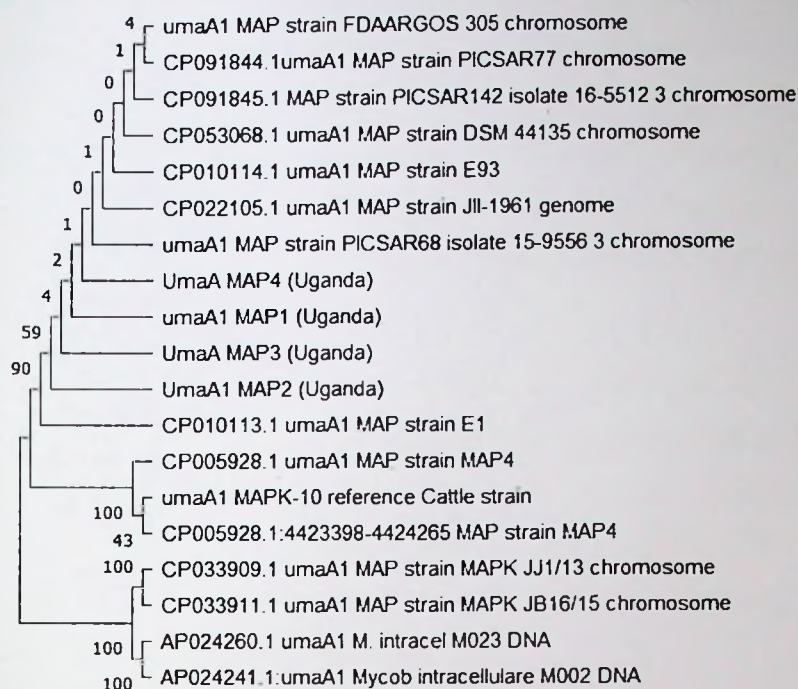


Figure 20: Phylogenetic tree of the *umaA1* gene for the four MAP isolates aligned with the MAP K-10 reference Cattle strain and other mycobacteria

8.4 Discussion

Understanding the pathogenesis of MAP, the causative agent of paratuberculosis in animals is key in designing strategies for the control and prevention of this devastating infection which causes enormous losses in the livestock industry especially dairy production (Whittington et al. 2019). In an attempt to decipher the mechanisms by which MAP causes disease, we performed an *in vitro* RAW 264.7 macrophage infection model in an earlier study (chapter seven) and observed the behaviour of different MAP isolates inside macrophages. Attributes such as MAP viability and quantification, cytokine and virulence gene expression at different time points were observed. From the analysis, it was discovered that the isolates behaved differently inside macrophages in terms of gene expression, quantity and viability. Isolate MAP4 in particular, behaved more diversely in ability to survive inside murine macrophages. It had lower viability and quantities compared to the other three isolates.

With this background, a genomic analysis of the four isolates was performed to examine whether the behavioral differences observed were arising as a result of polymorphisms within the different genes. In particular, we compared the putative virulence genes of the study isolates with the reference MAP-K10 genome to look for similarities or differences arising from polymorphisms within the genomes. Gene alignments and phylogenetic trees of the four study genomes with the reference strain and other mycobacteria were drawn to compare and infer phylogenetic relationships among them.

In a recent study, Colombatti and colleagues compared virulence levels of two strains of MAP Cattle type using *in vitro* macrophage infection and observed significant differences between them. Whole genome sequence analysis revealed differences in the genomic structure of the isolates in comparison to the reference MAPK-10 strain (Colombatti et al. 2022). Similarly, in the present study, differences were observed when putative MAP virulence genes of the study isolates were compared with those of the reference MAP K-10 strain.

The *impA* gene encodes the probable inositol monophosphatase and is believed to play a role in cell wall permeability and MAP persistence in macrophages (Nigou and Besra 2002). In the current analysis, the *impA* gene was found conserved as in the reference strain. However, some differences were exhibited when it was compared with mycobacteria of other species such as *M. lepraemurium* and *M. avium* subsp. *hominissuis*. The *umaA1* gene encodes a possible mycolic acid methyltransferase believed to play a role in bacterial cell biosynthesis (Li et al. 2005). It contrasted in the MAP-K10 reference strain and other mycobacteria such as *M. intracellulare* but was similar in all the study isolates. The *kdpC* gene was likewise the same in all the study isolates though it deviated from that of the reference. The *kdpC* gene is the probable potassium-transporting ATPase subunit believed to be involved in organ colonisation and granuloma formation in MAP pathogenesis (Shin et al. 2006). The *papA2* gene on the other hand is an acetyltransferase believed to be involved in mycobacterial virulence; in MAP it is associated with tissue colonisation (Shin et al. 2006). The *papA2* gene was the same as in the reference strain and in all the four study isolates. The *katG* gene is a probable catalase/oxidase believed to play a role in MAP survival inside macrophages when it counteracts the oxidative stress inside the macrophage (Granger et al. 2004). The *katG* gene was similar across the four study genomes but deviated from the reference strain.

All the putative virulence genes were similar in the study isolate except *fabG2_2* which was different in MAP4 isolate. The *fabG2_2* gene encodes the *fabG* protein in MAP (Li et al. 2005). It is proposed to play a role in bacterial survival in *in vitro* macrophage infection study (chapter seven), isolate attributes compared to the other three isolates. It is not clear if the *fabG2_2* gene could be responsible for the observed variations in virulence. Further research is needed to investigate into the behaviour of the isolates for their possible role in virulence.

Since most of the targeted putative virulence genes were similar, it is possible that other genes could be contributing to the variations in virulence. Phylogenetic trees and molecular observations are useful tools to predict the evolutionary trends in MAP strain diversity. Further research is however needed to identify the putative virulence genes as well as other genes in MAP pathogenesis.

8.5 Conclusion and Recommendations

The putative virulence genes of the field isolates exhibited high similarity to the reference strain as well as from other mycobacterial species. The *katG*, *impA*, *kdpC*, *katG*, *umaA1* and *fabG2_2* genes when compared with other mycobacteria. Only *impA* and *papA2* genes were conserved. The variations in virulence for some of the variations in virulence observed among isolates (chapter seven). However, MAP strain virulence may as well be regulated by other genes in the genome. It is proposed that further analyses be performed to identify the role of *fabG2_2* in MAP pathogenesis and further investigate strains with high virulence. This study depicts a natural host to aid strategies for the identification of *M. avium* paratuberculosis management.

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CHAPTER NINE

GENERAL DISCUSSION

9.1 Discussion of major research findings

This study has revealed that paratuberculosis exists in all the six districts of western Uganda where the investigation was carried out. The disease may be widespread to other areas in the region and the country as a whole given the high herd-level prevalence obtained (64%) with the combined ELISA and Recombinase Polymerase Amplification (RPA) tests. Among districts, Ntoroko registered the highest MAP prevalence with both ELISA and the RPA tests (true prevalence of 8.5% and 6.2% respectively); the district registered a herd-level prevalence of 78% and 67% using ELISA and RPA respectively. The high PTB prevalence in this district could be as a result of communal grazing practiced by some of the farmers as well as due to limited pastures and water especially during the dry seasons which forces some farmers to move cattle from one place to another hence spreading the disease. On the other hand, Kiruhura registered the lowest MAP prevalence with both ELISA and RPA tests (true prevalence of 2.5% and 1.4% respectively). This can be explained from the evidence that majority of the farms in this district are fenced hence there is limited movement and interaction of animals which limits the spread of the infection.

Among the risk factors associated with paratuberculosis was long dry spells. Areas that experience prolonged periods of little or no rain suffer from scarce feeds and water resulting in poorly fed and unthrift animals which compromises the immune system of the animal making them susceptible to infections including PTB. The prolonged dry seasons also result in herds being moved over long distances in search of water and pasture which increases avenues for disease transmission among herds. Ntoroko district is one of the areas that experience prolonged dry weather conditions and also had highest PTB prevalence rates. Other risk factors investigated such as grazing practices, breeds of cattle, calf feeding methods, manure handling, and tree shed cover, among others; did not have association with MAP occurrence in the present study. Some of these factors were found to have an association with PTB in previous studies such as calf feeding, manure management and recent purchase of heifers (Doré et al 2012; Tiwari et al 2009). Most of these factors are connected with contact with faeces, hence limiting this would be beneficial to curtail the spread of PTB. In the present study, majority of the farms were extensively managed which limits the contact of animals with the infective faecal material unlike in intensive animal husbandry practices. The disease is most likely spread by cattle movement because currently, PTB is not one of the diseases checked

before animals are permitted to move to different parts of the country. This implies that MAP is likely to be transmitted to wider areas and may cause a major impact on livestock production especially the dairy industry in the country.

In this study, two diagnostic methods were used to test for MAP; serological test for MAP antibodies using ELISA and a molecular RPA method to test for MAP DNA from faeces. Using more than one test method was shown earlier to have the advantage of identifying more positives than would otherwise have been detected (Clark et al. 2008). In the current study, 58 and 49 MAP-positive cattle were identified using ELISA and RPA respectively. Of these, only six tested positive for both tests, hence a total of 101 cattle were positive based on the two tests; implying that there was an overall incremental yield in positivity for both tests.

Mycobacterium avium subsp. *paratuberculosis* was not detected from environmental samples as none of the samples tested positive. Infected animals shed MAP in faeces in the environment which contaminate pasture and water for livestock (Lombard 2011). This study has demonstrated that MAP in the environment may not be detected based on single sampling due to low bacterial numbers in the sample. There were some limitations in the environmental study; the sampling needed to be done on multiple occasions over a period of time. Environmental samples should be collected from different locations on the farm over a period of time (one to three years) as previously described (Eisenberg et al., 2013). Multiple sampling on different occasions is done to increase the probability of detecting MAP in the sample. In addition to molecular diagnostic methods, samples need to be cultured to determine the c.f.u/g of the environmental sample.

To assess the virulence of the different MAP strains identified in the PTB survey, an *in vitro* infection model using RAW 264.7 murine macrophages was employed. The study has demonstrated that this is a reliable method to study MAP virulence attributes. It has been revealed that MAP causes a variety of gene expression profiles for different cytokines and virulence genes. These attributes depict the behaviour of MAP in the intracellular environment of the host macrophages. The four MAP isolates exhibited various gene expression profiles at different time points and this depicts their variable virulence. The number of MAP cells inside RAW 264.7 macrophages varied considerably as well as their viability at different time points. This further revealed variations in ability of isolates to survive in macrophages and their ability to replicate inside host cells.

Knowledge of mechanisms employed by MAP to survive the intracellular defence system of the host is important to design approaches for disease control and prevention.

From the four MAP isolates, MAP1 exhibited a high-virulence attribute whereas MAP4 isolate exhibited a low-virulent attribute in relation to their inflammatory cytokine response and viability during the *in vitro* murine infection model. For MAP1 isolate, IL-6 was significantly up-regulated after 24 h which is a MAP survival strategy (Abendaño, Juste, and Alonso-Hearn 2013). The isolate also exhibited high numbers of viable MAP cells during the infection, an indication for ability to replicate and survive intracellularly. On the other hand, MAP4 isolate had the lowest number of viable MAP cells intracellularly during the infection period, an indication of low survival ability and its cytokine expression profile was not suggestive of virulent attribute.

The different MAP isolates were sequenced by WGS analysis to investigate polymorphisms within virulence genes that could be possible sources of the differential virulence observed in the *in vitro* infection model. Putative virulence genes of MAP including; *impA*, *umaA1*, *papA2*, *katG*, *fabG2_2* and *kdpC* were identified and compared with the reference MAP K-10 strain. Among the field isolates, all the virulence genes were similar on alignment except the *fabG2_2* gene which exhibited deviations in the MAP4 isolate. Differences were observed when similar genes of the field isolates were compared with the reference strain and other related mycobacteria. Of the putative virulence genes, only the *impA* and *papA2* genes were conserved. These observations indicate that the isolates of this study possess genomic differences from the reference strain and other mycobacteria. Since most of the identified putative virulence genes were similar in all isolates, it implies that there may as well be other factors which also play a role and contribute to the differential virulence observed during the *in vitro* murine infection model.

9.2 Research outputs/Contributions

The PTB disease situation in the six districts of western Uganda is now known as well as the associated risk factors. This is important in guiding interventions for disease control and prevention in the country.

The RPA test is an efficient diagnostic tool which can be applied in MAP herd screening in addition to ELISA to improve the usually low sensitivity of the serological tests.

Environmental survey would be a valuable tool to determine PTB at the herd-level since it is cheap and quick to collect environmental samples. However, there is need to optimize the sample collection procedure and testing to achieve reliable results from this method.

Use of RAW 264.7 macrophages is a reliable method to study MAP behaviour in the intracellular environment and gives a reflection of the events that take place inside a natural host as this study has demonstrated.

Analysis of putative virulence genes has revealed genomic differences in the isolates from this study and others and this possibly explains their variable virulence. This is a useful approach to determine bacterial strain virulence and may aid strategies for identification of vaccine and drug targets.

This study has contributed to setting up a tissue culture unit in the reserach laboratory at COVAB, Makerere University which has set a basis for future *in vitro* experiments in the laboratory and in other laboratories that intend to carry out similar or related studies.

9.3 Conclusions

From this study, PTB is present in the districts that were surveyed with a high herd-level prevalence but medium to low animal-level prevalence. This shows that there is high rate of spread of PTB from herd to herd which implies a high exposure of cattle to MAP and this also exposes humans since MAP is a potential zoonosis.

All environmental samples from this study tested negative. However, this does not imply that MAP is not present in the environment of the surveyed herds. It could be due to low MAP copy numbers in the samples obtained that required additional tests to detect the organisms as well as to optimize the environmental sample collection procedure.

The attributes used to study MAP strain pathogenicity which included virulence genes and cytokine expressions as well as survival in macrophages were able to identify differences in expression of several genes (*katG*, *kdpC*, *papA2*, *umaA1*, *impA*) and cytokines (TGF- β , IL-10, IL-6, IFN- γ , TNF- α , IL-12P40) as well as survival of MAP in RAW 264.7 macrophages at different time points.

MAP1 isolate in particular exhibited high-virulence compared to the other isolates whereas MAP4 isolate exhibited the least virulent attribute in relation to their inflammatory cytokine response and survival inside macrophages. These are presented as useful tools to understand MAP behaviour in the intracellular host environment.

Further genomic analysis revealed polymorphisms within the *umaA1*, *katG*, *kdpC*, *papA2* and *fabG2_2* genes of the study isolates when compared with the reference strain as well as other strains. These are key observations which need further investigations to search for possible vaccine and drug targets that will aid in the management of paratuberculosis.

9.4 Recommendations and Future research areas

Surveys need to be conducted throughout the country to establish full data on the herd infection status of PTB which will guide interventions for disease control and prevention.

Since there is a high prevalence of paratuberculosis at herd level, measures should be taken to curtail its further spread among farms. Before animals are introduced into any herd, they should be screened to ensure that they are not shedding MAP. Paratuberculosis need to be included among diseases tested before animals are introduced into new herds for purposes of stocking.

Farmers and veterinarians need to be sensitised on the clinical presentation of PTB, the need to confirm suspected paratuberculosis cases and on measures to manage PTB at herd level.

The RPA method is recommended for MAP diagnosis and screening as well as for other diseases to improve disease management in the country. The test method is fast, all reagents are stable at ambient temperatures, it can be applied in low-resource laboratory settings even at the point of need and has less equipment requirements compared to serology and the other molecular diagnostic techniques.

There is need for more studies on MAP virulence and survival mechanisms using models that depict a typical natural host environment in addition to the *in vitro* cellular models.

Further genomic analyses are needed for the identified gene variations among different isolates to establish their contributions in the pathogenesis of paratuberculosis.

There is a need to carry out a robust study of MAP contamination and survival in the environment to determine the critical sources MAP infection for animals and the duration it takes MAP to survive within the environment in the context of Uganda.

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LIST OF APPENDICES

APPENDIX I: EPIDEMIOLOGICAL TOOL FOR THE SURVEY OF *M. AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTION AND ASSOCIATED RISK FACTORS AMONG CATTLE HERDS IN UGANDA

This questionnaire is intended for the collection of information that will promote our understanding of the role of different factors that promote the spread of Paratuberculosis or Johne's disease. It is not meant for the collection of information for tax assessments or any other political reason. As a respondent, you are free to participate or decline participation without any risk of punishment of whatever sort or loss of privileges. Due to the benefits that research contributes to society, we request for your voluntary participation to respond to these questionnaires.

Please respond to these questions and ask if it is not clear to you

1. Farmer's information

Name of the farmer: Village:.....

Parish: Sub county:

District:

2. Animal characteristics

i. How many cattle do you have? Total number: Female:

Males (bulls and steers):

ii. Age groups of the cattle kept; (a) Below 6 months..... (b) 6-12 month...

(c) 1-2 years (d) 2-3 years (e) Above 3 years ...

3. Herd characteristics

i. What cattle breeds do you keep on the farm?

ii. For how long have you kept these breeds of cattle in the farm?

iii. Has any of the animals suffered from prolonged diarrhea with body wasting?

Yes: No:

iv. If yes, how old was the affected cattle? the breed:

v. Were you able to treat and cure the affected cattle?

4. Johne's disease prevalence

- i. How many heads of cattle do you have?
- ii. Number of cattle sampled from the herd:
5. The ecology of *Mycobacterium avium* subsp. *paratuberculosis*
 - i. Rainfall or drought pattern in the area (Refer to meteorological data)
 - Do you experience long dry spells in your area? Y/N
 - How long is the long rainy season? the long dry season
 - ii. Vegetation cover of the farm?
 - Do you have tree shades on your farm? Y/N
 - How do you rate tree shades on your farm? a) Sparse:
b) Heavy canopy: c) Intermediate:
 - Do you graze on natural or planted pasture?
 - iii. Drainage of the farm
 - Do you have waterlogging on the farm? (Is the farm well drained?)
.....
6. Water source for the cattle

What is the source of water for the animals on the farm? (tick available water source)

 - i. Borehole
 - ii. Deep well
 - iii. Valley dam (a) Individually owned (b) Communally owned ...
 - iv. Stream(s)
 - v. River
7. Grazing practices (tick practice noted)

i. Paddocking ii. Communal grazing iii. Rangeland grazing iv. Transhumance
v. Grazing in rented area vi. Periodic bush burning
8. Stocking density estimation in paddocked farms
 - i. Number of cattle kept in the farm
 - ii. Farm size in hectares
 - iii. Stocking density
9. Stocking and restocking of the farms
 - i. Have you introduced new stock in the farm? Y/N

- ii. When was/were cattle introduced? In the last: 6 months One year ...
Two years Others (specify)
10. Breeding practices: Where do you get bulls for breeding?
 - i. Privately owned bull and is only used in the farm
 - ii. Shared bull from other farmers
 - iii. Artificial insemination
 - iv. Both artificial insemination and bull
11. Calf feeding practices: How do you feed calves in the farm? Tick appropriate
 - i. Calves suckle and walk with their dam during grazing
 - ii. Calves suckle and remain at the homestead
 - iii. Calves are bucket fed
12. Culling practices: How do you cull animals from your herd of cattle?
 - i. Slaughter them
 - ii. They are sold alive to any potential buyers
13. Common beliefs regarding poor animal health such as loss of body condition (unthriftiness)
 - i. What diseases associated with prolonged diarrhea and weight loss do you know?
.....
.....
 - ii. Do you know the causes of these diseases you have mentioned?
.....
 - iii. What traditional means of treatments do you have for the diseases mentioned?
.....
 - iv. Any other traditional knowledge that explains the cause of such a disease and how
traditional knowledge can be applied to treat these diseases
14. Other than cattle, which other livestock do you keep on the farm?
.....
15. Have any of these livestock also suffered from chronic wasting disease with loose stool that
failed to respond to treatment? Y/N
16. If Yes, which stock and how many?
.....
17. Did you ever find any solution to the problem? Y/N

18. Have you ever heard of Johne's disease or Paratuberculosis? Y/N
19. If Yes, from whom?
20. Are there wildlife within or around your premises? Y/N
21. If Yes, which species of wildlife?
22. How do wildlife affect farming practices on your farm?
.....
23. How do long dry seasons affect your husbandry practices?
.....
24. How do long rain seasons affect your farming practices?
.....
25. How do you handle accumulated manure on your farm? Tick appropriate
- i. Left in the farm/pasture
 - ii. Taken to plantations
 - iii. Used in plantations or sold

Thank you for your responses

**APPENDIX II: MAP INFECTION STATUS OF FARMS WHERE
ENVIRONMENTAL SAMPLES WERE COLLECTED**

District	Farm ID	MAP infection status of the herd (RPA/ELISA)	Boot swab (MAP DNA)	Water sample (MAP DNA)
Kabale	KB F3	Positive	Negative	Negative
Kabale	KB F13	Positive	Negative	Negative
Kabale	KB F7	Negative	Negative	Negative
Kabale	KB F8	Negative	Negative	Negative
Kabale	KB F9	Negative	Negative	Negative
Kabale	KB F18	Negative	Negative	Negative
Kabale	KB F16	Positive	Negative	Negative
Kabale	KB F12	Positive	Negative	Negative
Kabale	KB F6	Positive	Negative	Negative
Kabale	KB F17	Negative	Negative	Negative
Kabale	KB F4	Positive	Negative	Negative
Kabale	KB F11	Negative	Negative	Negative
Kabale	KB F5	Positive	Negative	Negative
Kabale	KB F1	Negative	Negative	Negative
Kabale	KB F10	Negative	Negative	Negative
Kabale	KB F15	Negative	Negative	Negative
Kabale	KB F14	Positive	Negative	Negative

Kiruhura	KR F11	Negative	Negative	Negative
Kiruhura	KR F4	Positive	Negative	Negative
Kiruhura	KR F9	Negative	Negative	Negative
Kiruhura	KR F13	Negative	Negative	Negative
Kiruhura	KR F15	Positive	Negative	Negative
Kiruhura	KR F3	Negative	Negative	Negative
Kiruhura	KR F16	Negative	Negative	Negative
Kiruhura	KR F10	Negative	Negative	Negative
Kiruhura	KR F8	Positive	Negative	Negative
Kiruhura	KR F14	Positive	Negative	Negative
Kiruhura	KR F6	Negative	Negative	Negative
Kiruhura	KR F7	Negative	Negative	Negative
Kiruhura	KR F1	Negative	Negative	Negative
Kiruhura	KR F2	Positive	Negative	Negative
Kiruhura	KR F5	Positive	Negative	Negative
Bushenyi	BU F21	Negative	Negative	Negative
Bushenyi	BU F7	Negative	Negative	Negative
Bushenyi	BU F10	Negative	Negative	Negative
Bushenyi	BU F16	Negative	Negative	Negative
Bushenyi	BU F20	Negative	Negative	Negative
Bushenyi	BU F23	Negative	Negative	Negative

Bushenyi	BU F12	Positive	Negative	Negative
Bushenyi	BU F17	Negative	Negative	Negative
Bushenyi	BU F8	Negative	Negative	Negative
Bushenyi	BU F3	Negative	Negative	Negative
Bushenyi	BU F4	Negative	Negative	Negative
Bushenyi	BU F15	Positive	Negative	Negative
Bushenyi	BU F1	Positive	Negative	Negative
Bushenyi	BU F24	Negative	Negative	Negative
Bushenyi	BU F9	Positive	Negative	Negative
Bushenyi	BU F8	Negative	Negative	Negative
Bushenyi	BU F22	Negative	Negative	Negative
Bushenyi	BU F13	Positive	Negative	Negative
Bushenyi	BU F3	Negative	Negative	Negative
Bushenyi	BU F19	Negative	Negative	Negative
Bushenyi	BU F15	Positive	Negative	Negative
Bushenyi	BU F11	Negative	Negative	Negative
Bushenyi	BU F2	Negative	Negative	Negative

APPENDIX III: LETTER FOR ETHICAL CLEARANCE



Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: A35ES

6th August 2018

Assoc. Prof. Julius Okuni
Principal Investigator
Makerere University
Kampala

Dear Assoc. Prof. Okuni,

I am pleased to inform you that on 31/07/2018, the Uganda National Council for Science and Technology (UNCST) approved your study titled, *Molecular Evolution of Mycobacterium avium subsp. paratuberculosis in Africa*. The Approval is valid for the period of 31/07/2018 to 31/07/2021.

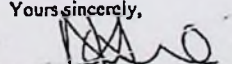
Your study reference number is A35ES. Please, cite this number in all your future correspondences with UNCST in respect of the above study.

Please, note that as Principal Investigator, you are responsible for:

1. Keeping all co-investigators informed about the status of the study.
2. Submitting any changes, amendments, and addenda to the study protocol or the consent form, where applicable, to the designated local Research Ethics Committee (REC) or Lead Agency, where applicable, for re-review and approval prior to the activation of the changes.
3. Notifying UNCST about the REC or lead agency approved changes, where applicable, within five working days.
4. For clinical trials, reporting all serious adverse events promptly to the designated local REC for review with copies to the National Drug Authority.
5. Promptly reporting any unanticipated problems involving risks to study subjects/participants to the UNCST.
6. Providing any new information which could change the risk/benefit ratio of the study to the UNCST for review.
7. Submitting annual progress reports electronically to UNCST. Failure to do so may result in termination of the research project.

Please, note that this approval includes all study related tools submitted as part of the application.

Yours sincerely,


Winfred Badanga Nazziwa

For: Executive Secretary

UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

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