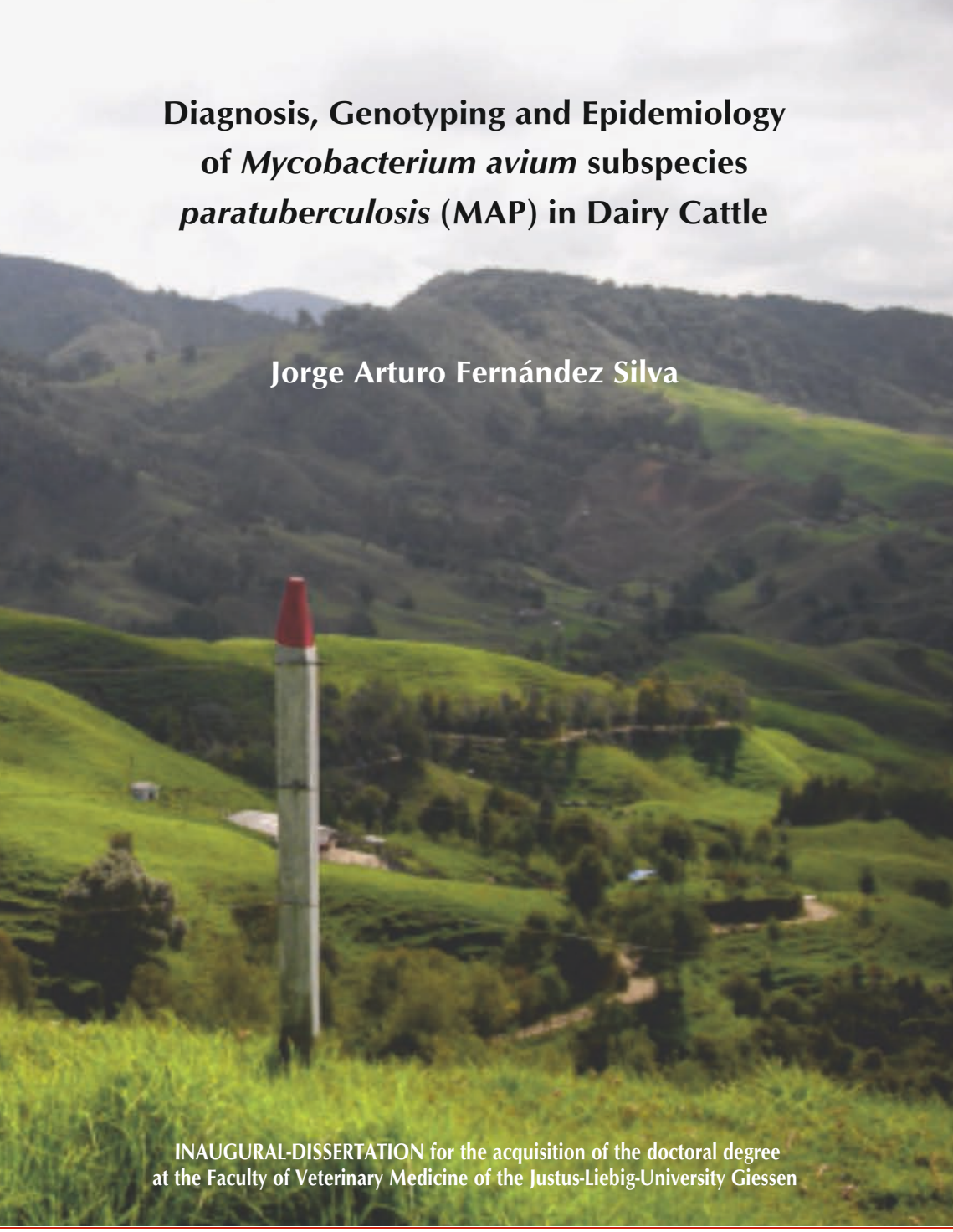
Diagnosis, Genotyping and Epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Dairy Cattle

Jorge Arturo Fernández Silva



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submitted by

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Veterinarian and Master in Public Health
from Medellín, Colombia

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- Fernández J., Abdulmawjood A., Akineden Ö., Bülte, M. 2009. Molekularbasierte Feintypisierung von *Mycobacterium avium* ssp. *paratuberculosis* aus unterschiedlichen Habitaten. 50. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der DVG, 29 Sep.–2 Oct. 2009, Garmisch-Partenkirchen, Germany.

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CONTENTS

LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion
AM	atypical mycobacteria
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CD	Crohn´s disease
CFU	Colony Forming Units
CI 95%	Confidence intervals of 95%
cm	centimeter
CMI	Cell-mediated immunity
Ct	Cycle threshold
CTAB	Cetyltrimethylammonium Bromide
D	Simpson´s index of discriminatory ability
DMSO	Dimethil Sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoabsorbent assay
e.g.	*exempli gratia* (for example)
et al.	*et alii* (and others)
g	gravity
Gr. pref.	Greek prefix
h	allelic diversity at a locus
HEYM	Herrold’s egg yolk medium
HPC	Hexadecylpyridinium chloride
HRM	High resolution melt (analysis)
IAC	Internal amplification control
IBD	Inflamatory bowel disease
i.e.	*id est* (that is)
INF	Interferon
INRA	Institut National de la Recherche Agronomique
INMV	INRA Nouzilly MIRU-VNTR
IS	Insertion Sequence

IS*1311*-PCR/REA	IS*1311*-based Polymerase Chain Reaction-Restriction Endonuclease Analysis
IS*900*-RFLP	IS*900* based-Restriction Fragment Length Polymorphisms
Kb	kilo base
LAM	lipoarabinomannan
LJ	Löwenstein Jensen medium
LSP	large sequence polymorphisms
M	molar
M.	*Mycobacterium*
M. L. fem. n.	Medieval Latin feminine noun
M. L. n.	Medieval Latin noun
MAA	*Mycobacterium avium* subsp. *avium*
MAC	*Mycobacterium avium*-intracellulare complex
MAH	*Mycobacterium avium* subsp. *hominissuis*
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MAS	*Mycobacterium avium* subsp. *silvaticum*
MB	Middlebrook
MGIT	Mycobacteria Growth Indicator Tube
min	minute
MIRU	Mycobacterial Interspersed Repetitive Unit
ml	milliliter
MLSSR	Multilocus Short Sequence Repeats
mm	millimeter
NaOH–OA	sodium hydroxide and oxalic acid
NBT/BCIP	nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate
n_c	Adjusted Rand's coefficient
NCBI	National Center for Biotechnology Information
nm	nanometer
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis

PP	percent of positivity
PPD	purified protein derivate
qPCR	quantitative PCR
RIDOM	Ribosomal Differentiation of Medical Microorganisms
RP	Rhineland–Palatinate
S/P	value of the sample / value of the positive control
SD	standard deviation
SDS	sodium dodecyl sulfate
Se	sensitivity
SNP	single nucleotide polymorphism
Sp	specificity
SS	suppershedder
SSC	saline sodium citrate
SSR	short sequence repeats
subsp.	subspecies
TBE	Tris/Borate/EDTA (buffer)
TE	Tris EDTA (buffer)
Th	T helper
TNF-α	Tumor Necrosis Factor-alpha
TR	tandem repeats
UV	ultraviolet
VNTR	variable number tandem repeat
W	Wallace coefficient
w/v	weight/volume
Wi	expected W value under independence
%	percentage
µl	microliter
µM	micromolar

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow–growing, mycobactin-dependent acid fast bacterium that causes Johne´s disease or paratuberculosis in dairy cattle (Sweeney, 1996). Paratuberculosis is a slow–developing disease characterized by chronic granulomatous enterocolitis and regional lymphangitis and lymphadenitis (Clarke, 1997). MAP has been also associated to the chronic human enteritis known as Crohn´s disease (CD), due primarily to the histopathological and clinical similarities of this disease and paratuberculosis of ruminants (Chacon et al., 2004). However, evidence to affirm or to deny its role as causal agent in at least some cases of Crohn´s disease is still insufficient (Nacy and Buckley, 2008). On the other hand, foods of animal origin and water have been found to be potential sources of human exposure to MAP (Gill et al., 2011).

The fecal-oral route is the main way to contract Johne´s disease in dairy cattle at the individual level and neonatal calves are more susceptible to MAP infection than other groups of age (Windsor and Whittington, 2010). Economic losses due to reduced milk production, increased cow replacement, lower cull–cow revenue and greater cow mortality are higher in paratuberculosis–positive herds compared to paratuberculosis–negative herds (Ott et al., 1999). Diagnosis of paratuberculosis is hampered by some imperfections of diagnostic tests and due to the special physiopathology of disease (Stevenson, 2010b). In general, MAP strains have been classified into three groups or types, type I (sheep), type II (cattle), and type III (intermediate). Despite of this classification, MAP has shown some degree of host preference but no host exclusivity, and different MAP types can be isolated from different animal species. Strain differentiation or sub-typing of MAP through genotyping is very useful to understand the origin of the infections and the disease transmission dynamics, to design more adequate control measures, and to improve diagnosis rates and the development of vaccines (Motiwala et al., 2006; Sohal et al., 2010).

In the present dissertation diverse aspects of the diagnosis, epidemiology, and genotyping of paratuberculosis in dairy cattle in Colombia and Germany were investigated. In the first part the establishment and evaluation of the MAP genotyping methods IS*900* based-Restriction Fragment Length Polymorphisms (IS*900*-RFLP), Mycobacterial Interspersed Repetitive Unit (MIRU), Variable Number Tandem Repeat (VNTR) and Multilocus Short Sequence Repeats

(MLSSR) was carried out; in the second part 14 dairy herds from Colombia were tested for serological, molecular and bacteriological diagnosis of MAP, for the determination of factors that influence the individual serological response to MAP, and for genotyping of MAP isolates; in the third part genotypes of MAP isolated from different host species in South America were compared to explore features of MAP-genotypes from these countries, as a contribution to global knowledge of MAP epidemiology; finally, the last part presents the results of the genotyping of MAP from dairy cattle in the federal state of Rhineland–Palatinate, Germany, using MIRU–VNTR and MLSSR, the evaluation of both methods, and the analysis of MAP molecular epidemiology at regional scale.

1. LITERATURE REVIEW

1.1. The Mycobacteria

Mycobacterium (*M.*) is the unique genus of Mycobacteria. In general, members of this genus are slender, rod-shaped bacteria, characteristically acid-fast, aerobic, slow growing, and free-living or pathogens of vertebrates (Anonymous, 1994; Biet et al., 2005). Members of the genus are straight or slightly curved rods, 0.2-0.7 x 1.0-10µm, sometimes branching. Filamentous or mycelium-like growth may occur, but it is readily fragmented into rods or cocci. No aerial hyphae are grossly visible. Mycobacteria are non-motile, non-sporing, and without conidia or capsules. They are aerobic and chemoorganotrophic. Growth is slow or very slow, visible colonies appear in 2-60 days or even later at optimal temperature. Colonies are often pink, orange, or yellow, especially when exposed to light, pigment is not diffusing, surface commonly dull or rough. Some species are fastidious and require supplements (e.g. MAP), or are not cultivable (e.g. *M. leprae*). They are catalase positive, arylsulfatase positive, and lysozyme resistant. Mycobacteria are widely distributed in soil and water; some species are obligate parasites and pathogens of vertebrates (Anonymous, 1994)

Species of *Mycobacterium* may be confused with the related genera *Corynebacterium*, *Nocardia* and *Rhodococcus*. However, the property of acid-fastness, due to waxy materials in cell wall, is particularly important for recognizing mycobacteria (Anonymous, 1994). The envelope of mycobacteria is composed of a variety of soluble proteins, carbohydrates, and lipids and basically three insoluble macromolecular components: mycolic acid, arabinogalactan, and peptidoglycan. Together, these insoluble macromolecules constitute the mycolylarabinogalactan peptidoglycan core of the cell wall, one of two lipopolysaccharides common to all mycobacteria (Inderlied et al., 1993) . Mycobacteria have been conventionally classified into broad taxonomic groups on the basis of pathogenicity for humans and animals, rate of growth at optimum temperatures, and effect of visible light on pigment production (Inderlied et al., 1993). Among the non-tuberculosis mycobacteria species classified by Runyon into four major groups (photochromogens, scotochromogens, nonphotochromogens and rapid growers), the best studied are those of the *M. avium-intracellulare* complex and *M. kansasii* pathogens (Biet et al., 2005).

1.2. The *Mycobacterium avium-intracellulare* complex (MAC)

The MAC comprises several species of slow-growing mycobacteria that are prevalent in environmental, veterinary and clinical settings. The MAC includes professional pathogens of birds and livestock, and opportunistic pathogens of humans, as well as organisms commonly found in soil and water (Turenne and Alexander, 2010). The MAC includes the species *M. intracellulare* and the *M. avium* (Biet et al., 2005), as well as the new species *M. chimaera*, *M. arosiense*, *M. colombiense*, and *M. vulneris* (Turenne and Alexander, 2010), Figure 1).

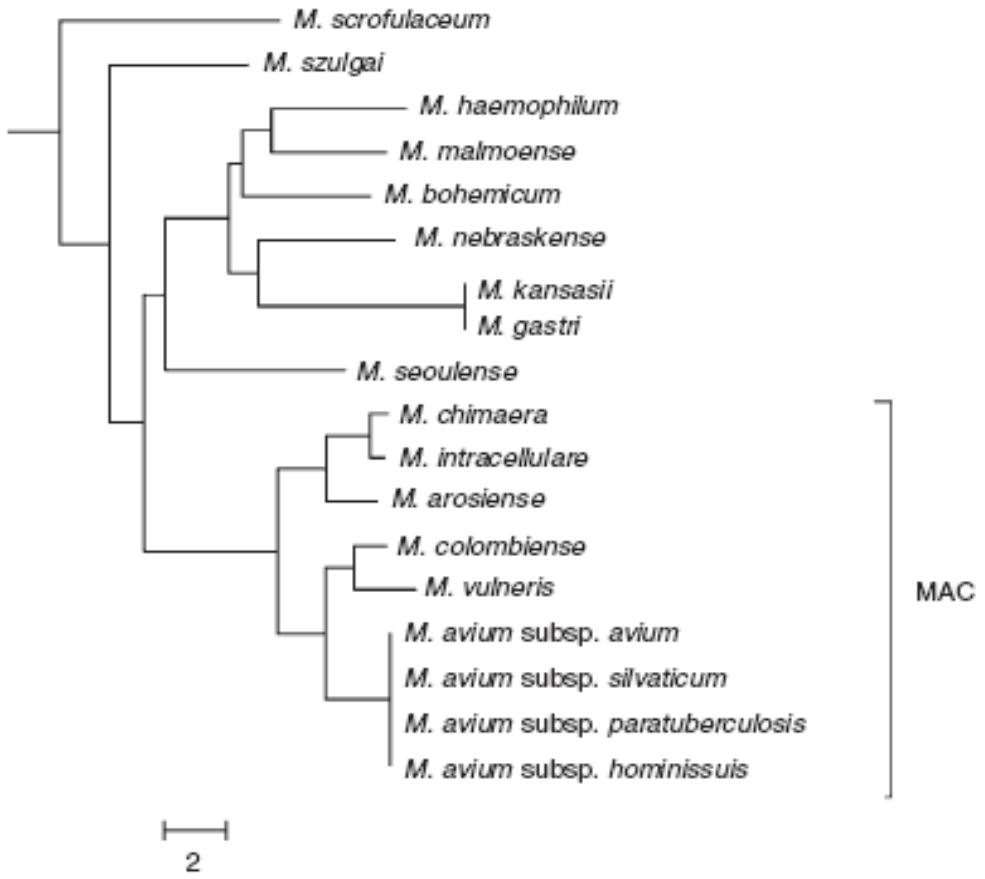


Figure 1. The *Mycobacterium avium-intracellulare* complex (MAC)
Mycobacterium avium phylogeny reconstruction of about 1400 base pair (bp) of the 16S rRNA gene. The species shown represent the subcluster that included MAC species in the context of a comprehensive 16S rRNA gene sequence alignment of all mycobacterial species. Scale represents the number of base pairs (bp) difference (Turenne and Alexander, 2010).

M. avium has four subspecies (subsp.) known as *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *silvaticum* (MAS), MAP, and *M. avium* subsp. *hominissuis* (MAH, (Thorel et al., 1990), Figure 1). The latter was proposed almost 10 years ago to separate the *M. avium* isolates predominantly recovered from humans and pigs (MAH) from those isolates

predominantly recovered from birds (MAA) based on several differences (Mijs et al., 2002). According to a recent study based on Multilocus Sequence Typing (MLST), MAH represents a diverse group of organisms from which the two MAP pathogenic clones (Sheep or type I and Cattle or type II) and MAA/MAS have evolved independently (Turenne et al., 2008). This evolution process appeared to have occurred after two different phases of acquisition of DNA and genomic deletion events, based on the investigation of the distribution of 25 large sequence polymorphisms (LSP) across a panel of genetically defined *M. avium* strains (Alexander et al., 2009). Nonetheless, the MAH subspecies has not been officially validated until now (Turenne and Alexander, 2010).

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

Mycobacterium avium subsp. *paratuberculosis* (MAP, (Thorel et al., 1990) [basonym *Mycobacterium paratuberculosis*, Bergey et al. 1923, paratuberculosis: Gr. pref. *para* beside, related; M.L. n. *tuberculosis* tuberculosis; M.L. fem. n. *paratuberculosis* tuberculosis-like, paratuberculosis (Anonymous, 1986)] is a slow growing, mycobactin-dependent, acid fast, weakly Gram-positive bacillus of 0.5-1.5µm length that causes paratuberculosis or Johne´s disease in cattle (Sweeney, 1996; Clarke, 1997). MAP full lineage is Bacteria; Actinobacteria; Actinobacteria (class); Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; *Mycobacterium*; MAC; *M. avium*; MAP (Anonymous, 2011a).

1.3.1. Phenotypical characteristics

Generation time of MAP has been estimated to be 1.3-4.4 days depending on the size of inoculum in liquid culture (Lambrecht et al., 1988; Whittington, 2010). MAP does not produce any detectable mycobactin, an iron-binding compound that is synthesized by most mycobacteria necessary for growth. MAP appeared to lose mycobactin dependence when subcultured; however, this was shown to be a result of mycobactin carried over from primary medium; removal of this contaminating cell-wall-associated mycobactin reestablished mycobactin dependence (Lambrecht and Collins, 1992). MAP mycobactin dependency is possibly related to the observation of a gene cluster encoding the biosynthetic enzymes used to build the mycobactin siderophore (mbtA-J) that is similar to other mycobacteria, with the notable exception that mbtA is truncated by approximately 150 amino acids in the MAP genome (Li et al., 2005).

1.3.2. Molecular characteristics

MAP (reference strain BAA-968 also known as K-10) has a single circular sequence of 4.829.781 base pairs (bp), with a G+C content of 69.3%, which is relatively constant throughout the genome (Li et al., 2005; Wynne et al., 2010). Approximately 1.5% or 72.2 kb of the MAP K-10 genome is comprised of repetitive DNA, including insertion sequences, multigene families, and duplicated housekeeping genes (Li et al., 2005), some of which have been used as targets in MAP molecular research (Table 1). A large segment of the MAP genome (greater than 30%) encodes sequences with no identified function (Paustian et al., 2010). The MAP genome has shown >95% sequence similarity between some MAP and MAA strains (Li et al., 2005). Genomic regions with altered nucleotide compositions are in many cases associated with mobile genetic elements and have been shown to be sites of LSPs (Paustian et al., 2010).

Table 1. Genomic elements of MAP routinely used for detection, typing or sub-typing

Genomic element	Type of genomic element	Features	References
F57	Sequence	620 bp size, 59% of GC content, only one copy in MAP genome	(Poupart et al., 1993)
HspX	Single-copy gene	Unique to MAP	(Ellingson et al., 1998)
251 and 255	Genes	MAP specific genes	(Bannantine et al., 2002)
IS*Mav2*	IS	Also present in *M. fortuitum*, *M. smegmatis* and two other *Mycobacterium* species	(Strommenger et al., 2001) (Mobius et al., 2008a)
IS*Mpa1*	IS	Belongs to the IS*110* family 1500 bp size, also found in some MAH isolates from pigs	(Olsen et al., 2004)
ISMAP*02*	IS	Six copies in genome. 1674 bp size, 80% identical to IS from *M. vanbaalenii* and *M. gilvum*	(Paustian et al., 2004) (Paustian et al., 2010)
IS*900*	IS	1451 bp ORF of 1197 bp between nucleotides 236 and 1432 Inserted in one direction at consensus regions in highly conserved loci	(McFadden et al., 1987) (Collins et al., 1989) (Green et al., 1989) (Bull et al., 2000)
IS*1311*	IS	Shares 85% homology with the IS1245 MAP types posses single nucleotide polymorphism (SNP) in position 233, 32, 64, and 65 of IS1311	(Whittington et al., 1998) (Sibley et al., 2007) (Whittington et al., 2001) (Sohal et al., 2009)

Table 1. continued

Genomic element	Type of genomic element	Features	References
SSR	Tandem repeat (microsatellite)	SSRs of 3-6 nucleotides frequently found in coding regions SSRs of 1, 2, 4 and 5nucleotides are found in non-coding regions	(Sreenu et al., 2007)
VNTR	Tandem repeat (minisatellites)	9-100 bp Dispersed in genomes of prokaryotic Also known as hypervariable regions	(Supply et al., 2000)
MIRU	Tandem repeat (minisatellites)	40-100 bp DNA Found as TR and dispersed in intergenic regions	(Supply et al., 1997)

MAP genome, as other genomes of prokaryotes, has two main types of nucleoid DNA structures: genes (or similar gene sequences) and extra gene sequences (Krawczak and Schmidtke, 1994), Figure 2).

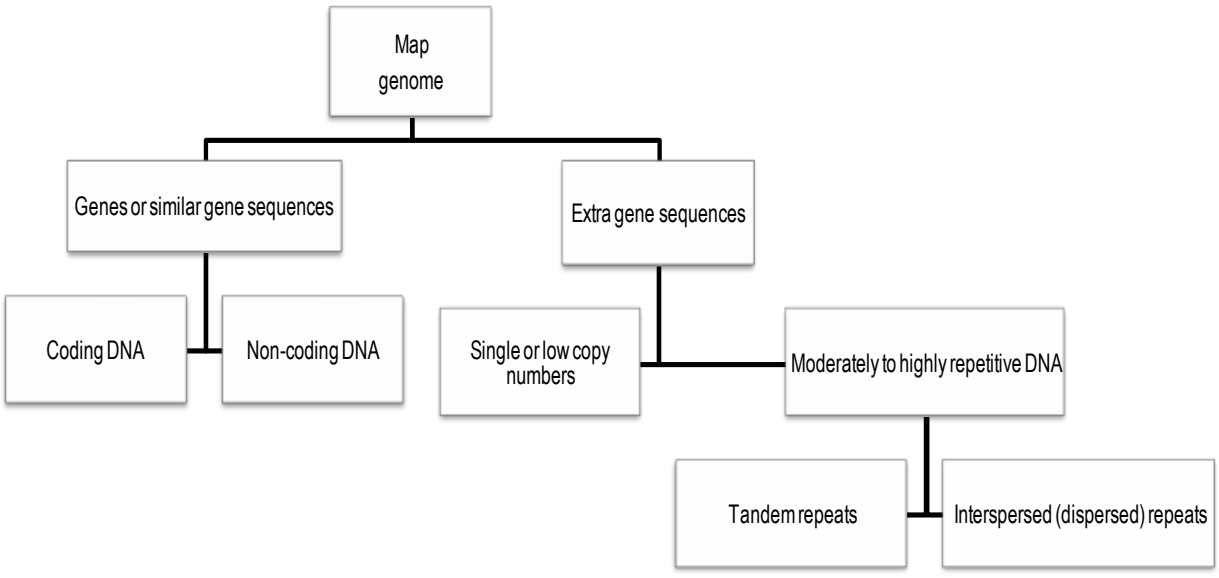


Figure 2. Organization of MAP genome
Genes (or similar gene sequences) can be found as coding DNA or non-coding DNA, while the extra gene sequences can be found in single or low copy numbers and moderately to highly repetitive-DNA. In turn, the moderately to highly repetitive DNA-sequences could be divided into tandem repeats and interspersed (dispersed) repeats according to the grade of repetition and to the location of repeats.

For the repetitive sequence regions with **tandem repeats (TR)** organization, the terms DNA-satellites, DNA-minisatellites, and DNA-microsatellites are also used. In general, satellites are found almost distributed in the whole genome and are of considerably variability and therefore of marked utility for genetic differentiation of individual organisms. This variability is based on the variable number of repetitions inside a specific locus. In this manner, variants are formed taking into account that for several mini- and microsatellites almost all individuals are heterozygote. This type of polymorphism is called variable number of tandem repeat (VNTR) polymorphism. Sometimes VNTRs are treated as equivalent to minisatellites, while microsatellites are called single sequences, short tandem repeats (STR) or short-sequence repeats (SSR, (Krawczak and Schmidtke, 1994; van Belkum et al., 1998).

SSRs are sequence motifs of 1-6 bp (Schlotterer, 2000) and consist of simple homopolymeric tract of a single nucleotide type: poly A, poly G, poly T, or poly C or a large or small numbers of several multimeric classes of repeats. These multimeric repeats are built from identical units (homogeneous repeats), mixed units (heterogeneous repeats), or degenerated repeat sequence motifs (van Belkum et al., 1998). SSRs provide targets with a relatively high clock speed of evolution. However, such repeats elements do not seem to change too frequently so as to result in SSR alteration during routine laboratory processing (van Belkum, 1999), Figure 3).

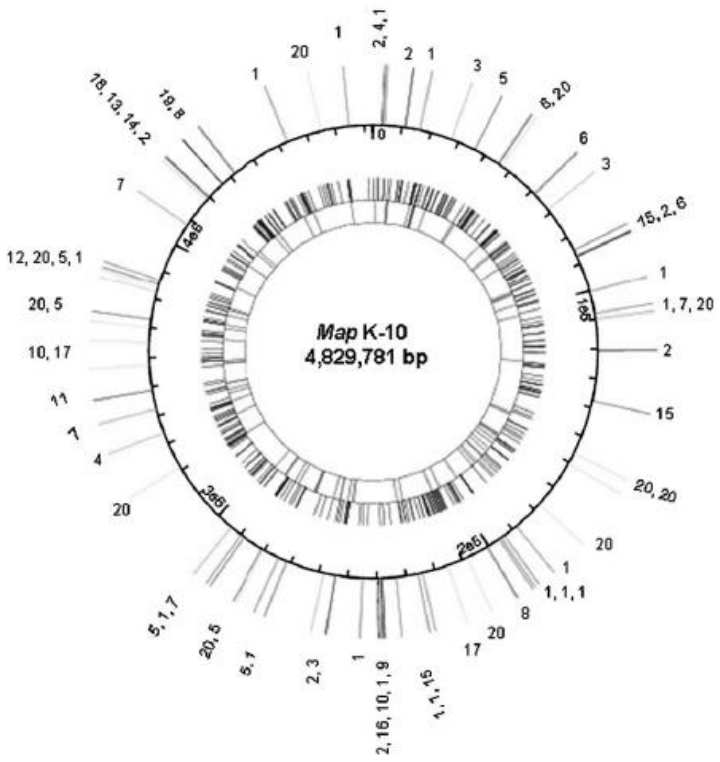


Figure 3. Circular representation of the repetitive sequences and IS elements.
From inside: Innermost histogram, SSRs; Second innermost histograms, VNTRs; black circle, scale; outermost histogram, Insertion Sequences (1: IS*900*; 2: IS1311; 3: IS*Mav2*; 4: IS_MAP01; 5: MAP02; 6: MAP03; 7: MAP04; 8: MAP05; 9: MAP06; 10: MAP07; 11: MAP08; 12: MAP09; 13: MAP10; 14: MAP011; 15: MAP12; 16: MAP13; 17: MAP14; 18: MAP015; 19: MAP16; 20: REP family, (Motiwala et al., 2006)

In general, variability and therefore polymorphisms is based on variable number of units in a specific region originated as the result of a mutation process in the DNA-replication known as slipped strand mispairing (Krawczak and Schmidtke, 1994; van Belkum et al., 1998). This process results in shortening or lengthening of the SSRs (van Belkum et al., 1998).

On the other hand, the **interspersed (dispersed) repeats** do not locate as tandem repeats, but in the whole genome more or less dispersed between non-repetitive sequences. The interspersed repeats elements of nucleoid can be subdivided in short interspersed nuclear elements (SINE) and long interspersed nuclear elements (LINE). This type of DNA could have been produced due to unequal crossing-over duplication mechanisms along the evolution (Krawczak and Schmidtke, 1994). Mycobacterial interspersed repetitive units (MIRU) are VNTRs that due to their particular characteristics have received this special designation (Collins, 2010). They are mini-satellite sequences of 77–101, 46–53 and 58–101 bp in length which are distributed throughout the genome as single copies or in multiple tandem repeats.

MIRU were first identified in *M. tuberculosis* shown to be present in up to 40 loci (Supply et al., 1997).

The repetitive sequences known as **Insertion Sequences (IS)** are short segments of DNA that act as transposable elements and play an important role for diagnosis. ISs are relatively short DNA segments capable of transposing within and between prokaryotic genomes, often causing insertional mutations and chromosomal rearrangements. Use of ISs as probes provides discrimination due to the tendency of these transposable elements to insert randomly and occupy multiple sites in the genome. In certain cases, the localization of specific insertion elements at defined places in the genome is sufficiently stable to allow them to be used as markers for species typing and for epidemiological purposes (Motiwala et al., 2006). Approximately 15% (72.2 kpb) of the MAP genome corresponds to ISs (Castellanos et al., 2011). A total of 58 ISs have been identified within the MAP genome, including about 17 copies of IS*900*, 7 copies of IS*1311* and 3 copies of IS*Mav2* (Li et al., 2005) Table 1, Figure 3).

1.3.3. Types of MAP

MAP strains have been classified into three groups: **Type I** (Sheep type or Type S), **Type II** (Cattle type or Type C), and **Type III** (Intermediate type) based on phenotypic characteristics (growth and pigmentation) and to different genotypic profiles. MAP strain types differ with respect to the ease with which they can be isolated on artificial media and their respective growth rates. MAP Type C strains are comparatively easy to isolate from clinical samples and will grow reasonably well on a range of solid or liquid media supplemented with mycobactin. These strains will typically produce detectable growth in 4–16 weeks, depending on the initial inoculum. MAP Type S strains typically grow more slowly and have fastidious requirements for culture on artificial media. Primary isolation of these strain types can take from 4 months to a year. The addition of egg yolk seems to be beneficial for the primary isolation of Type S strains, although Herrold's Egg Yolk Medium (HEYM), traditionally used for the isolation of MAP, does not support good growth of Type S strains (Whittington et al., 1999; de Juan et al., 2006b; Stevenson, 2010a). In general, MAP strain types cannot be differentiated according to colony morphology. The exceptions to this are the pigmented strains. These MAP strains produce a yellow or orange pigment that is stable during passage both *in vitro* and *in vivo*. They have been isolated from sheep, and the gut mucosa of infected animals shows a typical brilliant yellow color. To date, all pigmented MAP strains that have been typed have been

classified as Type I (Stevenson et al., 2002; Stevenson, 2010a). The classification by means of molecular based methods mainly embraces IS*900*-RFLP (Collins et al., 1990), IS*1311*-based Polymerase Chain Reaction-Restriction Endonuclease Analysis (IS*1311*-PCR/REA, (Whittington et al., 2001), Pulse Field Gel Electrophoresis (PFGE, (Stevenson et al., 2002; de Juan et al., 2006a), MIRU-VNTR (Castellanos et al., 2010b) and gyrB-PCR/REA (Castellanos et al., 2007b), among others (Table 2). Additionally to these methods, the use of high-resolution melt (HRM) analysis based on real-time PCR could also differentiate between Types I, II and III strains (Castellanos et al., 2010a). In addition to these types, one additional type ("Bison" type or Type B), apparently a subdivision of the Type C (Stevenson, 2010a), was determined using IS*1311*-PCR/REA and subsequent sequence analysis of the IS*1311*; according to this analysis, isolates of bison (*Bison bison*) correspond to a novel type based on a nucleotide variation at the base position 223 (Whittington et al., 2001). This type has been detected in isolates of other animal species from the United States and India (Sevilla et al., 2007; Yadav et al., 2008; Sohal et al., 2009).

Table 2. Nomenclature of MAP strain types determined by different procedures and their relation to the designated S-Type and C-Type (Stevenson, 2010a)

Typing method	Type S		Type C	References
	Type I	Type III	Type II	
IS*900*-RFLP	Sheep (S)	Intermediate (I)	Cattle (C)	(Collins et al., 1990) (Pavlik et al., 1999)
Pulsed-field gel electrophoresis	Type I	Type III	Type II	(Stevenson et al., 2002) (de Juan et al., 2005)
IS*1311* PCR-REA	Sheep (S)	ND[a]	Cattle (C) Bison (B)	(Marsh et al., 1999) (Whittington et al., 2001)
DMC-PCR[b]	Sheep type	ND	Cattle type	(Collins et al., 2002)
RDA-PCR[c]	Type I	ND	Type II	(Dohmann et al., 2003)
gyr-PCR[d]	Type I	Type III	Type II	(Castellanos et al., 2007b)
*inh*A-PCR[e]	Type I	Type III	Type II	(Castellanos et al., 2007a)
PPE-DGGE[f]	Type I	Type III	Type II	(Griffiths et al., 2008)
HRM[g]	Type I	Type III	Type II	Castellanos et al., 2010a

a ND, not differentiated from Type I by this assay.
b Digital microfluidic chip - polymerase chain reaction
c Representational difference analysis – polymerase chain reaction
d PCR analysis of the gyrA and gyrB genes
e PCR analysis of the inh-A gene
f PPE protein family - denaturing gradient gel electrophoresis
g High-resolution melt (HRM) analysis

The existence of the third group of MAP strains i. e. Type III (de Juan et al., 2005) or Intermediate (Type I, (Collins et al., 1990) has been controversial based on the results of a

whole-genome study, in which MAP isolates previously classified as Type III were grouped with the Type I or Sheep type strains, based on LSP distribution data (Alexander et al., 2009; Stevenson, 2010a). This grouping is consistent with the observation that Type I and Type III strains share similar phenotypic traits and host preferences (Stevenson, 2010a). There appear to be epidemiological trends associated with MAP strain types with respect to transmission, host preference and susceptibility to infection. However, the results of many past epidemiological studies need to be interpreted with caution since they often employed media that would not support growth of all MAP strain types (Whittington, 2010). This could easily result in a microbiological bias in these reports. Furthermore, many studies did not use molecular typing techniques that differentiated all MAP strain types (Stevenson, 2010a). Type I strains have been isolated predominantly, but not exclusively, from sheep and goats, suggesting a preference for these host species. MAP Type II isolates have a very broad host range and are commonly isolated from both domesticated and wildlife species, including non-ruminants. Type II is the most common MAP strain type isolated from cattle (Whittington et al., 2000; Bull et al., 2003b; Paustian et al., 2008; Stevenson, 2010a). MAP shows some degree of host preference but no host exclusivity and different MAP types can be isolated from different animal species (interspecies transmission, (Motiwala et al., 2006; Stevenson, 2010a).

1.3.4. Genotyping methods

Diverse methods to genotype (or sub-type) MAP have been developed in the last decades. These methods based predominately on the identification of specific repetitive sequences: IS, SSR, MIRU, and VNTR in the MAP genome (Castellanos et al., 2011), Table 3, Figure 3). Methods for MAP subtyping may be classified into three groups: Methods based on Total Genomic DNA Analysis, Analysis of Insertion Sequences, and Analysis of other Repetitive Sequences (Motiwala et al., 2006; Collins, 2010) Table 3).

Table 3. MAP sub-typing methods (Motiwala et al., 2006; Collins, 2010)

Total Genomic DNA Methods
Restriction endonuclease analysis (REA)
Pulse field gel electrophoresis (PFGE)
Amplified fragment length polymorphism (AFLP) analysis
Random amplified polymorphic DNA (RAPD-PCR) analysis
rRNA gene and spacer region analysis
Insertion Sequence Analysis
IS*900*-RFLP
IS*1311*-PCR/REA
IS*1311*-RFLP
Multiplex PCR for IS*900* loci (MPIL)
Other Repetitive Sequences
MIRU analysis
VNTR analysis [sometimes also called Multilocus Variable-Number of Tandem-Repeat Analysis – MLVA (Overduin et al., 2004)]
SSR analysis

MAP subtyping is an useful tool in epidemiological investigations in order to gain a better understanding of the origin of a paratuberculosis infection, identification of risk factors that influence transmission, characterization of the pathogenesis, and evaluation of regional control programs allowing a rational design of more adequate control measures, improvement in diagnostics and vaccine development (Motiwala et al., 2006; Sohal et al., 2010).

1.3.4.1. IS*900*-RFLP

RFLP is a technique by which organisms may be differentiated by analysis of patterns derived from restriction endonuclease digestion of their DNA followed by electrophoresis of DNA fragments (e.g. Southern blot technique). By applying this technique to MAP, genomic DNA is digested with a restriction enzyme that does not cut within IS*900*. The DNA fragments produced are separated by agarose gel electrophoresis, blotted on to nylon and hybridized to a probe made from part of the IS*900*-DNA sequence (Pavlik et al., 1999; Motiwala et al., 2006; Collins, 2010). As with other typing techniques that involve digestion of genomic DNA, the choice of restriction enzyme is important. Most studies have used one or more of the three enzymes *Bst*EII, *Pvu*II and *Pst*I. *Bst*EII gives slightly better discrimination than the other two

enzymes (Whipple et al., 1990; Pavlik et al., 1999; Stevenson et al., 2002), but the best discrimination is obtained by combining the results of two or three enzymes (Mobius et al., 2008b; Collins, 2010). Although RFLP has yielded excellent results in mycobacteria other than MAP, the method is time consuming, labor intensive and requires relatively large quantities of high quality DNA (Motiwala et al., 2006; Collins, 2010). The RFLP-based approach to the molecular typing of MAP is also limited by the very slow growing nature of most isolates and lack of growth of others, particularly from sheep in conventional culture. Besides, due to lack of polymorphism identified within the host-species in the major groups, IS*900*-RFLP analysis may have a limited role in epidemiological studies of Johne's disease (Motiwala et al., 2006; Collins, 2010).

1.3.4.2. MIRU

MIRU are a type of VNTR that due to their characteristics have received this name and could be treated as a synonym of VNTR (Collins, 2010). Actually, in the analysis of MIRU and VNTR loci in some studies, they are commonly called "MIRU-VNTR" independently of their original denomination as MIRU or VNTR (Thibault et al., 2008; Castellanos et al., 2010b; van Hulzen et al., 2011). Analysis of MIRU is performed based on amplification of specific loci using conventional PCR. MIRUs of MAP are composed by two sequences of consensus motifs A (24 bb) TGACGAGGAGCGGCGCAGATGGCA and B (29 bp) GGCGCCGGTGACGATGCAGAGCGTAGCGA (Bull et al., 2003b). There are 31 motifs A and 24 motifs B in the MAA genome and 26 motifs A and 20 motifs B in the MAP genome. All these motifs are clustered into 18 different loci, whereas only MIRU loci 1–6 showed differences between MAC members (Bull et al., 2003b). From these six MIRU loci, only MIRU-1 to MIRU-4 has been used in the molecular characterization of MAC members. MIRU-1 and MIRU-4 differentiated MAP from MAA, MAS, and *M. intracellulare*, but did not show any intra-species variability (Bull et al., 2003b). However, in a panel of MAP isolates from Germany, two allelic variants at MIRU-1 were reported (Mobius et al., 2008b; Castellanos et al., 2011).

1.3.4.3. VNTR

The five VNTR (or MIRU-VNTR) loci selected in early studies gave only very limited discrimination of MAP strains (Overduin et al., 2004). More recent studies used six (Romano et al., 2005) or eight (Thibault et al., 2007) VNTR loci for typing MAP strains of Type C. In two studies, the overall discrimination of VNTR typing was similar to that of IS*900* typing,

and combination of the two typing systems gave much better discrimination than either system separately (Thibault et al., 2007; Mobius et al., 2008b). Recently, one additional VNTR (VNTR-259) was proposed to subtype MAP (Castellanos et al., 2010b). The VNTR-method is primarily pursued by conventional PCR-amplification of several loci of MAP genome. The great attraction of VNTR typing is that, because it is based on PCR amplification, it requires only small amounts of sheared DNA, which can potentially be obtained from primary cultures (Collins, 2010).

1.3.4.4. MLSSR

In 2004, Amonsin et al. (2004) published the results of an *in silico* analysis, in which 78 loci with perfect repeats were found dispersed throughout the genome of MAP strain K-10. From these 78 loci, 11 loci were polymorphic and were reported to be more discriminatory than AFLP and MPIL for a collection of 33 MAP strains (Amonsin et al., 2004; Motiwala et al., 2006; Collins, 2010; Castellanos et al., 2011). This method originally termed "MLSSR Sequencing Approach" demonstrated a high degree of discrimination when applied on diverse MAP isolates (Motiwala et al., 2003). Although the allelic variation observed in the study focused on the number of copies of the SSRs, it was observed that some loci also revealed one or two base substitutions in some isolates; the majority of the nucleotide substitutions were found in an isolate recovered from a sheep (Motiwala et al., 2003). Since its first original description, MLSSR analysis has been used in the characterization of MAP isolates in several studies, revealing high discriminatory ability in the majority of them (Ghadiali et al., 2004; Motiwala et al., 2004; Corn et al., 2005; Motiwala et al., 2005; Harris et al., 2006; Cernicchiaro et al., 2008; Singh et al., 2009; El Sayed et al., 2009; Mobius et al., 2009; Stabel et al., 2009). Concerning stability, SSR of MAP strains appeared to be very stable based on results of recent studies, in which the four most discriminatory SSR-loci remained invariable in three MAP strains tested over ten subcultures (Harris et al., 2006) and in SSR sequences of 98 isolates of the bison type (Singh et al., 2009).

1.3.4.5. Combination of methods and homologous targets of subtyping methods

The combination of methods targeted to the same type of genomic structures of MAP with each other (e.g. repetitive elements: MIRU, VNTR, and MLSSR), or the combination of methods based on different genomic structures (e.g. repetitive elements and insertion elements: IS*900*-RFLP) using similar or different techniques (e.g. amplification and sequencing, or amplification and restriction) has shown an increment of the whole

discriminatory ability due to the addition of individual discriminatory powers of single methods (Sevilla et al., 2008; Thibault et al., 2008; Mobius et al., 2008b; Stevenson et al., 2009; Douarre et al., 2011). A recent review grouped all MIRU and VNTR loci targeting the same genomic regions reported previously in the literature (Castellanos et al., 2010b). According to this review the doubles or trios VNTR-3527 and MIRU-7 ARG; VNTR-1605 and MIRU-6 ARG; MIRU-2 and VNTR-292; VNTR-3249 and MIRU-5 ARG; VNTR-14 and VNTR-10; MIRU-3, VNTR-1658, and X-3; and VNTR-2495, VNTR-22, and VNTR-27 discovered and reported by several authors in different moments and in different publications correspond to the same target sequences (Castellanos et al., 2010b).

1.3.4.6. Subtypes of reference strains

The MAP reference strains K-10 (BAA-968) and ATCC 19698 have been subtyped in several studies. Using the IS*900*-RFLP method (endonuclease *Bst*E II), BAA-968 (K-10) produced the profile C1 or R01 (Cousins et al., 2000; Stevenson et al., 2002; Overduin et al., 2004; Thibault et al., 2007). Surprisingly, ATCC 19698 has been reported having two different IS*900*-RFLP (*Bst*E II) profiles (C1 and C5, (Stevenson et al., 2002; Borrmann et al., 2011). Using the MIRU-VNTR method both reference strains produced the profile 3751 corresponding to the repeat units found in MIRU loci 1 to 4, respectively (Bull et al., 2003b; Mobius et al., 2008b; Borrmann et al., 2011); the profile 22222 corresponding to the repeat units found in the VNTR loci 1067, 1605, 1658, 3527, and 3249, respectively (Overduin et al., 2004) and the profile 32332228 (or INMV 2) corresponding to the repeat units found in the VNTR loci 292, X3, 25, 47, 3, 7, 10, and 32, respectively (Thibault et al., 2007). However, using MLSSR, the reference strains showed different profiles, the K-10 reference strain revealed the genotype 14g (or >11g)-10g-5cg-5gc-5gc-5gcg-5ccg-5ggt-5tgc-5gcc-5ccg (Amonsin et al., 2004; Thibault et al., 2008), while the ATCC 19698 reference strain revealed the genotype 7g-11g-5cg-5gc-5gc-5gcg-5ccg-5ggt-5tgc-5gcc-5ccg (Thibault et al., 2008), corresponding to the numbers of repeats found in loci 1 to 11 in both reference strains, respectively.

1.3.4.7. Evaluation of subtyping methods

A subtyping method should be evaluated and validated with respect to a number of criteria that can be divided into performance and convenience criteria (van Belkum et al., 2007). Among the **performance criteria**, a good subtyping method should assess a marker that remains *stable* during the study period, and does not vary to a degree that confuses the

epidemiological picture. This marker should be testable in every isolate or should provide universal *typeability* of all isolates. It should also usefully *discriminate* among isolates, and this discrimination should be concordant with the epidemiological picture. Finally, the results of a good typing method should be *reproducible*, independently of the operator, place and time (van Belkum et al., 2007). According to Hunter and Gaston (1988), the performance criteria *typeability* and *reproducibility* are relatively easy to quantify and are often expressed as simple percentages. On the contrary, the *discriminatory power* of a typing method, i.e. its ability to distinguish between unrelated strains, demands the calculation of the Numerical Index of Discriminatory Ability of Typing Systems (*D*), developed based on the application of the Simpson's Index of Diversity. This index indicates the probability of two strains sampled randomly from a population belonging to two different types (Hunter and Gaston, 1988). The index is calculated according to the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

where *D* is the numerical index of discrimination, N the number of unrelated strains tested, s the number of different genotypes, and Xj the number of strains belonging to the jth genotype, assuming that isolates and strains will be classified into mutually exclusive categories (Hunter and Gaston, 1988). The index should ideally be 1.0 but, in practice, it should be at least in the order of 0.95 for a typing system to be considered more or less 'ideal'. A 5% probability of error is accepted by most professionals in the field (van Belkum et al., 2007). Grundmann et al. (2001), proposed the use of the large sample approximation for the calculation of confidence intervals (CI) for the Simpson's index, thereby improving the objective assessment of the discriminatory power of typing techniques (Grundmann et al., 2001). The confidence intervals are calculated according to the following formulas:

$$\sigma^2 = \frac{4}{n}[\sum \pi_j^3 - (\sum \pi_j^2)^2], \qquad (1)$$

Where j is the frequency nj/n, nj is the number of strains belonging to the jth type, and n is the total number of strains in the sample population.

$$CI = [D - 2\sqrt{\sigma^2}, D + 2\sqrt{\sigma^2}]. \qquad (2)$$

Where *D* is the Numerical Index of Discriminatory Ability of Typing Systems and σ^2 is the variance (Grundmann et al., 2001).

The **convenience criteria** include *flexibility*, *rapidity*, *accessibility*, *ease of use*, *costs*, and *suitability* for computerized analysis and storage of results (van Belkum et al., 2007).

Flexibility refers to the range of species (or subspecies) that are typeable with minimal modifications of the method; *rapidity* refers to the total time required to get from the bacterial isolates to the final typing results; *accessibility* depends upon the availability of reagents and equipment, as well as the skills required for a given method in a given laboratory; *ease of use* encompasses technical simplicity, workload, suitability for processing large numbers of isolates, and ease of scoring and interpreting the results (van Belkum et al., 2007).

On the other hand, typing methods generate "**partitions**", clusters or groups, result on the categorization of elements into groups formed by a given methodology on the global analysis of a data set, resulting in its classification. Consequently, in addition to the performance and convenience criteria for evaluation and validation, it is frequently needed to determine how well a given classification agrees with another (Carrico et al., 2006). For the purpose of comparing two sets of results of different microbial typing methods, an objective measure of agreement is needed. Several measures were developed for comparing two sets of partitions, taking different approaches to how partitions should be compared. Due to their ease of interpretation, Adjusted Rand's coefficient (n_c) and Wallace coefficient (W) have been proposed previously for partition analysis of typing methods (Carrico et al., 2006). Adjusted Rand´s coefficient (n_c) is estimated to access the global congruence of the two genotyping methods used, taking into account that the agreement between partitions could arise by chance alone (Carrico et al., 2006). The coefficient is calculated according to the following formula:

$$n_c = \frac{n(n^2+1) - (n+1)\sum n_i^2 - (n+1)\sum n_j^2 + 2\sum\sum \frac{n_i^2}{n}}{2(n-1)}$$

Where n represents the total sample size, ni the number of species belonging to the cluster i of partition A, and nj the number of species belonging to the cluster j of partition B.

The Wallace coefficient (W) can be more informative than Adjusted Rand by providing information about the partition relation. Wallace's coefficients provide an estimate of, given a typing method, how much new information is obtained from another typing method. A high value of Wallace's coefficient indicates that partitions defined by a given method could have been predicted from the results of another method, suggesting that the use of both methodologies is redundant. It can be defined as:

$$W_{AB} = \frac{N_{11}}{N_{11} + N_{10}} \qquad W_{BA} = \frac{N_{11}}{N_{11} + N_{01}}$$

where: (N11): number of point pairs in the same cluster under both A and B; (N10): number of point pairs in the same cluster under A but not B; and (N01): number of point pairs in the same cluster under B but not A. Pinto et al. (2008) extended this framework, proposing the calculation of the expected W value under independence (Wi) and the use of a confidence interval for W, adding statistical support for the comparison of these coefficients. These new measures reinforce the role of W in generating maps of types or sub-types equivalence between different typing methods, and facilitate the joint analysis of multiple typing methods (Pinto et al., 2008).

1.3.5. MAP in food

In general, transmission of MAP to humans most likely would occur via consumption of milk from herds that include infected animals and products prepared from such milk, consumption of meat and organ tissues from infected animals or animals contaminated by feces shed by infected animals, and drinking waters contaminated with MAP from feces of infected animals (National Advisory Committee on Microbiological Criteria for Foods, 2010; Grant, 2010; Gill et al., 2011). The main constraint for MAP detection in food is the current limitation in the methods to detect MAP and the absence of a standard method to detect viable cells (National Advisory Committee on Microbiological Criteria for Foods, 2010). The number of viable MAP cells in food that could arrive to the human consumer cannot be precisely estimated under current conditions due to limitations of detection of methods used. In a first instance, culture methods have the disadvantage of using decontamination procedure that has been demonstrated to reduce the real number of MAP viable cells. In a second instance, PCR, an additional common method used for MAP detection in food, does not have the ability to differentiate viable from non-viable MAP cells (Grant, 2010). So far, MAP has been isolated among others from raw milk of individual animals (Shankar et al., 2010) and of bulk milk containers (Foddai et al., 2011), from pasteurized milk (Gao et al., 2002), from infant food formula (Hruska et al., 2005; Hruska et al., 2011), from cheese (Ikonomopoulos et al., 2005; Stephan et al., 2007; Botsaris et al., 2010), ice cream and flavored milk drinks (Shankar et al., 2010); dressed carcasses (Meadus et al., 2008), muscle and organ tissues (Antognoli et al., 2008; Alonso-Hearn et al., 2009; Hasonova et al., 2009; Mutharia et al., 2010; Reddacliff et al., 2010; Okura et al., 2011) and retail meat (Jaravata et al., 2007).

1.3.6. MAP in the environment

MAP has been isolated from diverse sources including soil (Norby et al., 2007; Pribylova et al., 2011; Salgado et al., 2011a), amoeba (Whan et al., 2001; Whan et al., 2006; Mura et al., 2006), insects of the order Diptera (Fischer et al., 2005), and biofilms (Cook et al., 2010). Based on these findings MAP has been considered an environmental pathogen. The main source of this environmental contamination is the infectious animal that shed MAP in variable quantities from the intestine. In a previous study in North America, environmental samples were cultured positive in 78% of 80 herds known to be MAP-infected and in only one herd out of 28 uninfected herds; environmental samples were cultured positive in cow alleyways (77% of the herds), manure storage (68%), calving area (21%), sick cow pen (18%), water runoff (6%), and post weaned calves areas (3%, (Raizman et al., 2004). In Europe, a recent study revealed that all non-infected herds showed negative results, while 22 (71%) of the infected herds showed positive results in environmental samples. In this study the prevalence of the herds was taken into account revealing that nine infected herds with negative environmental sampling results had a low prevalence (0.04–4.04%). In addition, the study found that proportion of positive environmental sampling depended on prevalence and on sampling areas with 53.3 % positive results in lactating cow areas and 45.2% in milking areas. In this study was concluded that environmental sampling provides an efficient tool to determine MAP infection status or herd prevalence (Donat et al., 2011).

The isolation of MAP from the environment in and around cattle herds has been used as a strategy to screen herds for paratuberculosis (Lombard et al., 2006; Anonymous, 2010). MAP can survive in feces and can be cultured from dry, fully shaded locations for up to 55 weeks, and shorter periods in unshaded conditions; in addition MAP present dormancy in the environment, which could explain its appearance and reappearance in tested samples after a period of time (Whittington et al., 2004). MAP can also survive in water and/or sediment in the shade for up to 48 weeks compared to 36 weeks in a semi-exposed location. Survival in sediment was 12 to 26 weeks longer than survival in the water column. Survival in soil and fecal material in the terrestrial environment in the shaded location was only 12 weeks (Whittington et al., 2005). MAP on contaminated pastures can run off into watercourses when it rains (Pickup et al., 2005; Pickup et al., 2006). MAP has been recovered from entering a treatment plant and from solids extracted from the water during treatment (Pickup et al., 2006), as well as from untreated water samples (Whan et al., 2005). However, data on the

presence of MAP in drinking water appear to be lacking requiring more investigation (Gill et al., 2011).

1.3.7. MAP and Crohn's disease (CD)

Mycobacteria and MAP in particular have been long related to CD of humans (Chiodini, 1989). However, the role of MAP as part of the causal structure or as an opportunist in this human pathology remains controversial or lack enough evidence to be affirm or denied (Nacy and Buckley, 2008). CD is a debilitating, systemic syndrome with prominent gastrointestinal pathology (Behr, 2010). CD belongs to the Inflammatory Bowel Diseases (IBD), which also includes the Ulcerative Colitis (Chacon et al., 2004). Arguments pro and against the causal association of MAP and Crohn´s disease have been stated since the suggestion of a possible relationship between them (Sartor, 2005; Nacy and Buckley, 2008). One of the main arguments to associate MAP to human CD is the analogy, because MAP has been experimentally shown to cause an invasive IBD in a variety of hosts, it has been stated that as MAP causes IBD in ruminants, so too may it cause chronic bowel disease in humans. This argument of analogy, although incomplete, remains as a basic observation despite of the changes in the knowledge in mycobacterial disease and CD (Behr, 2010). In any case, reviews and meta-analysis from independent scientists, scientific associations, and from governmental organizations have concluded that with the current scientific evidence the role of MAP as the causal agent of CD cannot be refuted nor denied. Nonetheless, this relation (causal or coincidental) should not be ignored, taking into account the detection of MAP in food, water, and environment, that could represent permanent sources of exposure of MAP to humans (Anonymous, 2000; Feller et al., 2007; Waddell et al., 2008; Behr and Kapur, 2008; Nacy and Buckley, 2008; Mendoza et al., 2009)

1.4. Paratuberculosis in dairy cattle

Paratuberculosis is a slowly–developing infectious disease characterized by chronic granulomatous enterocolitis, and regional lymphangitis and lymphadenitis, leading to the typical clinical sign of progressive weight loss (Clarke, 1997). Incubation period may range from less than 6 months to over 15 years and clinical disease is the terminal stage of a slow chronic subclinical infection (Chiodini et al., 1984). Paratuberculosis occurs in multiple species (Anonymous, 2008b) and is a common disease in all countries with a significant dairy industry, especially in areas with a moderate and humid climate (Barkema et al., 2010).

1.4.1. Prevalence

The knowledge of the prevalence of MAP at the herd and animal level is often a key issue when decision or policy makers determine whether the infection should be considered important or not, and which measures to apply (Nielsen and Toft, 2009). In paratuberculosis prevalence estimations, infection frequency has been conventionally reported for three different levels: the herd-level, the animal-level (or cow-level), and the within-herd prevalence (Nielsen and Toft, 2009; Barkema et al., 2010). In addition, it is important to differentiate between apparent and true prevalence. The former is the simple proportion of infected animals to the population at risk, while the latter considers the characteristics of sensitivity and specificity of a given diagnostic test used for prevalence determination, and therefore it is considered more reliable than apparent prevalence (Smith, 2005; Nielsen and Toft, 2009). The apparent herd-level prevalence of MAP infection follows a negative binomial distribution, with a large proportion of farms having a relatively low prevalence and some farms, probably because of the lack of preventive measures, having a high prevalence. It is often suggested that both herd- and cow-level prevalence of MAP infection are increasing; however, this cannot be confirmed, because no sets of studies have been published using the same sampling strategy and laboratory method in the same region (Barkema et al., 2010).

A global (apparent or true) prevalence of paratuberculosis (at herd-level or at animal-level) is not available. In the 90´s, herd level prevalence of paratuberculosis in countries with a significant cattle industry was calculated at approximately 10%, while more recently it has been estimated to be 30–50% based in several studies (Barkema et al., 2010). In addition, MAP has been considered as an emerging pathogen with zoonotic potential (Skovgaard, 2007) and in some countries paratuberculosis has been reported as an emerging disease in different animal populations (Michel and Bastianello, 2000; de Lisle et al., 2003). Nevertheless, this emergence of paratuberculosis could be explained by the fact that in the last years techniques for detection have been improved and testing in some countries has been increased (Skovgaard, 2007).

True prevalence of paratuberculosis among cattle in Europe appeared to be approximately 20% and was at least 3–5% in several countries. Herd prevalence guesstimates appeared to be >50% (Nielsen and Toft, 2009). In the United States, results from serologic testing revealed that 3.4% of cows and 21.6% of dairy herds were infected with MAP (Wells and Wagner, 2000). In South America, information on prevalence and molecular epidemiology of MAP for

each country is practically unavailable. Few studies have reported consistent animal–level and herd–level prevalences, which are around 10–20% and 70%, respectively (Ferreira et al., 2001; Alfaro et al., 2006). Despite of this, prevalence information in South America has to be carefully analyzed due to interference of intradermal tuberculin test on paratuberculosis diagnosis still carried out in almost all countries, as part of control programs for bovine tuberculosis (Varges et al., 2008).

On the other hand, compared to the United States and Europe (Harris et al., 2006; Stevenson et al., 2009) molecular epidemiology of MAP in South America has remained relatively uninvestigated, excepting the studies of (Moreira et al., 1999) and (Romano et al., 2005) on MAP typing and subtyping. Although the study from Moreira et al. (1999) suggested differences in the predominant MAP genotypes in Argentina compared to Europe, using IS*900*–RFLP, this method alone is known to have insufficient discriminatory ability for MAP compared to MIRU–VNTR and MLSSR (Motiwala et al., 2006). The spread of paratuberculosis from its first recognition in Europe to herds of the New World may have coincided with the process of colonization and the subsequent growth of domestic animal agriculture (Manning and Collins, 2010). In addition, cattle importation (e.g. Holstein cattle) from Germany and United States has been a common practice in many Latin-American countries since the 19th century. Based on this, MAP should have followed a similar process of the well documented case of MAP brought to Iceland from Europe (Fridriksdottir et al., 2000).

Paratuberculosis in Colombia. Although Colombian cattle population was estimated in 26.8 million of heads for 2008 and dairy production has increased in the last 20 years to the point of become self-sufficient, it is still pursued under a complex scenario of limitations and opportunities, among which infectious diseases are a significant problem (Mojica et al., 2007). Although paratuberculosis is a notifiable disease in Colombia, no official control or eradication program for Johne´s disease is executed. Because of this the current epidemiological situation of this entity in Colombia is practically unknown. In Colombia, paratuberculosis was first reported in cattle in 1924, apparently in imported animals. After this, paratuberculosis research in cattle and sheep has been pursued sporadically and mainly focused on disease treatment and detection. In 1984 a prevalence of 11.25% and 2.88% was estimated for sheep (n=480) using the complement fixation test and the Ziehl-Neelsen staining of fecal smears, respectively (Mogollón G. et al., 1983). More recently,

paratuberculosis has been detected in sheep using Ziehl-Neelsen staining, intradermal test (avian purified protein derivate, PPD) and ELISA (Mancipe J. et al., 2009), and in dairy cattle using PCR and fecal culture (Zapata R. et al., 2010), as well as through clinical findings and histopathology (Ramirez et al. 2002, personal communication). These latter studies were very useful to confirm the presence of MAP in local cattle and sheep, but were carried out in only one single flock and were limited in delivering information on risk factors. In general, there is no data regarding the individual animal or herd management factors associated to paratuberculosis in Colombian dairy herds and it is considered that the epidemiological situation of bovine paratuberculosis for the country is unknown.

Paratuberculosis in Germany. Paratuberculosis is widely spread among cattle herds in Germany; in 2009, a total of 361 cases of bovine paratuberculosis were reported in the whole country (Kohler and Mobius, 2010). Paratuberculosis is notifiable in Germany, but there is no obligation to test animals with clinical signs of this disease and owners and veterinarians often refrain from submitting samples to diagnostic laboratories in order not to know if an animal or herd is paratuberculosis positive (Nielsen, 2009a). The German Guidelines to Control Paratuberculosis in Ruminant Flocks is based in three main components looking for standardization of control measures in the country, the reduction of clinical cases and thereby economic losses derived from an infection, the reduction of MAP spread, and the decrease of paratuberculosis prevalence (Anonymous, 2005). Paratuberculosis control in Germany is voluntary and is supported by the animal health insurances of some federal states (Kohler and Mobius, 2010). Despite of permanent paratuberculosis cases report, regional control programs (Luyven et al., 2002; Flebbe, 2002; Klawonn et al., 2002) and regional seroprevalence estimations (Bottcher and Gangl, 2004; Hacker et al., 2004) a whole country prevalence for Germany cannot be accurately calculated due to the diversity of diagnosis test used, and some critical issues detected in some of the regional studies carried out in the past (Nielsen and Toft, 2009). Specifically in the federal state of Rhineland–Palatinate, paratuberculosis is routinely diagnosed and several actions have been taken to improve diagnosis and control (Klawonn et al., 2002).

1.4.2 Transmission

In a recent revision, it was concluded from a meta-analysis that 9% of fetuses from subclinically infected dams and 39% of clinically affected dams were infected *in utero* with MAP (Whittington and Windsor, 2009). Epidemiologically, it has been determined that calves

born from seropositive dams to paratuberculosis had 6.6 times more likely to be seropositive compared with calves born from seronegative dams (Aly and Thurmond, 2005). Regarding the postnatal transmission, the fecal-oral route, especially at early life stage, is the main way to contract paratuberculosis in dairy cattle at the individual level (Sweeney, 1996; Clarke, 1997). Neonatal calves are more susceptibility to MAP infection than other groups of age (Windsor and Whittington, 2010). Neonatal calves acquire MAP by direct ingestion of MAP-contaminated feces, from the manure-contaminated teat and udder of the calf´s dam or indirectly via MAP-fecal-contaminated colostrum, milk, water, pasture, feedstuff or utensils (Sweeney, 1996; Manning and Collins, 2010; Fecteau and Whitlock, 2010). MAP has been identified in colostrum from subclinically infected cows (Streeter et al., 1995). Colostrum has been establish as a risk factor of MAP infection for calves (Nielsen et al., 2008) and the practice of feeding pooled colostrum or waste milk from cows has been considered to help the spread of infection to many calves (Fecteau and Whitlock, 2010). In conclusion, the major sources of MAP infection for an animal are infected animals (Manning and Collins, 2001). The prompt identification of infected animals and the elimination of factors that increase contamination of the environment and infection of new born calves with MAP are some of the most critical issues in a paratuberculosis control program. Although calves are more susceptible to a paratuberculosis infection than older heifers or adults, these can also get infected by the exposure to high and repeated doses of MAP in a contaminated environment, but are less likely than calves to develop clinical signs of Johne's disease (Windsor and Whittington, 2010; Fecteau and Whitlock, 2010). Although this risk is considered low, these animals could excrete organisms in their feces, particularly under nutritional, lactational or other stress (Windsor and Whittington, 2010). On the other hand, transmission to herds without previous history of paratuberculosis is mainly due to purchase of subclinical MAP-infected animals or sharing of breeding bulls between herds (Sweeney, 1996; Manning and Collins, 2010; Fecteau and Whitlock, 2010). Utensils or clothes contaminated with MAP could also be potential sources of infection, but these are considered insignificant compared to the introduction of apparent uninfected animals (Fecteau and Whitlock, 2010).

1.4.3. Immunopathology

The heterogeneity and variability of the response to paratuberculosis are the result of a complex immunological interaction dependent on the host rather than the infecting MAP. Paratuberculosis is a classical mycobacterial infection. Immunity to all mycobacteria infections is dependent on cell-mediated immune responses; humoral immune factors have

little or no protective value (Chiodini, 1996). Detailed knowledge of the risk factors and immune processes that determine whether exposure to MAP will lead to infection, regression, recovery or disease is undetermined (Windsor and Whittington, 2010).

Penetration of the mucosal barrier. Ingested MAP bacteria enter the intestinal wall of ruminants through the small intestine mucosa, primarily in the region of the ileum, via M cells, a specialized epithelial cell that lacks the brush-border microvilli, digestive enzymes and surface mucus commonly associated with enterocytes residing in the Peyer's patches, a lymphoid aggregate part of the gut-associated lymphoid tissue (GALT, (Momotani et al., 1988; Stabel, 2010). Intact and degraded MAP is transported in vacuoles across the M cells to macrophages or dendritic cells in subepithelial and intraepithelial areas of the Peyer's patches and in the adjacent lamina propria and it is immediately phagocytized (Momotani et al., 1988; Chiodini, 1996). After their uptake, MAP organisms are subject to the killing and degradation activities of the macrophage. Macrophages can migrate to other areas of the body, including the mesenteric lymph nodes and the peripheral circulation (Stabel, 2010).

Early infection. As an intracellular pathogen, MAP is able to survive within the macrophage by inhibiting maturation of the phagosome. Organisms may remain intact in the macrophages, thwarting the bactericidal mechanisms of the antigen-presenting cell (APC) or they may be processed and presented to T lymphocytes, engendering a domino effect on immunological responses (Stabel, 2000b; Stabel, 2010). The newly activated macrophages surround the lesion and begin the early formation of a granuloma. As the non-activated macrophages die and release their bacterial load, these liberated organisms are immediately phagocytized by the activated macrophages (Chiodini, 1996). Once the infected macrophages become activated by mycobacteria, they will produce interleukin-1 (IL-1), a cellular messenger that along with the presentation of major histocompatibility complex (MHC) class antigens on the surfaced of macrophages, activates T lymphocytes and other cytokines such as Tumor Necrosis Factor (TNF) -α and Interleukin (IL) -2 (Stabel, 2000b; Stabel, 2010). Infection with MAP initiates the upregulation of the cytokines mentioned above and of other pro-inflammatory cytokines, IL-6, IL-8, and IL-10 (Stabel, 2010). Subsequently, activated T cells produce IL-2, which results in clonal expansion of specific $CD8^+$ cytolytic T cells and $CD4^+$ T helper cell populations (Stabel, 2000a; Stabel, 2010). $CD4^+$ T helper (Th) cell populations can differentiate into either Th1 or Th2 subpopulations, based on the nature of the antigen presented (Stabel, 2000b).

The Th1 lymphocyte population produces IL-2, TNF-β, and Interferon (INF) -γ, cytokines which direct cell-mediated immune function (Stabel, 2010). In contrast, the Th2 subpopulation of lymphocytes is responsible for induction of humoral immune function via cytokines IL-4, IL-5, IL-6 and IL-10 (Stabel, 2000b). The differentiation of $CD4^+$T-cell population is skewed towards a Th 1 T-cell subpopulation in the early stages of MAP infection, characterized by the secretion of the Th-1-associated cytokines, including INFγ (Stabel, 2010). Early lesions are likely to be controlled exclusively by GALT, with little or no stimulation of peripheral immunity. As a result, all measures of immune responsiveness based on peripheral blood will be negative (Chiodini, 1996). In some animals, an effective immune response is not elicited in all foci and the infection becomes progressive. The manner in which the progressive phase is manifested systematically differs between animals, thus creating the pleomorphic responsiveness in animals observed with use of diagnostic assays (Chiodini, 1996).

Transition from early to late infection- intermediate immunologic events. $CD4^+$ T cells appear to be the primary source of IFN-γ in mycobacterial infections including MAP, but $CD8^+$ and γδ T cells also produce IFN-γ (Stabel, 2010). Regardless of T cell phenotype ($CD4^+$, $CD8^+$, or CD4-/CD8- γδ T cells), IFN-γ appears to be the most critical cytokine for controlling mycobacterial infections. Granulomatous lesions in the intestine are a hallmark characteristic of MAP infection, and the composition of cell types within the lesions is correlated with stage of infection. The localized immune response at the site of lesions has been shown to change in accordance with lesion severity (Stabel, 2010). With the inability to contain infection, MAP continues to proliferate and the host continues to recruit mononuclear phagocytes to the inflammatory foci. As a result, the granulomatous lesion expands (Chiodini, 1996).

Late infection. The progression of paratuberculosis from a subclinical to clinical state is associated with a switch from Th1 to Th2 immune response. The production of Th2 regulatory cytokines, IL-4, IL-5 and IL-10, supports a humoral immune response characterized by the expansion of B lymphocytes, immunoglobulin secretion and control of Th1-mediated responses, in which IL-4 and IL-10 play specific roles in the suppression of IFN-γ production by $CD4^+$ Th1 cells (Stabel, 2010). Humoral responses in paratuberculosis seem to show an inverse relationship with cell-mediated responses in cattle. Serum antibody

concentrations usually raise later that CMI in the course of an infection and can be detected by the complement fixation test, agar gel immunodifusion (AGID) test or enzyme-linked immunoabsorbent assay (ELISA). Immunoglobulin (Ig) G, IgM and weak IgA responses are found in the serum of chronically infected cattle. In the same way, immune histological labeling of bovine intestine also showed an increase IgG^+ and IgM^+ cells but not IgA^+ cells (Clarke, 1997). The role that B cells play in mycobacterial infections is not well understood. However, in addition to the secretion of antibody, B cells act as antigen-presenting cells and play a role in the activation of $CD4^+$ Th2 cells (Stabel, 2010).

1.4.4. Clinical course

The cardinal clinical sign of paratuberculosis in cattle is the chronic progressive weight loss with chronic or intermittent diarrhea (Clarke, 1997). In paratuberculosis the clinical animal is considered the “tip of the iceberg” because it has been estimated that for every advanced case of Johne’s disease on a farm, it is likely that 15-25 other animals are infected and from these infected animals only 25-30% will be detected with the most sensitive molecular testing techniques (Whitlock and Buergelt, 1996; Fecteau and Whitlock, 2010). In general, four stages of disease have been determined for paratuberculosis, depending on the severity of clinical signs, the potential shedding organisms into the environment, and the ease with which the disease may be detected using current laboratory methods (Whitlock and Buergelt, 1996; Tiwari et al., 2006; Fecteau and Whitlock, 2010). The relation between the pathogenic events, transmission levels, and the associated clinical signs along a typical paratuberculosis course are presented in Figure 4.

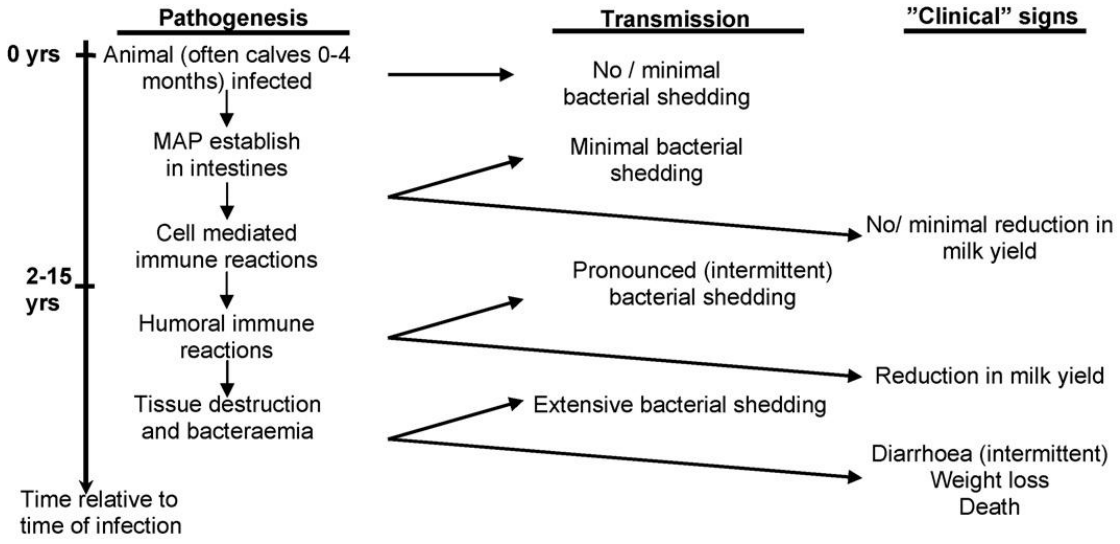


Figure 4. Schematic presentation of various stages of MAP infection and their effects.
This presentation may represent the typical picture, but deviations are likely to occur (Nielsen and Toft, 2008)

In the **first stage or "silent" infection**, animals (calves, heifers, young stock and adult cattle) present no clinical signs, but are possibly shedding infectious organisms undetectable with any diagnostic test. In this phase, the infection can spread to adjacent lymph nodes or eventually into disseminated infection (Fecteau and Whitlock, 2010). Infected cattle seem identical to noninfected herdmates regarding growth, weight gain, and outward appearance. Neither routine nor special clinicopathologic tests detect infection. The only way to detect infected cattle at this early stage is by culture of multiple intestinal tissues (Sweeney et al., 2006a). These animals, however, may shed organisms into the farm at level below threshold of detectability of inclusive culture and PCR (Whitlock and Buergelt, 1996; Fecteau and Whitlock, 2010). Calves infected experimentally with MAP develop both a measurable humoral and cell-mediated immune response to MAP (Waters et al., 2003).

In the **second stage or subclinical disease**, MAP persists and multiplies in subepithelial macrophages leading to a chronic transmural inflammatory reaction (Clarke, 1997; Manning and Collins, 2001). Animals (unapparent carrier adults) do not show visible clinical signs typical for Johne's disease (weight loss or diarrhea), but they may have detectable antibodies to MAP and or/ altered cellular immune response like increase IFN-γ and may be prone to other diseases such as mastitis and infertility (Whitlock and Buergelt, 1996). Most animals shed MAP in their feces, contaminating the environment (Whitlock and Buergelt, 1996; Fecteau and Whitlock, 2010). A small percentage of these infected cattle (15-25%) may be

detected by fecal culture subsequently removed from the herd (Whitlock and Buergelt, 1996). The rate of disease progression through the second stage is highly variable and is most likely influenced by a wide range of factors, which may include: age at initial exposure to MAP, the dose of MAP at initial exposure, the frequency of re-exposure over time, genetic factors of both the host and the organism, environmental factors, nutritional factors, production effects and a variety of other stressors. Most cattle in the second stage will be culled from the herd for reasons unrelated to paratuberculosis, such as infertility, mastitis, lameness or reduced milk production (Whitlock and Buergelt, 1996; Fecteau and Whitlock, 2010).

The **third stage or clinical disease** follows a prolonged incubation period of 6 to over 15 years, with a majority of cases occurring between 3-5 years of age (Chiodini et al., 1984). Clinical disease may be precipitated by parturition, lactation or other stresses (Chiodini et al., 1984; Clarke, 1997). Animals have gradual weight loss and diarrhea, with periods of normal consistency. Thirst is usually increased. Vital signs are normal. Appetite is normal and intermittent diarrhea is often present for weeks (Fecteau and Whitlock, 2010). Serum and plasma biochemical changes associates with paratuberculosis are predictable and characteristic of the clinical stages of disease (Brady et al., 2008), but are not specific to be useful as diagnostic tests for Johne's disease (Whitlock and Buergelt, 1996). MAP population in the mucosal cell is very high. The absorptive capacity of the bowel is abrogated, resulting in weight loss associated to a protein-loosing enteropathy (Fecteau and Whitlock, 2010). The infection becomes disseminated with MAP detectable in several extra-intestinal sites (Antognoli et al., 2008). These animals have a higher frequency of transmitting MAP *in utero* and have a higher frequency of MAP isolated from milk (Fecteau and Whitlock, 2010). Most of the animals at this stage test positive on fecal culture and have increased antibody detectable by serological tests (Whitlock and Buergelt, 1996). Cattle at this stage rarely remain in herd long and are culled due to weight loss, decreased milk production and unresponsive diarrhea (Whitlock and Buergelt, 1996; Fecteau and Whitlock, 2010).

In the **fourth stage or advanced clinical disease**, animals are diarrheic, lethargic, weak, and emaciated, being culled from the herd due to decreased milk production and severe weight loss (Whitlock and Buergelt, 1996). Intermandibular edema is characteristic of this phase of disease. Animals can progress quickly from the second to the fourth stage in few weeks but a more gradual progression is most typical. Most animals are sent to slaughter at this point and

may not pass inspection for human consumption. Otherwise, death occurs as result of dehydration and cachexia (Whitlock and Buergelt, 1996; Fecteau and Whitlock, 2010).

1.4.5. Shedding patterns

Cattle shed MAP from their gastrointestinal tract independently from the clinical course of disease in different forms. MAP *active* shedding occurs when a systematically infected animal excretes MAP into the intestinal tract. MAP *passive* shedding takes place when cattle have detectable MAP in fecal samples following oral ingestion of MAP, similar to the described passing through phenomenon (Sweeney et al., 1992). Some low shedders and even moderate shedders might be false positives caused by passive instead active shedding which represent a bottleneck in paratuberculosis diagnosis (Fecteau and Whitlock, 2010). The term "suppershedder" (SS) refers to a cow shedding more than 10000 MAP CFU/g of manure. The phenomenon has always existed but was relatively recently recognized. Most SS dairy cows are asymptomatic, with no evidence of diarrhea or weight loss, yet excrete huge number of MAP organisms into the environment. Preliminary estimates suggest that 10% of heavy shedders (or about 2-3% of all culture-positive cattle at a single time point) may be SSs, excreting >10 billion MAP CFU per cow per day. It has been suggested that high proportions of low shedders could reflect passive shedding after consumption of feedstuffs or water contaminated by a small number of SSs. One SS excretes more MAP organisms into the environment than 2000 moderate or 20000 low low-shedder cows. SS cows contribute to passive fecal shedding of MAP by uninfected cows (Fecteau and Whitlock, 2010).

1.4.6. Economic impact

Paratuberculosis infection causes significant direct economic losses (decreased milk production, premature voluntary culling, mortality, and reproductive losses) in dairy herds (Tiwari et al., 2008). For an average Canadian dairy herd with 12.7% of 61 cows seropositive for MAP, the mean loss was $2992 (95% C.I., $143 to $9741) annually, or $49 per cow per year. Additional culling, decreased milk production, mortality, and reproductive losses accounted for 46%, 9%, 16%, and 29% of the losses, respectively (Tiwari et al., 2008). Groenendaal et al. (2002) estimated annual losses per animal in an infected herd to be €19 and US$35, respectively, in a Dutch and a Pennsylvanian dairy herd (Groenendaal et al., 2002). Economic losses due to reduced milk production, increased cow replacement, lower cull–cow revenue and greater cow mortality are higher in paratuberculosis–positive herds compared to paratuberculosis–negative herds (Ott et al., 1999). Paratuberculosis has also negative impact

in production and reproduction on dairy cattle, due to the reduction in milk production and cow longevity (Hendrick et al., 2005b), and due to the higher non-return for infected cows compared to negative cows from negative herds (Marce et al., 2009). Farm-level economic consequences related to control measures (management-related practices to control Johne´s disease were marginally economically attractive ($3 /animal/ year) in infected herds (Groenendaal et al., 2002; Groenendaal and Wolf, 2008). Vaccination against paratuberculosis has been found to be economically useful but only if it is used combined with other prevention and control strategies (van Schaik et al., 1996; Groenendaal and Galligan, 2003). In general, economic loss due to Johne´s disease in infected dairy herds is relatively low compared with other major diseases such as subclinical mastitis, fertility or lameness. However due to the right-skewed distribution of individual animal MAP prevalence within herds, Johne´s disease can cause considerable losses in some herds (Groenendaal and Galligan, 2003; Barkema et al., 2010).

1.4.7. Diagnosis

Ante-mortem diagnosis of paratuberculosis is challenging because of disease nature and some limitations of diagnostic tests. These limitations demand to adequately define the purpose of diagnosis in order to applied the most appropriate diagnostic procedure (Collins et al., 2006; Nielsen and Toft, 2008; Nielsen, 2010; Stevenson, 2010b). In general, it has been concluded that the quality of design, implementation and reporting of evaluations of tests for paratuberculosis has been generally poor (Nielsen and Toft, 2008). ELISA, bacteriological cultivation of fecal samples, and PCR are test widely used for the *ante-mortem* diagnosis of paratuberculosis in cattle herds (Clark, Jr. et al., 2008; Nielsen and Toft, 2008; Stevenson, 2010b). Although ELISA is the test most widely used, the isolation of MAP from an animal by culture is still considered the golden standard of Johne´s disease diagnosis (Chiodini et al., 1984; Collins, 1996; Whittington, 2010). In addition, sampling all or a representative proportion of adult cattle in every herd, environmental sampling, serial testing, and the use of two to three diagnostic tests has been recommended for herd screening and to increase the accuracy of MAP diagnosis (Chacon et al., 2004; Collins et al., 2006; Stevenson, 2010b).

1.4.7.1. Indirect methods

Indirect (immune-based) methods for paratuberculosis diagnosis are mainly based on the occurrence of an immune response to infection by MAP. The immune-based diagnostics are primarily based on detection of IFN-γ and IgG antibodies using antigens derived from MAP,

but this immune response has yet been not fully characterized affecting the use and interpretation of test results (Nielsen, 2010).

1.4.7.1.1. Cell-mediated Tests

There are two tests available for the detection of a CMI response to MAP infection: the intradermal tuberculin test or skin test using johnin or avian purified protein derivate (PPD); and the IFN-γ test (Nielsen, 2010; Stevenson, 2010b). The skin test was used more frequently in the past but its current use is limited (Kohler et al., 2001; Nielsen, 2010). The introduction of the *in vitro* IFN-γ assay has replaced the skin test in current studies using the CMI response in the diagnosis of MAP infection (Paolicchii et al., 2003; Huda et al., 2004; Antognoli et al., 2007). Sensitivity (Se) and specificity (Sp) estimates for IFN-γ tests have not been reported for detection of subclinical infected animals (Nielsen, 2010), but it has been considered that this could be the best option to test this group (Stevenson, 2010b). For animals deemed to be MAP infectious (shedding MAP with or without symptoms), Se estimates for IFN-γ tests are between 0.13 and 0.85; Sp estimates are between 0.88 and 0.94 (Nielsen and Toft, 2008).

1.4.7.1.2. Serology

One of the most widely used immune-based tests is ELISA, which detects antibodies in serum and milk samples (Harris and Barletta, 2001). ELISA tests performed on milk and serum samples have shown only moderate agreement, but proportion of positive results for milk and fecal samples were not significantly different, suggesting that milk ELISA is a convenient method of detecting paratuberculosis in dairy herds (Hendrick et al., 2005a). Multiple antibody ELISAs have been evaluated, and Se and Sp estimates vary greatly within and between tests (Kohler et al., 2008; Fry et al., 2008; Nielsen and Toft, 2008). Overall, the Se of antibody ELISA for detection of MAP-infected animals is low (about 5–30%), but it increases with increasing age (Nielsen and Toft, 2006). Sp estimates are generally above 95% for commercial ELISAs (Kohler et al., 2008; Fry et al., 2008; Nielsen and Toft, 2008). The ability of antibody ELISAs to detect MAP infectious animals depends on the test frequency, the test make, and the cut-off chosen to deem the ordinal ELISA response ‘positive’ or ‘negative (Nielsen, 2010; Stevenson, 2010b). Sensitivity of ELISA is the highest for animals with lepromatous lesions, those with clinical symptoms, or those that shed large number of bacteria (Kohler et al., 2008; Nielsen and Toft, 2008). Some infected cows produce antibodies several years prior to continuous shedding of detectable amounts of MAP. However, in other animals,

antibodies may not be detectable during the early stages of infection, when MAP shedding is minimal (Nielsen, 2010), Figure 5).

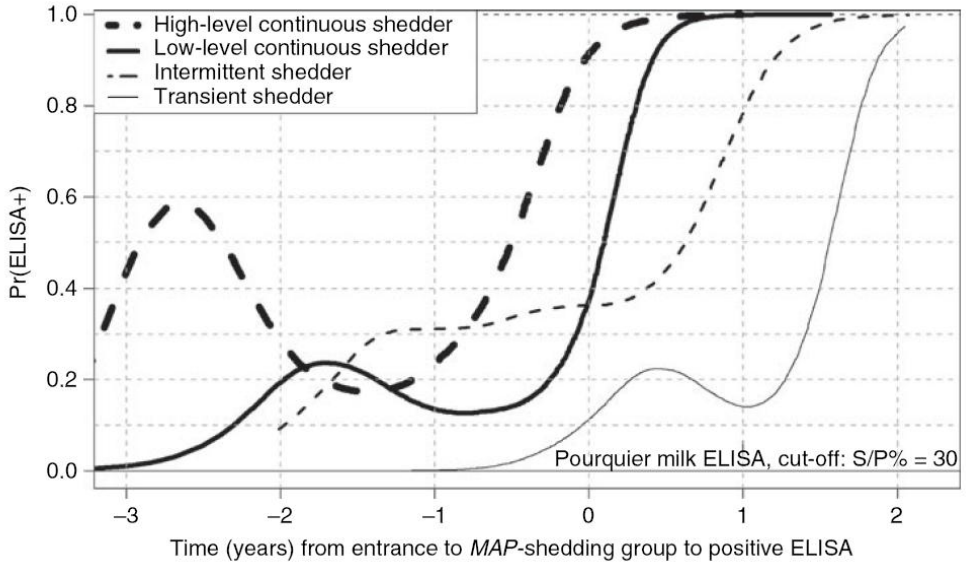


Figure 5. Probability of testing positive by a commercial antibody ELISA at various time points relative to the start of MAP shedding

Percentage of cows testing positive using the Pourquier ELISA relative to when they started shedding high, low, intermittent or transient levels of MAP. For cows not shedding MAP (light grey flat line), the median time in a test period was zero. Pr: ELISA-positive animals (corresponding to a percent, (Nielsen, 2009b; Nielsen, 2010).

A fundamental component of ELISA and of all immune-based diagnostic methods for paratuberculosis is the antigen employed. Some of them are not MAP specific and false positive results can therefore occur (Kohler et al., 2008; Nielsen, 2010; Stevenson, 2010b), while others could be associated with specific antibody reactivity (Koets et al., 2001). Single antibody ELISA results may be of limited value (Kohler et al., 2008), except for prevalence estimates and herd certification, because animals with various antibody profiles are in different stages of MAP infection. Instead, the results from frequent testing can better reveal the stage of infection, by following trends in results with time (Collins et al., 2006; Stevenson, 2010b). However, there is limited information on test evaluation in the context of repeated testing (Nielsen, 2010).

1.4.7.2. Direct methods

1.4.7.2.1. Examination of Ziehl-Neelsen stained fecal smears

A presumptive diagnosis of clinical paratuberculosis can be confirmed using microscopic examination of Ziehl-Neelsen stained fecal smears (ZN-test) for the presence of clumps of acid-fast organisms. However, MAP organisms were detected only in 56% of 177 culturally positive faecal samples and was therefore concluded that ZN-test was an unreliable method for the detection of MAP organisms (Ris et al., 1988). In another study, the presence of acid-fast bacteria was detected in 49.3% of the samples from clinically affected cattle and in only 19.3% from subclinically infected cattle; in this study it was concluded that ZN-test is unreliable in diagnosing Johne's disease (Zimmer et al., 1999). More recently, ZN-test and serum ELISA were evaluated in cattle suspected of clinical paratuberculosis, revealing lower values of Se and Sp, as well as lower predictive values than ELISA; this study has concluded that ELISA is preferred to the ZN-test to confirm the presumptive diagnosis of clinical paratuberculosis and that little diagnostic information can be gained by performing the ZN-test in addition to the ELISA (Weber et al., 2009).

1.4.7.2.2. Polymerase Chain Reaction (PCR)

The detection of MAP genes by PCR has shown advantages (speed, identification of agent, lack of contamination) and disadvantages (moderate sensitivity, high cost, special equipment and skilled personal required (Collins, 1996). However, due to recent developments, PCR is being suggested for herd screening (Collins et al., 2006; Anonymous, 2010), and it has been recently brought to discussion as a possible new golden standard for paratuberculosis (Stevenson, 2010b). On the other hand, the PCR technique is rapid and specific, and in contrast to culture–based diagnostic, no additional tests are required to confirm the identity of the organism detected (Collins, 1996). The most popular target gene for detection of MAP is the multi-copy element IS*900* (Bull et al., 2003a; National Advisory Committee on Microbiological Criteria for Foods, 2010; Bolske and Herthnek, 2010; Stevenson, 2010b; Gill et al., 2011). However, mycobacteria other than MAP have been found to carry IS*900*-like elements with nucleotide sequences that are up to 94% identical to the nucleotide sequence of MAP IS*900* (Englund et al., 2002). Some PCR systems that target IS*900* also can give false-positive results with DNA from mycobacteria other than MAP and with DNA from other types of organisms (Mobius et al., 2008a). In response to the uncertainty about the specificity of PCR systems that target IS*900* for identification of MAP, several other target sequences for

use in MAP identification systems have been proposed: ISMap02, IS*Mav2*, hspX, locus 255, and F57 (Table 1). The F57 sequence appears to have been the most widely used of these targets. Both single-round and nested PCR systems that target the F57 sequence have been reported to be highly specific for MAP (Tasara and Stephan, 2005; Mobius et al., 2008a).

PCR performed very well as confirmatory test on cultures, but its application to clinical samples has been problematic, mainly due to the problems associated with DNA extraction from complex matrices as milk, feces and blood and the presence of PCR inhibitors (Stevenson, 2010b). Limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, matrix tested, and PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, and multiplex PCR, (National Advisory Committee on Microbiological Criteria for Foods, 2010). PCR formats and techniques for enrichment or concentrating MAP are variable, having advantages or disadvantages depending on the matrices used for MAP detection and the way they are performed (Mobius et al., 2008a; Bolske and Herthnek, 2010; Stevenson, 2010b).

In conventional PCR, only the amount of final DNA product is measured, a process referred to as end point analysis (Bolske and Herthnek, 2010). In the nested PCR, the running of two consecutive PCR reactions with two different sets of primers, using the product of the first reaction as template in the second, enables a number of potential advantages to be achieved, including specificity and sensitivity. Nested PCR is more demanding of time and labor than conventional PCR and the transfer of products from the first run to the second poses a greater risk for cross-contamination, which is the main disadvantage with nested PCR (Bull et al., 2003a; Mobius et al., 2008a; Bolske and Herthnek, 2010; Gill et al., 2011). In real-time PCR or quantitative PCR (qPCR) the instrument monitors the reaction progress throughout every temperature cycle by measuring the light emitted from a fluorophore incorporated with the DNA product, by using either a fluorescent dye that intercalates with all double-stranded DNA or target-specific fluorescent probes (Wittwer and Kusukawa, 2004; National Advisory Committee on Microbiological Criteria for Foods, 2010; Bolske and Herthnek, 2010). There are different probe technologies, but the most widely used is the so-called Taq- Man probe. The number of cycles needed for the fluorescence to reach a certain threshold level (or Cycle threshold), often referred to as the Ct-value, and is a concrete measure that lends itself to comparison across samples and quantification of target DNA. The lower the Ct value, the more target DNA in the sample (Wittwer and Kusukawa, 2004; Bolske and Herthnek, 2010).

The use of several sets of primers designed for different targets (multiplex PCR) enables simultaneous testing of a sample for several target sequences. In qPCR, the fluorophores in the respective Taqman probe can be chosen to emit light of different wavelengths, measured in separate channels (Bolske and Herthnek, 2010).

In PCR an internal amplification control (IAC) is a nontarget DNA sequence present in the very same sample tube, which is coamplified simultaneously with the target sequence (Hoorfar et al., 2004; Brey et al., 2006). In a PCR without an IAC, a negative response (no band or signal) could mean that there was no target sequence present in the reaction. But could also mean that the reaction was inhibited, due to malfunction of thermal cycler, incorrect PCR mixture, poor DNA polymerase activity, or not least the presence of inhibitory substances in the sample. Conversely, in a PCR with IAC, a control signal should always be produced even through there was no target sequence present. When neither IAC nor target signal is produced, the PCR reaction fails (Hoorfar et al., 2004). Incorporating an IAC to each reaction minimizes the likelihood of false-negative results in both conventional and real-time PCR (National Advisory Committee on Microbiological Criteria for Foods, 2010). Often multiplex PCR is used to co-amplify an IAC molecule (sometimes referred to as a ‘mimic’), to monitor PCR inhibition (Bolske and Herthnek, 2010).

1.4.7.2.3. Culture

Cultivation and identification of MAP is the definitive diagnostic test for Johne´s disease at individual and herd level; thus it is standard practice to use culture to confirm a presumptive diagnosis in individual animals (Whittington, 2010). Nevertheless, although culture still remains as the golden standard, its sensitivity lies around 30% in subclinically infected cattle (Nielsen and Toft, 2008), mainly because of the intermittent shedding of microorganisms and diverse features of the culture techniques (Whitlock et al., 2000). This means that, the sensitivity of fecal culture could be high for detection of symptomatic, but low for the detection of subclinical infected animals. The specificity is considered to be almost 100%, if isolates are confirmed as MAP (Nielsen and Toft, 2008). Disadvantages of culture are slow detection, generally 12 to 16 weeks or longer, detection of only animals excreting MAP in feces and the relatively high cost compared to other tests (Collins, 1996). According to Whittington (2010), there are four critical steps in the cultivation of MAP: 1. decontamination of clinical samples to destroy or suppress irrelevant, mostly rapidly growing microbes, which include both bacteria and fungi, present in vast numbers in feces. 2. Prolonged incubation in

appropriate media containing antimicrobial agents to suppress any remaining contaminants for long enough for MAP to emerge. 3. Recognition of MAP colonies on solid media or a particular sign of growth in broth media. 4. Identification of MAP by phenotypic and/or genotypic means.

Clinical samples, especially feces, present a particular problem due to their high load of enteric bacteria, and most protocols require removal of the particulate matter with which many microbes associate. Several chemical products have been used to reduce or eliminate contaminants of fecal samples. A combination of NaOH and Oxalic Acid (NaOH–OA) was initially introduced and is more commonly used in Europe (Jorgensen, 1982; Kalis et al., 1999), while hexadecylpyridinium chloride (HPC) was used first in the USA and has been used in almost all countries of the world (Whittington, 2010). There is a dramatic loss of MAP when samples are prepared for culture. The antimicrobials that are used to prepare samples or included in culture media may have a deleterious effect on viability, recovery or growth of MAP (Johansen et al., 2006). To increase the analytical sensitivity of fecal culture, MAP may be concentrated during or after decontamination of the sample. Later modifications included centrifuging the decontamination solution, combining both of these earlier methods and incorporating three decontaminants (Stabel, 1997; Whittington, 2010). However, a progressive loss of viable organisms occurs with each step in sedimentation and centrifugation protocols, as only part of the material from one step is taken forward to the next step (Whittington, 2010).

Both solid and liquid media can be used to cultivate MAP, but not all media support the growth of all strains (de Juan et al., 2006b; Cernicchiaro et al., 2008; Whittington et al., 2011). Two media have been found to be the most suitable: Löwenstein Jensen medium (LJ) in some European countries and HEYM elsewhere. Other media as Modified Middlebrook (MB) 7H10 agar, MB 7H11 agar and BACTEC 12B medium appear to performed well and within reasonable incubation periods (Whittington et al., 2011). MB 7H10 or MB7H11 agar and MB 7H9 broth are also suitable base media, but, for optimal growth of MAP, egg yolk must be added (Whittington et al., 2011). Common Type S strains of MAP do not grow well or even at all on HEYM, LJ (Whittington et al., 2011) or in Mycobacteria Growth Indicator Tube (MGIT) medium. In most circumstances mycobactin is an essential component of media for primary culture of MAP, but there may be sufficient carry-over of mycobactin to new media to disguise this need during subculture (Lambrecht and Collins, 1992). Sodium

pyruvate was reported to stimulate the growth of MAP and is included in both the LJ and the HEYM media used by some laboratories (Eamens et al., 2000). However, some isolates grew better on HEYM without sodium pyruvate than on HEYM with it (Whittington et al., 2011). Antimicrobials such as malachite green, cycloheximide, amphotericin B, vancomycin, nalidixic acid, chloramphenicol, penicillin G, polymyxin B, trimethoprim, azlocillin and ampicillin have been included in various media but thorough evaluation of their potential to inhibit growth of MAP is mostly lacking (Whittington, 2010).

Culture protocols have evolved independently in different laboratories over the years. In some European countries, the NaOH–OA decontamination protocol is used in conjunction with LJ medium (Kalis et al., 1999). In the USA and most other countries, sedimentation methods with HPC are combined with HEYM or liquid media (Stabel, 1997; Grant et al., 2003). In Denmark a comparison between HEYM and LJ using only the NaOH–OA decontamination protocol found that HEYM medium had slightly greater sensitivity than LJ (Nielsen et al., 2004).

1.4.8. Risk factors

Risk factors are factors that are associated with an increased likelihood of a disease occurring (Smith, 2005). Prevention and control of paratuberculosis demands a high level of knowledge about the magnitude of disease presentation and about factors that influence its entrance and perpetuation in herds. Herd practices can increase or decrease the probability of MAP to enter or to circulate in a given dairy cattle population. These practices vary not only between countries or agro ecological zones, but also between regions or even herds. This situation leads to the need of a local determination of specific factors associated with the paratuberculosis status of animals or herds in a specific region. Many and different individual animal and management herd factors have been identified to influence the paratuberculosis infection status in dairy cattle. Most of these studies have been conducted at the herd level, and have used mainly serological results to establish the paratuberculosis diagnosis of animals and the subsequent identification of risk factors (Johnson-Ifearulundu and Kaneene, 1999; Hacker et al., 2004; Dieguez et al., 2008). However, other studies have used other diagnostic methods as PCR (Ansari-Lari et al., 2009), fecal culture (Obasanjo et al., 1997) or more than one diagnostic method (Kobayashi et al., 2007) to determine paratuberculosis status or prevalence and paratuberculosis risk factors.

1.4.9. Control

Control of paratuberculosis is based on the fact that if no control measures are taken the prevalence of Johne´s disease will further increase, seriously affecting the quality of domestic livestock. In addition, the economic impact of paratuberculosis on production and reproduction, the suspected but unconfirmed causal relationship between MAP and Crohn´s disease, and the detection of relative small quantities of MAP in food and water, are sufficient reasons to support control programs to improve animal health and welfare and to reduce the exposure of the food chain to MAP under a precautionary principle (Bakker, 2010). Almost all control programs for paratuberculosis based on reducing within-herd transmission of MAP. However, control programs for paratuberculosis have been based on different diagnostic and intervention approaches depending on the objectives pursued at regional or national scale (Nielsen, 2009a; Bakker, 2010; Whitlock, 2010; Kennedy and Citer, 2010). Control measures are normally based on a combination of management changes reducing the risk of transmission between animals that shed MAP and other no-infected (young) animals, and the removal of infectious animals by early detection (test and cull) or vaccination strategies (Benedictus et al., 2000). Overall disease status of herd, region or country is an important determinant of what option for control are applicable, if any measure will be applied (Bakker, 2010).

In general, control programs for paratuberculosis in Europe are voluntary in almost every country pursuing one, they are mostly regional, include several initiatives, and are based on individual serum or individual or bulk milk ELISA, fecal culture and less frequently PCR or even ZN-test (Nielsen, 2009a; Bakker, 2010). Another factor common to almost all control programs for paratuberculosis is the acknowledgment of the need to introduce management changes to reduce transmission and the need to warm participating herd owners that controlling paratuberculosis will take considerable time and effort (Bakker, 2010).

Vaccination to control bovine paratuberculosis has been relatively little used in the frame of control programs, compared to sheep and goats, mainly due to reasons regarding interference with skin testing for bovine tuberculosis, failure to prevent infection, and the presence of large lesions at the inoculation site (de Lisle, 2010). However, several vaccine types (kill-whole-cell-based, live attenuated whole-cell-based, and improved whole-cell-based) have been used in cattle and have demonstrated to be: effective on cellular and humoral immunology response (Muskens et al., 2002), highly profitable (van Schaik et al., 1996), and effective to reduce

MAP shedding (Juste et al., 2009). Nevertheless, a recent meta-analysis has concluded that it is impossible to prevent infection in dairy cattle only by implementation of vaccination programs, the reduction of pathological findings and decrease of positive culture results indicate a lower risk of infection for vaccinated animals, and the decrease of positive fecal culture results after vaccination can possibly reduce the environmental load in infected herds (Kohler et al., 2010).

2. MATERIALS AND METHODS

2.1. Establishment and evaluation of MAP genotyping methods

The objective of this study was the establishment and evaluation of methods for differentiation (typing and subtyping) of MAP isolates.

2.1.1. Mycobacterial strains and isolates

Two MAP reference strains, 10 MAP field isolates, and one MAH isolate from different host species and clinical samples were analyzed in this study (Table 4). Reference strains and isolates were selected from the mycobacteria collection of the Institute of Veterinary Food Sciences of the Justus–Liebig–University Giessen, Germany. Independently of the medium used for primary isolation, mycobacteria were subcultured for this study on HEYM slants supplemented with mycobactin J (Becton Dickinson, Heidelberg, Germany), and were incubated at 37°C for a maximum of 20 weeks. All isolates and strains grown in this period of incubation and none of them showed pigmentation. MAP isolates were not tested for MAP-type differentiation and were assumed cattle-type, type C or type II, due to the compatible phenotypical characteristics (especially rapid growth) observed on HEYM.

Table 4. Isolates and strains used for establishment and evaluation of MAP subtyping methods

Identification	Country of isolation	Host species	Isolated from
K-10 (BAA-968) ATCC 19698 (J27)	USA	Cattle	Feces
IPRK 297 RK 18 218/133 518 464 MW6	Germany		
JD 131	Scotland	Sheep	Tissue
Pat 7 III A Pat 30 III A[a]	Germany	Human	Bowel biopsy
Hum 1 Hum 2	England		

[a] MAH isolate

2.1.2. DNA isolation and MAP confirmation

Genomic DNA was extracted from all mycobacterial strains using the method described by (van Soolingen and Hermans, 1995; Pavlik and Bartos, 2008). Briefly, one loop full of cells were transferred into a tube containing 400 µl of Tris EDTA (TE) buffer, heated for 20 min at 80°C and cooled at room temperature. 50 µL of 10 mg/ml lysozyme were added and incubated for at least 1 h at 37°C. Subsequently 75 µl of 10% Sodium Dodecyl Sulfate (SDS)/proteinase K mix were added, shortly vortexted and incubated for 10 minutes at 65°C. Before the addition of 100 µl of a Cetyltrimethylammonium Bromide (CTAB)/NaCl pre-warmed solution, 100 µl of 5 M NaCl were added. This solution was vortexted and incubated for 10 min at 65°C. DNA was purified and precipitated with 750 µl chloroform/isoamyl alcohol and a volume of isopropanol, respectively. Subsequently the mix was place for 30 min at -20°C. After centrifugation the supernatant was removed and the precipitate was washed with 1 ml of cold 70% ethanol, centrifuged two times and all traces of ethanol removed. The pellet was lead to dry at room temperature for 10 min and was dissolved in TE buffer and stored at 4°C until used. DNA concentration was measured with an UV-Spectrophotometer (BioMate 3, Thermo Electric, Wisconsin, USA) to achieve a precise quantification of the quantity and quality of the DNA to be used in further analysis.

The DNA obtained on the first DNA-isolation procedure was used for both MAP confirmation and subtyping. All mycobacterial isolates were tested for MAP confirmation using the F57 and IS*Mav2*-real-time PCR and the IS*900*-conventional PCR described above (see 2.2.7.). One mycobacterial isolate (Pat 30 III A) was negative to MAP specific F57 / IS*Mav2*-real-time PCR and IS*900*-conventional PCR. This isolate was identified as *Mycobacterium avium* subspecies based on the PCR amplification results of the 16S rRNA gene and the 16S-23S rRNA internal transcribed spacer (ITS) for similarity–based species identification, GenBank accession number EF059903.1 and EF059904.1, respectively (Füllgrabe, 2009). This isolate was later identified as MAH based on the results of the MIRU-VNTR analysis in the frame of the present dissertation.

2.1.3. IS*900*-RFLP

Probe preparation. IS900-RFLP analysis was performed based on the protocols from (Bauerfeind et al., 1996; Pavlik et al., 1999; Pavlik and Bartos, 2008; Anonymous, 2008a). The IS900 probe labeling for RFLP was performed by using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer´s instructions and using the primers suggested by (Pavlik et al., 1999). The MAP reference strain K-10 was used as template for the probe amplification and labeling.

Southern blot. Approximately 1.5 µg of DNA was digested by restriction endonucleases *BstE* II, *Pst* I, and *Bam*H I (Roche, Mannheim, Germany) according to the manufacturer´s instructions, with exception of the duration of incubation that was prolonged to 6 hours according to the standard protocol by (Pavlik et al., 1999). Electrophoresis of the digested DNA was carried out in a 0.8% 1x Tris/Borate/EDTA (TBE) buffer agarose gel with measures 15 x 20 cm and with 16 slots. The DNA molecular weight marker III, digoxigenin-labeled (Roche, Mannheim, Germany) was mixed with DNA loading buffer and used during gel electrophoresis. After an initial run of 10 min at 100 V (4 V/cm), the voltage was switched to 45 V (1.8 V/cm) until end of the electrophoresis. The whole electrophoresis was run for 17 h. After electrophoresis the gel was stained with ethidium bromide for 20 min and checked and scanned with a camera on an UV transilluminator. DNA was transferred from agarose gel to nylon membranes positively charged (Roche, Mannheim, Germany) using an upward capillary transfer procedure and 20x Saline Sodium Citrate (SSC, Roche, Mannheim, Germany) as transfer buffer according to standardized protocols (Anonymous, 2008a).

Hybridization steps (pre-hybridization and hybridization) of probe to target were carried out in Stuart Scientific roller bottles (Bibby Scientific Limited, Staffordshire, UK) and in an Stuart Scientific Hybridization Oven/Shaker SI20H (Bibby Scientific Limited, Staffordshire, UK) at 42°C, and using the hybridization solution for nucleic acid blots with digoxigenin-labeled probes DIG Easy Hyb (Roche, Mannheim, Germany). After hybridization the DNA was fixed by UV cross linking (Ultraviolet Crosslinker, Hoefer, San Francisco, USA) at 300 mJ/cm2, 3 min as suggested by (Bauerfeind et al., 1996). Subsequently, two stringent washes, low-stringency wash (2x SSC, room temperature, 5 min, two times) and high-stringency wash (0.3x SSC, 4% SDS, 55°C, 10 min, two times) were performed to disrupt undesired hybrids (Pavlik and Bartos, 2008). For localizing the Probe-Target hybrids with Anti-DIG, the DIG Wash and Block Buffer Set, including a washing buffer, maleic acid buffer, blocking solution,

and detection buffer (Roche, Mannheim, Germany) was used. For visualizing the Probe-Target hybrids (digoxigenin–labeled DNA), the DIG Nucleic Acid detection kit (Roche, Mannheim, Germany) based on an enzyme immunoassay and enzyme-catalyzed color reaction with Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Chromogenic Method) was used according to the manufacturers' protocol. Results obtained were photographed with a digital camera and analyzed using the software Bionumerics version 5.1 (Applied Maths, Sint-Marteens-Latem, Belgium). RFLP profiles obtained were classified based on previous publications according to the endonuclease used, *BstE* II (Pavlik et al., 1999), *Pst* I (Whipple et al., 1990), and *Bam* HI (Cousins et al., 2000).

2.1.4. MIRU-VNTR

For the MIRU-VNTR establishment, the loci MIRU-1, MIRU-2, MIRU-3, MIRU-4 (Bull et al., 2003b), MIRU-4-ARG, MIRU-5-UK, MIRU-6, MIRU-7, VNTR-8, and MIRU-11-UK/VNTR-11 (Romano et al., 2005), VNTR-1067, VNTR-1605, VNTR-1658, VNTR-3527, and VNTR-3249 (Overduin et al., 2004) were analyzed in the 13 mycobacterial isolates and strains following the procedures according to (El Sayed et al., 2009).

2.1.4.1. PCR amplification of MIRU-VNTR

The final PCR reaction volume (30µL) contained GeneAmp 10x PCR Buffer (Applied Biosystems, Darmstadt, Germany), dNTP-Mix (10 µM each, Roche, Mannheim, Germany), 0.2 µM of each primer (Eurofins MWG, Martinsried, Germany), 10% Dimethil Sulfoxide (DMSO, Roth, Karlsruhe, Germany), 1U of AmpliTaq Gold Polymerase 5U/µl (Applied Biosystems, Langen, Germany), and 3 µl of DNA. A master mixture blank (without DNA) was included as control in every PCR reaction. Seven micro liters of every PCR product were mixed with 2µl of loading buffer, and electrophoresed in a 1.5% 1x TBE buffer agarose gel.

2.1.4.2. Determination of the number of repeats per locus of MIRU-VNTR

Calculation of the number of repeats per locus for MIRU-VNTR was initially performed according to the size of the amplicon determined by electrophoresis in 1.5% agarose gel. MIRU-VNTR profiles were confirmed by the number of repeat units in selected sequences of representative alleles visualized using the software Bionumerics version 5.1 (Applied Maths, Sint-Marteens-Latem, Belgium), the freeware software Cromas lite ver. 2.01 (Technelysium Pty Ltd., Brisbane, Australia), and the software CodonCode Aligner (CodonCode

Corporation, Dedham, USA) for visualization of sequences. MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus.

2.1.4.3. Purification of PCR products

The amplicons of PCR reactions from the MIRU and VNTR were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) suitable for fragments raging from 100 bp to 10 kb, using a microcentrifuge, and following the instructions of the kit fabricants.

2.1.4.4. Sequencing of purified PCR products

Sequencing procedures of PCR products were carried out by contract with an independent laboratory (Sequence Laboratories, Göttingen, Germany). The single reads sequencing service "Hot-shot" with a reading length of approximately 1000 bases was used on all studies requiring this sequencing step. According to the sequencing service providers, 97% of clean DNAs with no excessive secondary structures deliver 99% exact readings. For the submission of PCR products, all preparation steps were carried out according to the instructions of the service provider available at http://www.seqlab.de/.

2.1.5. MLSSR

2.1.5.1. PCR amplification of MLSSR

The MLSSR analysis was carried out by amplification of the SSR found in locus 1, 2, and 8 according to primers and PCR conditions reported by (Amonsin et al., 2004). The final PCR reaction volume (30µL) contained GeneAmp 10x PCR Puffer (Applied Biosystems, Darmstadt, Germany), deoxyribonucleotide triphosphate (dNTP)-Mix (10 µM each, Roche, Mannheim, Germany), 0.2 µM of each primer (Eurofins MWG, Martinsried, Germany), 10% DMSO (Roth, Karlsruhe, Germany), 1U of AmpliTaq Gold Polymerase 5U/µl (Applied Biosystems, Langen, Germany), and 3 µl of DNA. A master mixture blank (without DNA) was included as control in every PCR reaction. Seven micro liters of every PCR product were mixed with 2µl of loading buffer, and electrophoresed in a 1x TBE buffer agarose gel.

2.1.5.2. Determination of the number of repeats per locus of MLSSR

For the calculation of the number of repeats per locus, PCR products were purified and sequenced as described above for MIRU and VNTR. MLSSR genotypes were expressed as

the combination of the number of repeats found in the loci amplified by PCR and visualized using the software Bionumerics version 5.1 (Applied Maths, Sint-Marteens-Latem, Belgium) or the freeware software Cromas lite ver. 2.01 (Technelysium Pty Ltd., Brisbane, Australia) or CodonCode Aligner (CodonCode Corp. Dedham, MA, USA) for visualization of sequences. If the number of g–repeats at locus 2 were more than 11, g–repeats for this locus were denoted as >11g as suggested previously (Thibault et al., 2008).

2.1.6. Evaluation and calculations

For evaluation of the established subtyping methods, the performance and convenience criteria described by (van Belkum et al., 2007) were used. This assessment was based on a combination of objective (quantitative) and subjective (qualitative) estimates. For the estimation of the discriminatory ability of the subtyping methods individually and combined, the Simpson's index of diversity (*D*) and CI 95% for the index were used as described above. The index was calculated using the online tool for quantitative assessment of classification agreement (available at http://darwin.phyloviz.net/ComparingPartitions/). For evaluation in terms of *convenience*, flexibility, rapidity, accessibility, ease of use, costs, and suitability for computerized analysis and storage of results, subjective aspects were considered and evaluated.

2.2. Diagnosis, genotyping and epidemiology of MAP in dairy cattle in Colombia

The objectives of this study were the diagnosis of MAP in adult cows using serological, bacteriological and molecular methods, the identification of individual and herd management factors associated with the serological individual response to paratuberculosis, and the molecular characterization (subtyping) of MAP isolates in 15 Colombian dairy herds.

2.2.1. Selection of herds and animals for determination of MAP infection (Screening)

Fourteen dairy herds of 9 districts located in and around a municipality of a dairy region in Colombia were sampled for detection (screening) of MAP in November 2007 (Table 5, Figure 6). The municipality is located in the Andean region of Colombia; it has an area of 296 km^2, an altitude of 2500 m, a mean annual temperature of 14°C, and a cattle population of approximately 21500 animals. The herds were selected attempting a representation of all productive districts of the municipality. The calculation of the number of animals per herd to be sampled was carried out based on a procedure previously reported (Johnson-Ifearulundu and Kaneene, 1998). Of these 14 herds, only one herd had presented sporadic clinical cases compatible with paratuberculosis confirmed by PCR and histopathology (Zapata R. et al., 2010). The number of animals estimated to be tested in every herd was randomly sampled.

Table 5. Herds sampled for detection of MAP in Colombia, screening

District	Herd	Herd cattle population	Number of samples	Current tuberculosis status
Monterredondo	1[a]	102	20	Free
Playas	2	75	19	Free[b]
Zona Urbana	3	128	21	Undet[c]
Labores	4	300	29	Undet.
	5	100	19	Undet.
	6	176	25	Undet.
	7	102	23	Undet.
El Yuyal	8	140	20	Free
	9	74	22	Undet.
Santo Domingo	10	181	23	Reactive
Amoladora	11	83	20	Undet.
	12	75	20	Undet.
Zafra	13	67	21	Undet.
Zancudito	14	96	25	Undet.
Total		**1699**	**307**	

[a] Herd with history of diagnosis and clinical cases of paratuberculosis.

[b] Free status expired in October 2010

[c] Undet. Undetermined

Figure 6. Political MAP and location of Colombia
The star shows the approximate location of herds selected for MAP diagnosis in this study

2.2.2. Collection of samples and information

Blood and fecal samples were taken. Serum and feces were frozen at -20°C until analysis at the Institute of Veterinary Food Science of the Justus–Liebig–University Giessen (Germany) in January 2008. Information about age and some features of the animals, as well as herd management practices were collected.

2.2.3. ELISA–A (Svanovir Para–TB Ab, Svanova Biotech AB)

All serum samples (n=307) were tested with the ELISA–A test. The ELISA-A is an indirect serum ELISA test based on detection of lipoarabinomannan (LAM, Svanovir Para–TB Ab ELISA Kit, Svanova Biotech AB, Uppsala, Sweden). The interpretation of ELISA-A results was done following instructions of the test fabricants. Briefly, the percent of positivity (PP) by ELISA was estimated according to the optical density values (OD) obtained at 450 nanometers (nm) and using the formula PP= Mean OD value (sample or Negative control) / Mean OD value (Positive control) x 100. A positive animal by ELISA was defined as an animal with a PP $\geq$ 53 and a negative animal was defined as an animal with a PP $\leq$ 31

negative. ELISA-A was carried out in single preparation. A herd was considered positive if at least one animal tested positive by ELISA–A.

2.2.4. ELISA–B (Paratuberculosis antibody verification, Institute Pourquier)

Positive and doubtful serum samples with ELISA–A (n=39) were tested with ELISA–B described above (Figure 7). The ELISA–B is an indirect ELISA test based on detection of antibodies to MAP protoplasmic antigens. The test includes a pre–absorption step with *M. phlei* (ELISA paratuberculosis antibody verification, Institute Pourquier, Montpellier, France). ELISA–B test was applied in duplicate to serum samples positive and doubtful by ELISA–A. Animals with ELISA-B results of Value of the Sample to Value of the Positive Control relation (S/P) more than or equal to 70% were considered as positive, between 60%-70% as doubtful, and less than or equal to 60% as negative. S/P% was obtained using the formula S/P= (corrected OD. 450 nm value of the sample /mean corrected OD 450 nm value of the positive control) x 100.

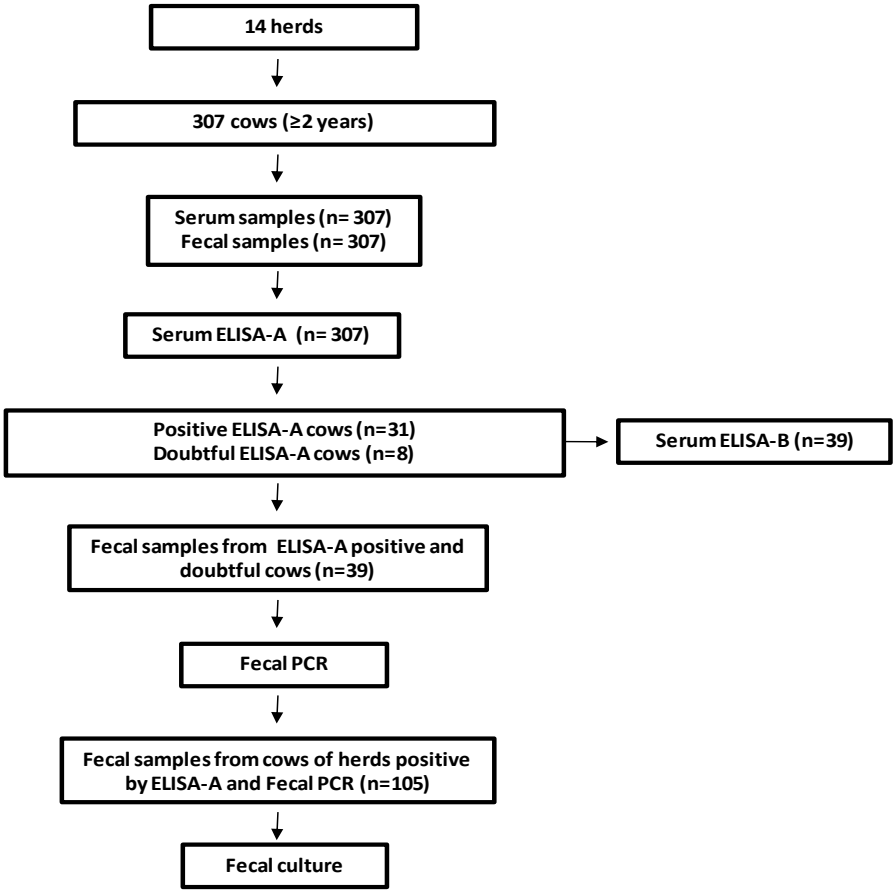


Figure 7. Overview of the study design for the screening of MAP in dairy cattle in Colombia

2.2.5. DNA isolation from fecal samples

DNA isolation from fecal samples of ELISA–A–positive animals was carried out in duplicate using a commercial DNA preparation kit (High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany). Briefly, 1.5 g of bovine feces was put in a 15 ml sterile, non-pyrogenic centrifuge tube (Sarstedt, Nümbrecht, Germany). Five ml of a buffer for stabilization (Stool Transport and Recovery-S.T.A.R. buffer, Roche, Mannheim, Germany) was added to fecal sample and homogenized. This suspension was subsequently centrifuged for 1 min by 1000 x g and 1 ml of the supernatant was put in a 2 ml conical sample tube (Biozym Scientific, Hess. Oldendorf, Germany) containing ceramic beads, size range 1.4-1.6 mm, Genotype ZY (Zirkonoxid-Beads, Yttrium stabilized, Sigmund Lidner, Warmensteinach, Germany). A mechanical cell disruption step was carried out in an automated biological sample lyser (Precellys 24, Bertin technologies, Montigny-le-Bretonneux, France) to achieve efficient cell lysis. The mixture was subsequently incubated at 95°C for 10 min and centrifuged 5 min by 5000 x *g*. 200 microliters of the supernatant was added to a 1.5 ml reaction tube containing 5 µl of lysozyme (Merck, Darmstadt, Germany) solution. Further processing was done according to kit´s protocol for isolation of nucleic acids from bacteria and yeast. DNA isolation was always carried out in duplicate.

2.2.6. DNA isolation from Mycobacteria

DNA isolation from mycobacteria for MAP confirmation by PCR and subtyping of MAP isolates was carried out using a commercial preparation kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany). This preparation included overnight lysis buffer incubation at 37°C, proteinase K/AL-buffer incubation for 90 min at 56°C, and final incubation for 15 min at 95°C, as a modification of the protocol of the commercial kit.

2.2.7. PCR

2.2.7.1. F57 and ISMav2-real-time PCR

DNA from fecal and slurry samples as well as from pure mycobacterial culture was tested in duplicate for MAP detection, identification or confirmation with the real-time PCR method targeted to specific MAP F57 and IS*Mav2* described by (Schonenbrucher et al., 2008). The real-time PCR method also included an IAC to avoid the misinterpretation of false negative results (Schonenbrucher et al., 2008). In the PCR system, a positive MAP control (DNA of a positive MAP strain), a non-MAP negative control (DNA of a non-MAP mycobacteria) and a master-mix blank control were also included.

2.2.7.2. IS900-conventional PCR

DNA from fecal and slurry samples and from bacterial culture was tested in duplicate for MAP detection, identification or confirmation with the conventional nested-PCR targeted to IS*900* described by (Bull et al., 2003a). Additional to the samples, a positive and a negative preparation control, as well as a blank control were included. In the PCR system, a positive MAP control (DNA of a positive MAP strain), a non-MAP negative control (DNA of a non-MAP mycobacteria) and a master-mix blank control were also included.

2.2.8. Fecal culture

Fecal samples from all animals of herds positive by ELISA–A and by PCR (n=105), regardless of individual result, were cultured using the culture methods 1, 2, and 3.

2.2.8.1. Culture method 1 (0.75% HPC and HEYM)

In this method three grams of feces were added to a 50 ml sterile tube (Sarstedt, Nümbrecht, Germany) containing 30 ml of a 0.75% HPC weight/volume (w/v) solution. This suspension was manually mixed by shaking and vortexting, and let in vertical position for 5 min at room temperature to allow the precipitation and sedimentation of big particles. Approximately 20 ml of the upper portion of the supernatant was transfer to another 50 ml sterile tube, in which the whole suspension was agitated for 30 min at 200 U / min. Tubes were place in vertical position in the dark for 24 h at room temperature. Decontaminated pooled fecal samples were centrifuged at 900 x *g* during 30 min, supernatant was discarded and two HEYM slants, supplemented with mycobactin J (Becton Dickinson, Heidelberg, Germany) were inoculated with 300µL of the decontaminated pellet (Anonymous, 2007). The slants were incubated at 37°C for a minimum of 16 weeks and checked at 1-2-week intervals for mycobacterial growth or contamination with undesirable germs.

2.2.8.2. Culture method 2 (4% NaOH - 5% oxalic acid and LJ)

In this method two grams of feces were added to a 50 ml sterile tube (Sarstedt, Nümbrecht, Germany) containing 50 ml of a solution of 4% NaOH (w/v) and were homogenized by shaking and vortexting. Particulate matter was allowed to settle for 15 min. The supernatant was transferred into a new 50 ml sterile tube and shaken for 10 min on a horizontal shaker. After centrifugation (3,000 x g, 15 min, 20°C) the pellet was resuspended in 20 ml of 5% oxalic acid (w/v) by thorough repeated agitation on a vortexer and was then shaken for 15 min

on a horizontal shaker. The suspension was centrifuged as before, the pellet was resuspended in 4 ml of sterile saline (0.15 M NaCl) and used as inoculum, for which 200µl aliquots were transferred to each of two slants of LJ containing mycobactin J and a mix of polymyxin B, amphotericin B, carbenicilin and trimetoprim (Bioservice, Waldenburg, Germany, (Glanemann et al., 2004). All slants were incubated for 6 months and were checked at 1-2-week intervals for mycobacterial growth or contamination with undesirable germs.

2.2.8.3. Culture method 3 (0.75% HPC and modified MB 7H11)

In this method one gram of feces was homogenized with 10 ml of distilled water in a Stomacher plastic bag for 30 seconds. The resultant homogenates were mixed with 10 ml of 1.5 % HPC. The treated homogenates were left overnight at room temperature to allow particulate materials to settle. After this, the supernatants were centrifuged at 3800 x g for 30 min. at 4°C and each pellet was resuspended in 10 ml of distilled water. The centrifugation step was repeated and each pellet resuspended in 1 ml of distilled water. The suspension was transferred to a microcentrifuge tube and centrifuged at 6500 x g for five minutes. The final pellet was resuspended in 0.5 ml sterile distilled water. After this, two slants of modified MB7H11 medium with whole egg and Amphotericin B supplemented with mycobactin J (Bioservice, Waldenburg, Germany) were inoculated in duplicate with 0.25 ml of the prepared suspension (Greig et al., 1997). All slants were incubated for 6 months and were checked at 1-2-week intervals for mycobacterial growth or contamination with undesirable germs.

Colonies with compatible mycobacterial morphology were tested for acid fastness by the Ziehl–Neelsen stain of smears method (Becton Dickinson, Heidelberg, Germany) following standards procedures of the fabricants. The mycobacterial isolates were tested for MAP confirmation by the F57 and IS*Mav2*-real-time PCR and IS*900*-conventional PCR methods described above (see 2.2.7). Acid–fast mycobacteria that tested negative for MAP by MAP-PCRs were further examined to determine their identity by PCR amplification of the 16S rRNA gen.

2.2.9. rRNA similarity–based species identification

Mycobacterial isolates that tested negative for MAP by F57 and IS*Mav2*-real-time PCR and by IS*900*-conventional PCR were further examined to determine their identity by similarity–based species identification. For this purpose the PCR amplification of a fragment of the 16S

rRNA gen was carried out as described by (Kuhnert et al., 1996). PCR products were purified as described above and shipped for sequencing. Sequences obtained were compared for similarity–based species identification using the databases Ribosomal Differentiation of Medical Microorganisms (RIDOM) from the University of Würzburg, Würzburg, Germany (available at http://www.RIDOM.de, (Harmsen et al., 2002), and the Basic Local Aligment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI, available at http://www.ncbi.nlm.nih.gov). Similar isolates sharing the distinct phenotypical characteristics of growth and pigmentation of acid fast mycobacteria already indentified were not further tested again for 16s rRNA similarity–based species identification, due to economical reasons and because the specific species determination of all mycobacterial isolates different from MAP was beyond the scope of all studies.

2.2.10. Purification of PCR products

The amplicons of PCR reactions from the similarity–based species identification by 16S rRNA gen amplification, MIRU, VNTR, and MLSSR methods were purified as described above (see 2.1.4.3).

2.2.11. Sequencing of purified PCR products

Sequencing procedures of PCR products were carried out as described above (see 2.1.4.4.)

2.2.12. Factors associated with the MAP serological status

For the exploratory determination of factors associated to the paratuberculosis status in the present study, the result of the ELISA-A test described above was chosen and considered the outcome or dependant variable in the further analysis. Doubtful results by ELISA-A were considered negative and not taken into account in the present study. To determine factors that could have influenced the individual serological result to paratuberculosis in the study population, information regarding individual animal features and herd management practices was collected. These animal features and herd management practices have been reported as factors associated to paratuberculosis status in previous studies on the same type of animals (dairy cattle) and using the same diagnostic test (ELISA).

For the collection of information, two questionnaires were administered to herd managers or herd owners during collection of serum samples that were used later for paratuberculosis infection determination by ELISA-A. The first questionnaire asked for information (interval-

scaled and categorical variables) regarding individual factors of animal such as age, whether born in the farm, parity, and individual daily average milk production (See Annex 1). A second questionnaire asked for information (interval-scaled and categorical variables) regarding herd management practices related to the maintenance or transmission of MAP within and between herds (See Annex 2). Questions regarding herd management practices were grouped in four categories: the first category referred to general information of herd (herd size, access to veterinary assistance, herd average milk production per day, and whole herd cattle population); the second category concerned the information about the presentation of disease in herd (currently presentation of symptomatic animals in herd, presentation of Johne's disease in the last 2 years, and treatment of symptomatic animals); the third category related to the management factors affecting MAP transmission between herds (cattle purchase, own animals graze in foreign pastures, foreign animals graze in own pastures, existence of specific calving place); and the fourth category included the factors regarding housing and hygiene (housing type of calves before weaning, feed type of calves before weaning, manure spread on pastures, and birds sighting in feedstuff store).

2.2.13. Selection of herds and animals for determination of MAP infection (Confirmation)

Between November and December of 2009, five dairy herds were selected to be examined for MAP (Table 6). Of these five herds, four herds (herds 1, 2, 3, and 4) tested ELISA and PCR positive but culture negative for MAP two years before in the screening in 2007 (see 2.2.1.). The four herds previously tested in 2007 (screening) and selected again in 2009 for confirmation (herds 1, 2, 3 and 4) have never followed any structured or consistent control program for prevention or control of paratuberculosis before the screening in 2007, or in the period between both screening in 2007 and confirmation in 2009. However, culling of animals with non-responsive diseases (including animals with compatible signs of Johne's disease in herd 1) or low productive or reproductive performance was done permanently. The remaining herd (herd 5) had a cow with weight loss and nonresponsive diarrhea compatible with paratuberculosis, but did not have a history of Johne's disease or a previous diagnosis of MAP. From the herds tested, only herd 2 has purchased animals before the screenig study in 2007 and between both studies. Herd 5 has purchased animals in the last two years before the sampling of the present study. Between herd 3 and herd 4, which belong to the same farmer, cattle exchange occurs usually. All herds raise their own replacement heifers (Table 6).

Table 6. Information on herd management of five herds examined for MAP in Colombia, 2009

Herd[a]	District	Number of cattle purchased in the last two years		Raising of own replacement heifers
		2007 (screening)[b]	2009 (confirmation)	
1	Monterredondo	0	0	Yes
2	El Yuyal	10	5	Yes
3	El Yuyal	0	0	Yes
4	Santo Domingo	0	0	Yes
5	Santa Bárbara	N.A.[b]	4	Yes

[a] Herd 2 and herd 4 belong to the same farmer and cattle exchange between both herds occurs frequently.

[b] N.A.: not applicable. This herd was not sampled in 2007 (screening).

2.2.14. Collection of samples and information

Blood and fecal samples were taken from all adult dairy cows (≥2 years) in every herd. In herd 2, 110 cows were sampled for feces, but only 53 of them were sampled for serum due to reluctance of farmer to sample all animals. In one herd (herd 1) that had slurry pit collecting liquid manure and wastewater from the herd's milking parlor, slurry samples were additionally taken from three different places of the pit. From one animal of the same herd (herd 1), a section of large intestine (colon) and a mesenteric lymph node were obtained after euthanasia and necropsy due to advanced clinical symptoms compatible with Johne's disease. Information about age was collected from all animals with exception of six animals, from which farmers did not have available data at the moment of sampling.

2.2.15. ELISA-C (ID Screen Paratuberculosis Indirect, IDVET)

Serum samples (n=329) were tested with the ELISA-C test (Figure 8). The ELISA-C used is an indirect ELISA test based on detection of antibodies to MAP extract (ID Screen Paratuberculosis Indirect, IDVET, Montpellier, France, ELISA-C). The test includes a pre–absorption step with *M. phlei*. For each sample, the S/P percentage was calculated as follows using the corrected sample and control values: S/P= OD sample – OD Negative Control / OD Positive Control – OD Negative Control x 100. Samples presenting a S/P % less than or equal to 60% are considered negative, greater than 60% and less than 70% are considered doubtful, and greater than or equal to 70 % are considered positive. ELISA-C was carried out in single

preparation. In this study, a herd was considered MAP-positive, if at least one animal of herd tested positive by ELISA-C.

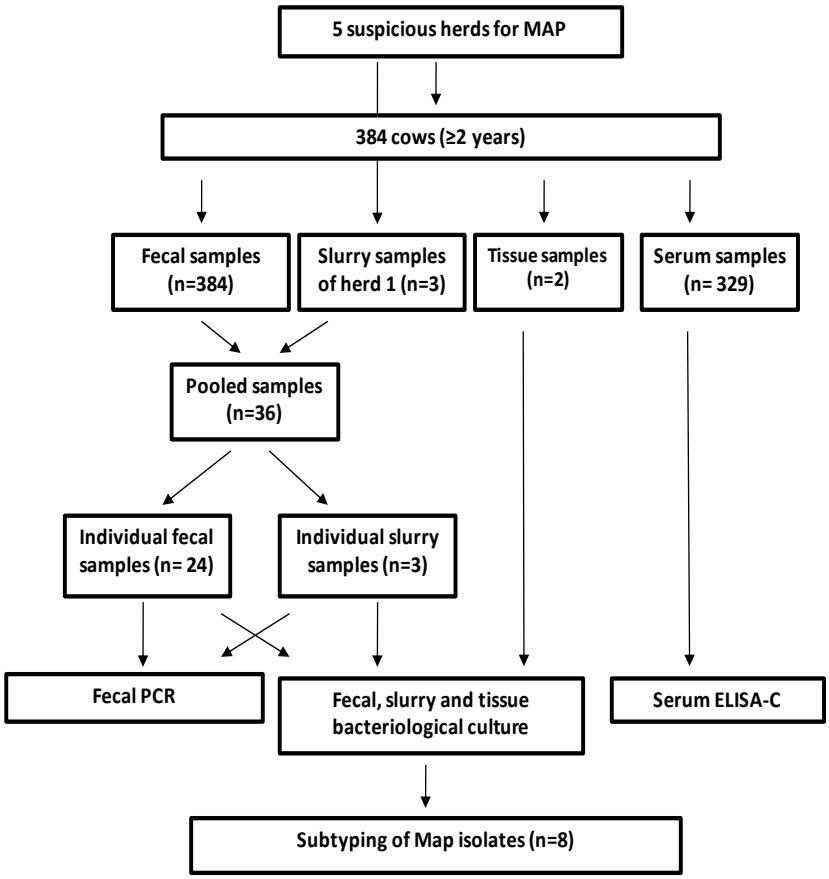


Figure 8. Overview of the study design for the confirmation of MAP in dairy cattle in Colombia

2.2.16. Pooling procedure of fecal and slurry samples

Fecal samples (n=386) were examined on the basis of a strategic pooling procedure. Fecal samples were sorted on the basis of birth order of the animals, and 2g of feces from each cow was mixed at the laboratory into pooled fecal samples of 8-12 cows per pool.

2.2.17. Fecal culture

After the pooling procedure of fecal and slurry samples, culture method 1 was carried out. If HEYM slants inoculated with pooled fecal or slurry samples showed mycobacterial growth, single fecal samples were cultured individually using culture method 1. The individual samples from a negative pool were assumed negative and not tested individually, except for the fecal samples from ELISA-C positive animals of herd 2, which were cultured individually.

2.2.18. Tissue culture

Tissue samples (colon and mesenteric lymph node) were prepared, decontaminated and inoculated in duplicate onto HEYM slants (Anonymous, 2007). Briefly, the colon tissue was cut open and the mesenteric lymph node was released from adipose tissue. Both samples were cut up separately and approximately 1g of the respective tissue material was put in a stomacher bag with 7 ml of 0.9% (w/v) HPC (0.9% HPC) and was homogenized for 6 min in the stomacher. The homogenized tissue was put in a 50 ml sterile tube (Sarstedt, Nümbrecht, Germany) and shaken at room temperature, 200 U / min for 5-10 min. After that, tubes were place in vertical position in the dark for 24 h at room temperature. After decontamination, the tubes were centrifuged at 1880 x *g*, at 20°C for 20 min. The supernatant was discarded and the sediment was resuspended in phosphate buffered saline (PBS) Buffer pH 7.2 and vortexted. Finally two HEYM slants, supplemented with mycobactin J (Becton Dickinson, Heidelberg, Germany) were inoculated with 300 µl of the decontaminated pellet. Slants were incubated at 37°C for maximum 20 weeks and checked at 1-2-week intervals for mycobacterial growth or contamination (Anonymous, 2007). Contamination rate was estimated in 8.3% (3/36) for the fecal and the slurry pooled samples, and 3.7% (1/27) for the individual fecal samples (including fecal cultures from ELISA-C positive animals of herd 2) and the tissue samples. In all contaminated samples, only one slant of the duplicate was affected. In case of mycobacterial growth, MAP was confirmed by the PCR methods described above.

2.2.19. DNA isolation

DNA isolation from fecal and slurry samples was carried out using the method described above for fecal samples (see 2.2.5). DNA from bacteria isolated on fecal culture was extracted as described above (see 2.2.6)

2.2.20. PCR

PCR was carried out only on individual fecal and slurry samples that were part of positive fecal and slurry pooled samples by culture, and to fecal samples of four positive ELISA-C animals of herd 2 (n=27, Figure 8). For this analysis the methods described before were used (see 2.2.7.)

2.2.21. MIRU-VNTR

Based on the results of MIRU-VNTR obtained in the establishment and evaluation of subtyping methods described above (see 2.1.), the loci MIRU-1, MIRU-2, MIRU-3, MIRU-4 (Bull et al., 2003b); VNTR-292, VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32 (Thibault et al., 2007), VNTR-1658 (alias MIRU-3, and X3, (Overduin et al., 2004), and VNTR-259 (Castellanos et al., 2010b) were used to subtype the isolates of Colombia.

2.2.21.1. PCR amplification of MIRU-VNTR

For all loci, primers used were those suggested by the authors mentioned above, except for the PCR conditions of MIRU-1, VNTR-7, and VNTR-10 carried out according to (Mobius et al., 2008b), and of VNTR-25 and VNTR-47 according to (Castellanos et al., 2010b). For the PCR amplification of VNTR-32, 5µL of Betain (Sigma-Aldrich, Schenelldorf, Germany) was additionally added to the mix as suggested by (Thibault et al., 2007).

2.2.21.2. Determination of the number of repeats per locus of MIRU-VNTR

MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus in the order MIRU-1, MIRU-2, MIRU-3, MIRU-4, VNTR-292, VNTR-1658, VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32, and VNTR-259. The Institut National de la Recherche Agronomique-INRA Nouzilly MIRU-VNTR (INMV) nomenclature, as defined by (Thibault et al., 2007), was taken into account for ease comparison with previous studies. For this purpose only the results of loci VNTR-292, VNTR-1658, VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, and VNTR-32 were considered.

2.2.22. MLSSR

For subtyping of the MAP isolates, MLSSR analysis of loci 1, 2, 8, and 9 was carried out according to (Amonsin et al., 2004), see 2.1.5.

2.2.23. Statistical analysis

The descriptive analysis of age, estimation of standard deviation (SD) and determination of confidence intervals 95% (95% CI) were carried out by using the program packages BMPD release 7.0 (Dixon, 1993) and BIAS (Biologische Analyse von Stichproben) for Windows, release 8.2 (Hochheim-Darmstadt, Germany). The estimation of the testing agreement

between ELISA and culture (Cohen's kappa-κ coefficient) were done with the program Win-Episcope 1.0 (Zaragoza, Spain). True prevalence was estimated based on the apparent prevalence obtained by ELISA-C using sensitivity (42%) and specificity (99%) values determined previously on asymptomatic infected animals (Kohler et al., 2008). The relation age vs. ELISA results was analyzed descriptively, according to age classes arbitrarily defined.

For the exploratory determination of factors associated to the MAP serological status, the animal was the unit of statistical analysis. For the statistical analysis, the statistical program package BMDP release 7.0 (Berkeley, USA, (Dixon, 1993) was used. On first instance, interval-scaled variables were descriptively analyzed grouped by MAP outcome. After that, categorical variables were analyzed using two-way frequency tables (univariate analysis) with calculation of the Pearson Chi-square and the Fisher Freeman Halton test for raw statistical significance determination. Finally, all variables (interval-scaled and categorical) were analyzed using a stepwise logistic regression (multifactorial analysis). In the logistic regression, the result of ELISA test was used as dependent variable, while categorical and interval-scaled variables were used as independent variables. In the model, a $p < 0.10$ and a $p > 0.15$ were used for entry and for removal from the model, respectively. In addition, exact logistic regression was performed for some variables using the logistic regression analysis software LogXact ver. 9.0.0 (Cambrige, MA, USA, (Cytel Inc., 2010). Factors were considered to be significant at significance level of 5% ($p \leq 0.05$).

2.3. Genotypes of MAP from South American countries

The objective of this study was the analysis of the genotypes of MAP determined so far in South American isolates by a combination of the subtyping methods MIRU–VNTR and MLSSR.

2.3.1. MAP genotypes and isolates

Thirty–five South American genotypes of MAP determined by a combination of MIRU–VNTR and MLSSR were included for analysis in the present study. From these 35 genotypes, 24 were determined in MAP strains originally isolated from cattle, deer, and environmental samples in Argentina (n=14), Colombia (n=8) and Venezuela (n=2). The genotypes from Argentina and Venezuela were taken from the studies of Thibault et al. (2007) and Thibault et al. (2008), while the genotypes from Colombia were taken from the results of the present dissertation (see 3.2.3.4). The remaining 11 genotypes were determined in MAP isolates from Chile in the present study (Table 7). These isolates were sub–cultured on slants of Herrold's Egg Yolk Agar Medium (HEYM) supplemented with mycobactin J (Becton Dickinson, Heidelberg, Germany). The slants were incubated at 37°C for 20 weeks.

2.3.2. DNA isolation and identification

Genomic DNA of subcultured isolates was extracted from pure culture using a commercial preparation kit as described above (see 2.2.6.). For the molecular confirmation of isolates as MAP, the F57 and IS*Mav2*-real-time PCR was used as described above (see 2.2.7.1.).

Table 7. Origin of genotypes and isolates of MAP from South American countries

Strain	Country[d]	Host or Source[e]
258	CL	C
299	CL	C
300	CL	C
292	CL	C
134	CL	C
174	CL	C
175	CL	C
79	CL	H
18	CL	H
F2	CL	P

Table 7. continued

Strain	Country[d]	Host or Source[e]
3208	CL	Sh
Cow 1[a]	CO	C
Cow 2[a]	CO	C
Cow 3 – A [a]	CO	C
Cow 3 – B [a]	CO	C
Cow 3 – C [a]	CO	C
Cow 4 [a]	CO	C
Slurry 1 [a]	CO	Sl
Slurry 2[a]	CO	Sl
222[b]	AR	C
224[b]	AR	C
226[b]	AR	C
227[b]	AR	C
229[b]	AR	D
269[b]	AR	C
291[b]	AR	D
304[b]	AR	D
225[c]	AR	C
280[c]	AR	C
299[c]	AR	C
300[c]	AR	C
301[c]	AR	C
309[c]	AR	C
234[c]	VE	C
284[b]	VE	C

[a] Genotypes taken from the results of the study determination of MAP infection (confirmation, see 3.2.3.4).
[b] Genotypes taken from the publication of Thibault et al. (2008).
[c] Genotypes taken from the publication of Thibault et al. (2007).
[d] Country: CL Chile, CO Colombia, AR Argentina, VE Venezuela.
[e] Source: H hare, C cattle, Sl slurry, Sh sheep, P pudu, D deer

2.3.3. MIRU –VNTR

MIRU–VNTR analysis was performed by PCR amplification as described above (see 2.2.21).

2.3.4. MLSSR

MLSSR analysis was carried out by PCR amplification of locus 1, locus 2 and locus 8 (see 2.1.5.).

2.4. Molecular characterization of MAP in Rhineland–Palatinate, Germany

This study had three objectives. The first objective was the molecular characterization of MAP isolated from dairy cattle symptomatic of paratuberculosis in herds located in the state of Rhineland–Palatinate (RP), Germany, in the neighboring states of Saarland, North Rhine–Westphalia, and Hesse, and in Luxembourg using MIRU–VNTR and MLSSR. The second objective was the evaluation of both methods in terms of discriminatory ability, congruence, and predictability. The third objective was the analysis of MAP molecular epidemiology at a regional scale.

2.4.1. MAP isolates

Eighty-two MAP isolates obtained from 71 dairy herds located in the German federal state of RP were genotyped by MIRU–VNTR and MLSSR. In addition, isolates from two dairy herds located in the neighbor German federal states of Saarland (n=4), one dairy herd in North Rhine–Westphalia (n=1) and one dairy herd in Hesse (n=1), as well as from two dairy herds in Luxembourg (n=3) were also included (Table 8, Figure 9).

Table 8. MAP isolates from 78 dairy cattle herds of Rhineland–Palatinate genotyped by MIRU-VNTR and MLSSR

Isolate	Acronym	Herd	Ort	County
D 9356/05 W..,	538	24	Friesenhagen	Altenkirchen (Westerwald) (AK)
D 13764/06 W…,	RK 101	13	Burglahr	
D 163/07 R…,	RK 107	40	Katzwinkel	
D 8907/02 E..,	425	37	Hetzerath	Bernkastel-Wittlich (WIL)
D 3031/02 K..,	449	38	Hontheim	
D 13713/04 L..,	513	36	Hetzerath	
D 2607/2/07 K…,	RK 94	43	Landscheid	
D 4157/07 S…,	RK 103	66	Sellerich-Hontheim	
D 16186/7/03 S..,	473	63	Rimsberg	Birkenfeld (BIR)
D 11521/06 S…,	RK 95	12	Bundenbach	
D 11683/06 S…,	RK 110	12	Bundenbach	
D 1950/07 S…,	RK 112	27	Gollenberg	
D 7158/02 S..,	421	11	Briedeler-Heck	Cochem-Zell (COC)
D 12291/26/03 G…,	462	10	Brenningen	
D 6253/04 A..,	490	74	Winnweiler	Donnersbergkreis (KIB)
D 11934/06 B…,	RK 99	78	Schweisweiler	

Table 8. continued

Isolate	Acronym	Herd	Ort	County
D 2381/02 S..,	410	19	Dingdorf	
D 3649/02 E..,	413	56	Nimshuscheid	
D 8168/02 B..,	423	35	Herzfeld	
D 17764/02 E..,	441	7	Bleialf	
D 17766/02 A..,	442	65	Seiwerath	
D 12459/03 M..,	464	18	Dingdorf	
D 17228/03 B..,	475	21	Eisenach	
D 1858/04 D..,	483	52	Mützenich	
D 5965/04 L..,	489	32	Großkampenberg	
D 6586/35/04 H..,	492	45	Lauperath	
D 6696/04 H..,	494	2	Oberlascheid	
D 9639/04 E..,	497	71	Wascheid	
D 9695/15/04 B..,	498	35	Herzfeld	
D 11492/04 H..,	508	2	Oberlascheid	
D 13711/04 S..,	512	34	Hersdorf	
PA 29/05 H..,	517	42	Lambertsberg	
PA 231/05 R..,	521	9	Brandscheid	
PA 563/05 S..,	524	44	Lasel	Eifelkreis Bitburg-Prüm (BIT)
PA 682/05 W..,	526	29	Gondenbrett	
D 1229/05 L..,	527	67	Taben-Rodt	
D 1765/05 S..,	528	6	Bleialf	
D 9687/05 P..,	540	23	Euscheid	
D 2243/07 S…,	RK 97	6	Bleialf	
D 10991/06 H…,	RK 102	61	Pronsfeld	
D 6652/07 M…,	RK 104	69	Üttfeld	
D 12431/06 I…,	RK 105	8	Brandscheid	
D 11987/06 L…,	RK 106	46	Lichtenborn	
D 13768/06 I…,	RK 108	8	Brandscheid	
D 10756/06 M…,	RK 109	3	Bauler	
D 5267/07 K…,	RK 111	30	Gondenbrett	
D 5840/07 B…,	RK 113	64	Rommersheim	
D 11787/06 P…,	RK 114	5	Birtlingen	
PA 684/05 W…,	RRG 3/Da	28	Gondenbrett	
PA 1103/05 H…,	RRG 6/Ln	2	Auw	
PA 1103/05 H…,	RRG 6/Da	2	Auw	

Table 8. continued

Isolate	Acronym	Herd	Ort	County
PA 1328/05 D…,	RRG 7/Ln	75	Wißmannsdorf	
PA 1328/05 D…,	RRG 7/Da	75	Wißmannsdorf	
PA 338/05 B…,	RRG 9/Ln	35	Herzfeld	
PA 28/05 S…,	RRG 10/Ln	6	Bleialf	
D 3031/03 K..,	452	72	Wentheim	Germersheim (GER)
PA 92/04 H..,	477	15	Dunzweiler	
D 1628/04 T..,	479	54	Neunkirchen	Kusel (KUS)
D 5835/04 H..,	488	48	Matzenbach	
PA 130/06	RRG 2/Da	47	Septfontaines	
PA 1154/05 M…,	RRG 4/Ln	76	Huldange	Luxemburg (LU)
PA 1154/05 M…,	RRG 4/Da	76	Huldange	
PA 562/05 B..,	523	68	Thür	Mayen-Koblenz (MYK)
PA 42/05 A..,	518	60	Perl	
PA 42/05 A…,	RRG 1/Ln	60	Perl	Merzig-Wadern (MZG)
PA 42/05 A…,	RRG 1/Da	60	Perl	
DPA 15/05 R..,	515	59	Ottweiler	Neunkirchen (NK)
PA 27/05 N..,	516	77	Neitzert	
D 6639/05 J..,	534	20	Ehlscheid	Neuwied (NW)
D 127/05 M..,	519	22	Espenschied-Lorch	Rheingau-Taunus-Kreis (RÜD)
D 15769/02 W..,	438	4	Beulich	
D 4063/02 L..,	450	39	Hungenroth	Rhein-Hunsrück-Kreis (SIM)
D 4470/05 S..,	532	53	Mutterschied	
D 12139/02 F..,	430	16	Dausenau	Rhein-Lahn-Kreis (EMS)
D 7786/05 B..,	536	50	Miehlen	
PA 153/05 M..,	520	51	Monschau	Städteregion Aachen (AC)
PA 528/04 G..,	482	41	Kirschbacherhof	Südwestpfalz (PS)
D 10368/27/04 F..,	506	1	Althornbach	
D 8390/02 S..,	424	14	Damflos	
D 10987/02 G..,	426	49	Meuerich	
D 11513/02 H..,	428	57	Nittel	
D 928/02 N..,	446	33	Gusenburg	Trier-Saarburg (TR)
D 928/03 M..,	451	25	Geisenbach	
D 6460/04 K..,	491	31	Gräfenhom	
D 14012/04 P..,	514	70	Vierherrenborn	

Table 8. continued

Isolate	Acronym	Herd	Ort	County
D 10712/06 B…,	RK 100	73	Wincherigen	
D 15738/03 M..,	471	26	Gillenfeld	Vulkaneifel (DAU)
D 13820/06 W…,	RK 96	58	Ormont	
D 16031/03 D..,	472	62	Rennerod	
D 8920/04 K..,	495	17	Dernbach	Westerwaldkreis (WW)
D 9962/1/04 T..,	502	55	Niederroßbach	

The isolates were obtained from samples (feces or tissue) of diarrheic animals considered as suspicious for Johne´s disease in different districts of RP. The samples were sent by field veterinarians to the Federal State Investigation Centre RP, Koblenz, Germany (Landesuntersuchungsamt) for MAP analysis. The samples were tested and confirmed as MAP-positive using bacteriological and molecular methods in the Landesuntersuchungsamt and sent to the Institute of Veterinary Food Sciences at the Justus Liebig University Giessen (Giessen, Germany) between 2004 and 2007.

From the 91 MAP isolates, 59 (64.8%) were originally sent to Giessen as lyophylisate, 20 (22%) were sent as fecal samples positive for MAP, and 12 (13.2%) were received as tissue samples (lymph node or intestine). Herrold's Egg Yolk medium (HEYM) with mycobactin J, Amphotericin B, Nalidixic acid, and Vancomycin (Becton Dickinson, Heidelberg, Germany) were always used for primary isolation, subcultivation, and propagation in Koblenz and in Giessen.

The HEYM slants were incubated at 37°C for a maximum of 20 weeks. During incubation none of the isolates presented pigmentation and all grew in less than 16 weeks. Originally, 104 isolates (MAP-positive clinical samples and lyophylisates) were sent from Koblenz to Giessen and were subcultivated on HEYM. However, due to lack of growth, 13 isolates could not be analyzed. Eight MAP strains out of 91 were isolated in herds included in two studies to test the efficacy of the commercial vaccines Neoparasec (Rhone–Merieux, Lyon, France, n=1) and Silirum (CZ Veterinaria, Porriño, Spain, n=7).

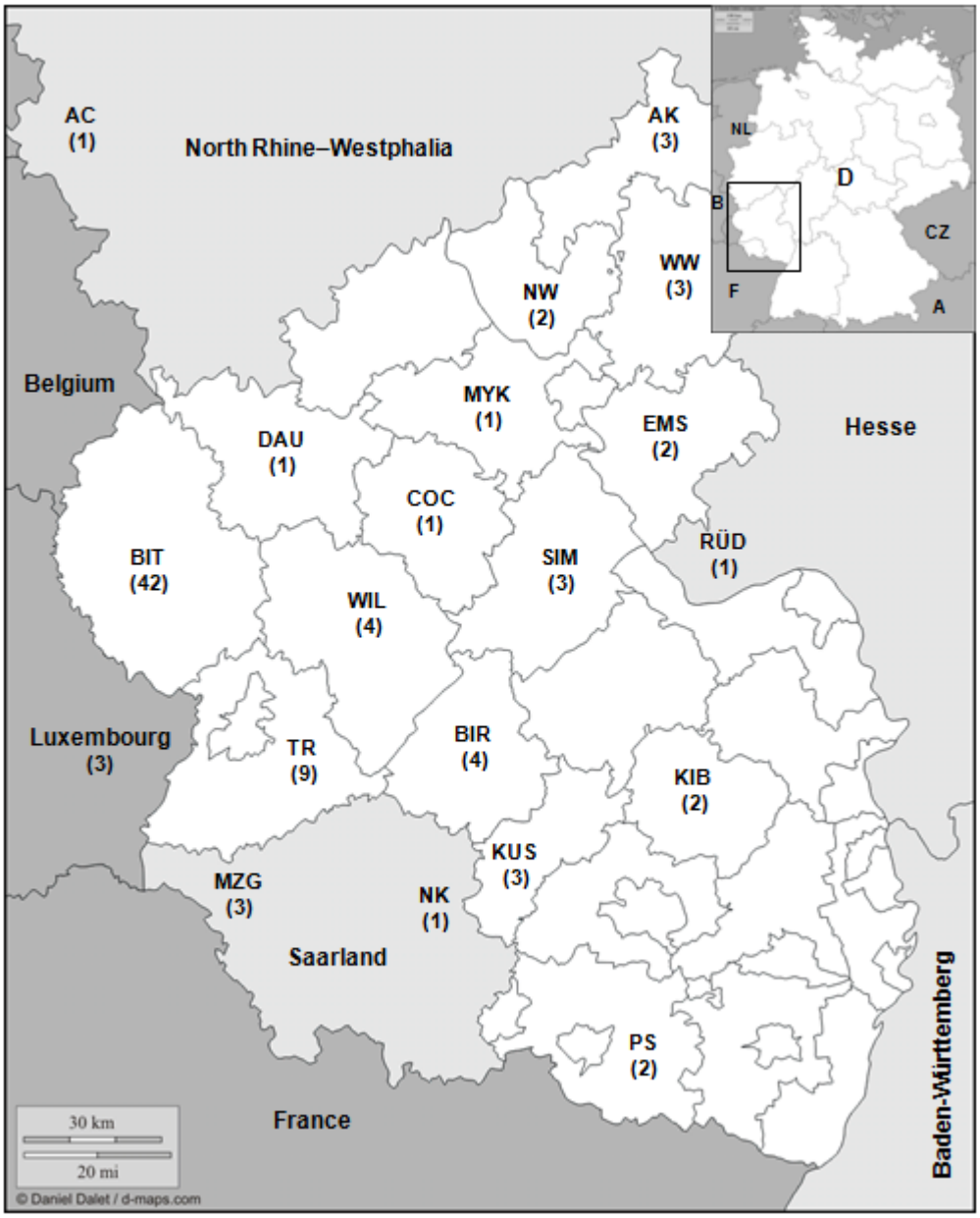


Figure 9. Counties of Rhineland–Palatinate where MAP was isolated.
German federal states of North Rhine–Westphalia, Hesse and Saarland, as well as Luxembourg are also visible. Numbers in parentheses corresponds to the number of strains isolated in every county. For full-name of counties, see Table 8. **D** Germany, **F** France, **B** Belgium, **NL** The Netherlands, **CZ** Czech Republic, **A** Austria.

2.4.2. DNA isolation

Genomic DNA of subcultured isolates was extracted from pure culture using a commercial preparation kit as described above (see 2.2.6.).

2.4.3. MAP identification

For the molecular confirmation of isolates as MAP, the F57 and IS*Mav2*-real-time PCR was used as described above (see 2.2.7.1.).

2.4.4. MIRU –VNTR

MIRU–VNTR genotyping was performed by PCR amplification of the loci MIRU–1 (Bull et al., 2003b), MIRU–VNTR 1658 (alias X3 and MIRU–3, (Overduin et al., 2004), MIRU–VNTR 292 (alias MIRU–2), MIRU–VNTR 25, MIRU–VNTR 47, MIRU–VNTR 3, MIRU–VNTR 7, MIRU–VNTR 10, MIRU–VNTR 32 (Thibault et al., 2007), and MIRU–VNTR 259 (Castellanos et al., 2010b), see 2.2.21.)

2.4.5. MLSSR

MLSSR analysis was carried out by PCR amplification of locus 2 and locus 8 (see 2.2.22).

2.4.6. Calculations

The Simpson's index of diversity or numerical index of discrimination (*D*) was used to estimate the discriminatory ability of subtyping methods individually and combined (Hunter and Gaston, 1988). For the Simpson's index of diversity, only the results of epidemiologically unrelated MAP isolates were included in the calculation. This means that if two isolates or more from the same herd produced the same genotype, these isolates were considered to be a clone and were counted only as one for the index calculation. Confidence intervals of 95% (CI 95%) for the Simpson's index of diversity were calculated according to (Grundmann et al., 2001) to determine whether methods were independent. Adjusted Rand´s coefficient (n_c) was estimated to access the global congruence of the two genotyping methods used, taking into account that the agreement between partitions could arise by chance alone, as previously described (Carrico et al., 2006). The Wallace coefficient (W) with C.I. 95% and the expected W value under independence (Wi) were calculated to obtain additional adirectional information of the use of MIRU–VNTR and MLSSR and vice versa, as previously described (Pinto et al., 2008). All indexes were calculated using the online tool for quantitative assessment of classification agreement (available at http://darwin.phyloviz.net/ComparingPartitions/). The allelic diversity (h) at a locus was calculated for MAP epidemiologically unrelated isolates in studies with a significant epidemiological component according to (Selander et al., 1986).

3. RESULTS

3.1. Establishment and evaluation of MAP genotyping methods

3.1.1. IS*900*-RFLP

IS*900*-RFLP using the endonucleases *BstE* II, *Pst* I, and *Bam*H I was applied to all strains and isolates. However, IS*900*-RFLP profiles from all strains could not be obtained due to insufficient quality and quantity of DNA or to inactivity of the endonucleases to digest DNA in some isolates (Figure 10, lane 6). A total of six IS*900*-RFLP profiles (C1, C18, C17, C5, Cnew, and Cnew2) were identified among the MAP isolates after digestion with *BstE* II (Figure 10).

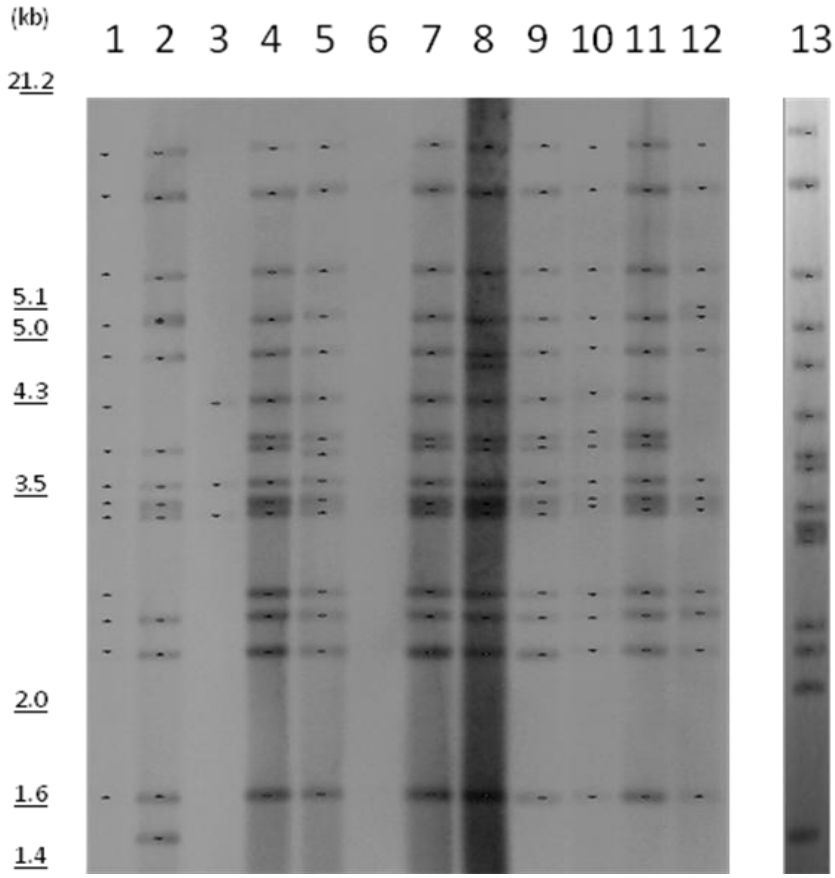


Figure 10. Results of IS*900*-RFLP with the endonuclease *BstE* II.

Lane 1: Hum 2 (profile C5), Lane 2: Hum 1 (profile Cnew2), Lane 3: Pat 30 III A (profile MAH), Lane 4: Pat 7 (profile C1), Lane 5: JD 131 (profile C17), Lane 6: MW6 (no profile determined), Lane 7: 464 (profile C1), Lane 8: 518 (profile) C18, Lane 9: 218/133 (profile C1), Lane 10: RK 18 (profile C1), Lane 11: IPRK 297 (profile C1), Lane 12: ATCC 19698 (profile Cnew), Lane 13: K-10 (profile C1).

All MAP-profiles obtained by IS*900*-RFLP correspond to the MAP strain type C, type II, or cattle type (Table 14). The "*Cnew2"* obtained by one MAP isolate using *BstE* II was not published at the time of performance of this study and was identified following the same identification system employed by (Mobius et al., 2008b). Fifty percent (6/12) of the MAP isolates and strains showed a C1 profile, while the remaining strains and isolates showed diverse profiles. Two different profiles (1 and 3) were obtained with the enzyme *Pst* I, while three different profiles (1, 11, and 16) were obtained with the enzyme *Bam*H I (Data not shown). Fifty percent (6/12) of the profiles obtained with the endonuclease *Bam*H I correspond to the profile 1 and four corresponded to the profile 11. The profile 16 was found in one single isolate. The pattern "16" obtained in one MAP isolate using *Bam*H I was not previously published at the time of performance of this study and was identified a new type following the same identification system employed by (Cousins et al., 2000). The C1 profile obtained with the endonuclease *BstE* II were subdivided into two different profiles by endonuclease *Bam*H I and also by endonuclease *Pst* I. The profile produced by the MAH isolate used for control (profile not reported by any MAP isolate) was denominated MAH to avoid confusion with the MAP profiles (Table 14). From the point of view of technique, one isolate (isolate 518) presented excess of background, probably related to the low quality (impurity) of the DNA (Figure 10, lane 8).

3.1.2. MIRU

Analysis of the number of MIRU present in four specific loci, MIRU-1, MIRU-2, MIRU-3, and MIRU-4 (Bull et al., 2003b), revealed differences between the MAP and MAH isolates (Table 9). However, only MIRU-2 and MIRU-3 were able to differentiate within the MAP isolates (Table 9, Figure 11). The second group of MIRU, MIRU-4-ARG, MIRU-5-UK, MIRU-6, MIRU-7, and MIRU-11 UK/VNTR-11 (Romano et al., 2005), was able to differentiate between MAP and MAH, but was unable to differentiate between MAP isolates (Table 10, Figure 12).

Table 9. MIRU profiles (loci 1 to 4) obtained during the establishment and evaluation of MAP subtyping methods

Isolate / strain	Locus [a]				
	1	**2[b]**	**3[b]**	**4**	**Profile[c]**
K-10	3	7	5	1	2
ATCC 19698	3	7	5	1	2
IPRK 297	3	7	5	1	2
RK 18	3	5	5	1	3
218/133	3	9	7	1	4
518	3	9	5	1	1
464	3	9	7	1	4
MW6	3	5	5	1	3
JD 131	3	9	5	1	1
Pat 7 III A	3	7	5	1	2
Pat 30 III A	1	5	5	5	5
Hum 1	3	7	5	1	2
Hum 2	3	7	5	1	2
Number of profiles	**2**	**3**	**2**	**2**	**5**
D	**0.15**	**0.69**	**0.28**	**0.15**	**0.77**
C.I. (95%)	**-0.09-0.4**	**0.58-0.8**	**0.005-0.55**	**-0.09-0.4**	**0.59-0.95**

[a]Loci and methods according to (Bull et al., 2003b)

[b]Loci showing polymorphism among MAP isolates and strains.

[c]Profile is based on the combination of results of all loci with and without polymorphisms.

Table 10. MIRU profiles (loci 4ARG to 11UK) obtained during the establishment and evaluation of MAP subtyping methods

Isolate / strain	Locus[a]						
	4 ARG	**5 UK**	**6**	**7**	**8**	**11 UK**	**Profile[b]**
K-10	2	1	3	5	4	4	1
ATCC 19698	2	1	3	5	4	4	1
IPRK 297	2	1	3	5	4	4	1
RK 18	2	1	3	5	4	4	1
218/133	2	1	3	5	4	4	1
518	2	1	3	5	4	4	1
464	2	1	3	5	4	4	1
MW6	2	1	3	5	4	4	1
JD 131	2	1	3	5	4	4	1
Pat 7 III A	2	1	3	5	4	4	1
Pat 30 III A	4	2	4	3	6	8	2
Hum 1	2	1	3	5	4	4	1
Hum 2	2	1	3	5	4	4	1
Number of profiles	**2**	**2**	**2**	**2**	**2**	**2**	**2**
D	**0.15**	**0.15**	**0.15**	**0.15**	**0.15**	**0.15**	**0.15**
C.I. (95%)	**-0.09-0.4**	**-0.09-0.4**	**-0.09-0.4**	**-0.09-0.4**	**-0.09-0.4**	**-0.09-0.4**	**-0.09-0.4**

[a]Loci and methods according to (Bull et al., 2003b; Romano et al., 2005)

[b]Profile is based on the combination of results of all loci with and without polymorphisms.

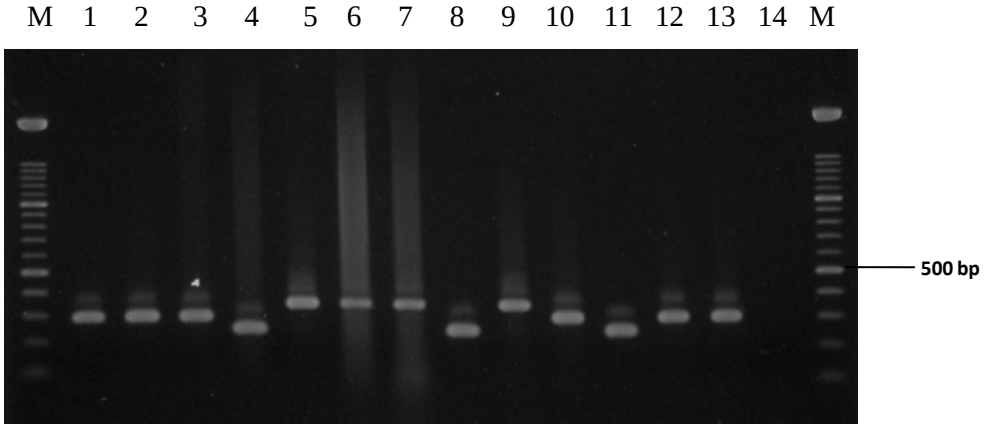


Figure 11. MIRU-2 PCR results showing size polymorphism among MAP isolates.
Lane 1: K-10, Lane 2: ATCC 19698, Lane 3: IPRK297, Lane 4: RK18, Lane 5: 218/133, Lane 6: 518, Lane 7: 464, Lane 8: MW6, Lane 9: JD131, Lane 10: Pat7IIIA, Lane 11: Pat30IA (MAH), Lane 12: Hum1, Lane 13: Hum2, Lane 14: master mixture blank, M: 100 base pair ladder DNA Molecular Weight Marker XIV (Roche, Mannheim, Germany).

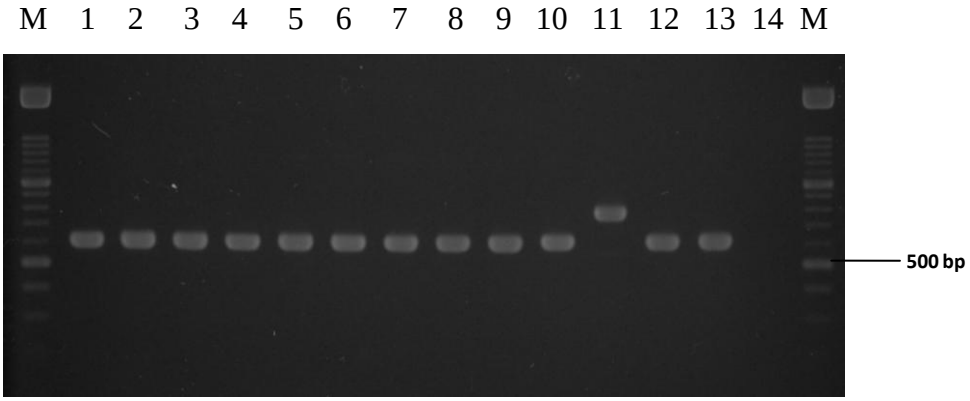


Figure 12. MIRU-11 PCR results showing absence of size polymorphism among MAP isolates. Lane 1: K-10, Lane 2: ATCC 19698, Lane 3: IPRK297, Lane 4: RK18, Lane 5: 218/133, Lane 6: 518, Lane 7: MAP 464, Lane 8: MW6, Lane 9: JD131, Lane 10: Pat7IIIA, Lane 11: Pat30IA, Lane 12: Hum1, Lane 13: Hum2, Lane 14: master mixture blank, M: 100 base pair ladder DNA Molecular Weight Marker XIV (Roche, Mannheim, Germany).

3.1.3. VNTR

The VNTR genotyping system, including loci VNTR-1067, VNTR-1605, VNTR-1658, VNTR-3527, and VNTR-3249 (Overduin et al., 2004) was useful to differentiate between the MAP isolates and MAH. However, only VNTR 1658 was able to distinguish between MAP isolates, but not between MAP and MAH, which was achieved by the other loci (Table 11, Figure 13 and Figure 14).

Table 11. VNTR profiles obtained during the establishment and evaluation of MAP subtyping methods

Isolate / strain	Locus[a]					
	1067	**1605**	**1658[b]**	**3527**	**3249**	**Profile[c]**
K-10	2	2	2	2	2	I
ATCC 19698	2	2	2	2	2	I
IPRK 297	2	2	2	2	2	I
RK 18	2	2	2	2	2	I
218/133	2	2	3	2	2	II
518	2	2	2	2	2	I
464	2	2	3	2	2	II
MW6	2	2	2	2	2	I
JD 131	2	2	2	2	2	I
Pat 7 III A	2	2	2	2	2	I
Pat 30 III A	3	3	2	1	3	III
Hum 1	2	2	2	2	2	I
Hum 2	2	2	2	2	2	I
Number of Profiles	**2**	**2**	**2**	**2**	**2**	**3**
D	**0.15**	**0.15**	**0.28**	**0.15**	**0.15**	**0.43**
C.I. (95%)	**-0.09-0.40**	**-0.09-0.40**	**0.005-0.55**	**-0.09-0.40**	**-0.09-0.40**	**0.13-0.74**

[a]Loci and methods according to (Bull et al., 2003b)

[b]Locus showing polymorphism among MAP isolates and strains.

[c]Profile is based on the combination of results of all loci with and without polymorphisms.

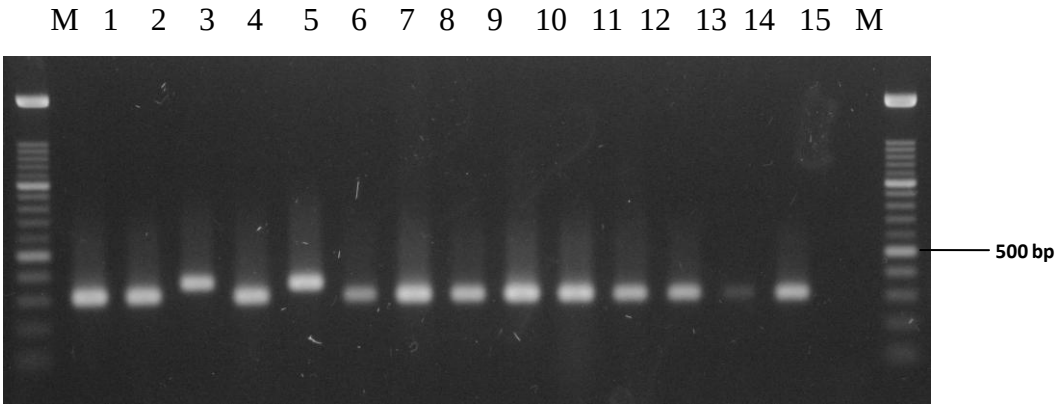


Figure 2. VNTR-1658 PCR results showing polymorphism among MAP isolates, but absence between MAP and MAH.

Lane 1: K-10, Lane 2: ATCC 19698, Lane 3: 218/133, Lane 4: 518, Lane 5: 464, Lane 6: MW6, Lane 7: Niebüll (*not included in the present study*), Lane 8: JD131, Lane 9: Pat 30 III A (MAH), Lane 10: Hum1, Lane 11: Hum2, Lane 12: Pat7IIIA, Lane 13: RK18, Lane 14: IPRK297, Lane 15: master mixture blank, M: 100 base pair ladder DNA Molecular Weight Marker XIV (Roche, Mannheim, Germany).

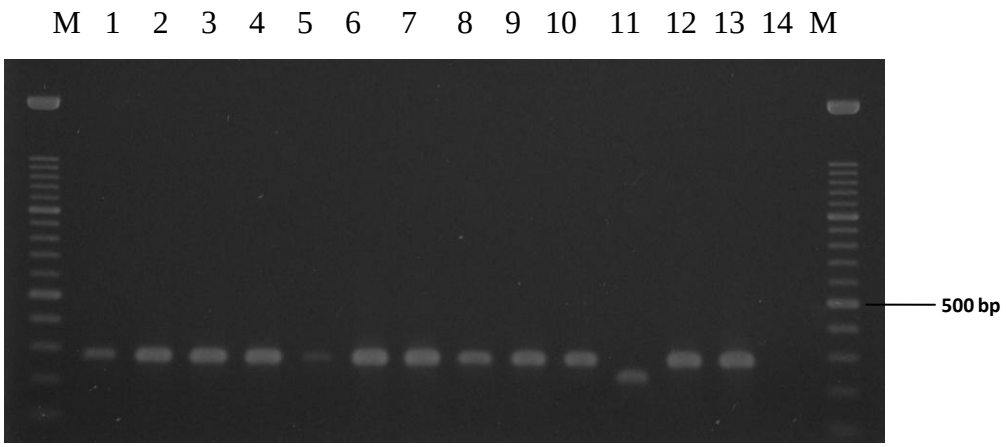


Figure 3. VNTR-3527 PCR results showing absence of size polymorphism among MAP isolates. Lane 1: K-10, Lane 2: ATCC 19698, Lane 3: IPRK297, Lane 4: RK18, Lane 5: 218/133, Lane 6: 518, Lane 7: 464, Lane 8: MW6, Lane 9: JD131, Lane 10: Pat7IIIA, Lane 11: Pat 30 III A (MAH), Lane 12: Hum1, Lane 13: Hum2, Lane 14: master mixture blank, M: 100 base pair ladder DNA Molecular Weight Marker XIV (Roche, Mannheim, Germany).

3.1.4. MLSSR

The MLSSR analysis revealed polymorphisms in the three loci analyzed (Table 12). From these loci, locus 2 delivered the highest number of alleles (n=4), while locus 1 and locus 8 revealed three alleles each one (Table 12, Figure 15)

Table 12. MLSSR profiles obtained during the establishment and evaluation of MAP subtyping methods

Isolate / Strain	Locus (repeat type)			Profile
	1 (g)	2 (g)	8 (ggt)	
K-10	>11	10	5	A
ATCC 19698	7	9	5	B
IPRK 297	7	11	4	C
RK 18	7	11	4	C
218/133	7	>11	4	D
518	7	9	4	E
464	7	>11	4	D
MW6	7	>11	4	D
JD 131	7	11	4	C
Pat 7 III A	10	12	4	F
Pat 30 III A	4	8	3	G
Hum 1	7	10	5	H
Hum 2	7	10	5	H
Number of Profiles	**4**	**6**	**3**	**8**
D	**0.423**	**0.872**	**0.564**	**0.910**
C.I. (95%)	**0.01-0.75**	**0.81-0.94**	**0.36-0.76**	**0.83-0.99**

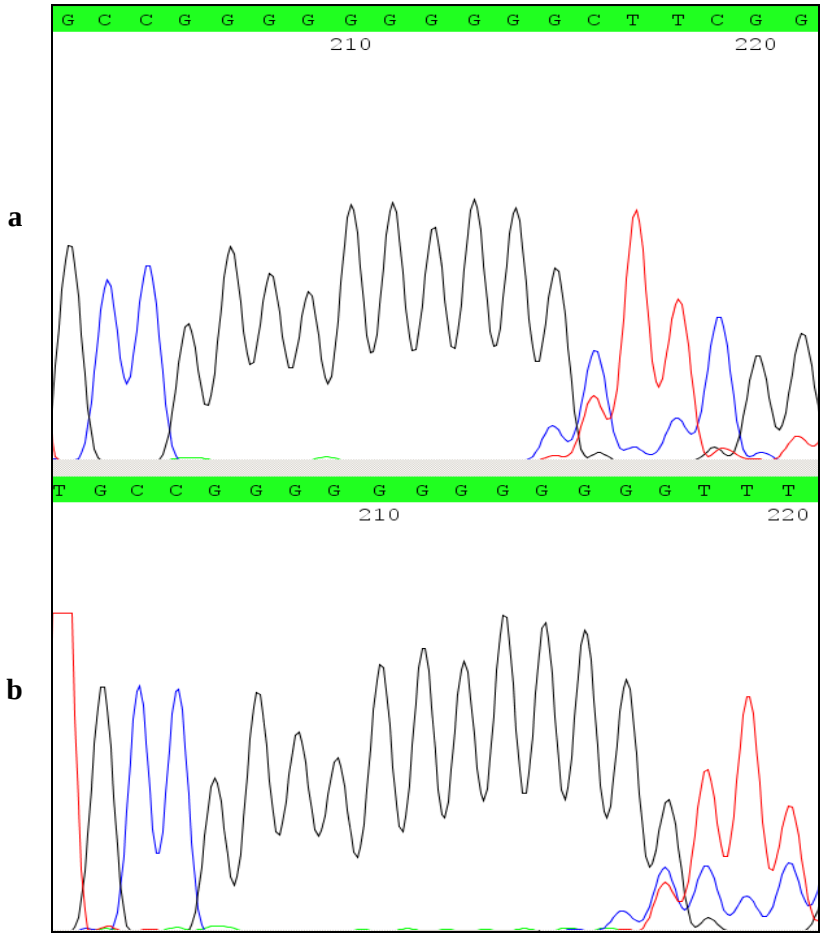


Figure 45. Representative MLSSR results (locus 2) after sequencing of PCR products
a. SSR results of MAP reference strain K-10 (10g repeats). **b.** SSR results of MAP isolate 7IIIA (12g repeats).

3.1.5. Evaluation

Performance and convenience criteria of methods used in these studies were assessed based on a combination of objective (quantitative) and subjective estimates as follows.

3.1.5.1. Performance criteria

Stability

- IS*900*-RFLP. Difficult to assess due to the lack of cell material in some isolates.
- MIRU-VNTR. Very stable, profiles remained unchanged through time.
- MLSSR. Stable. Although stability has been previously reported, SSR-2 alleles of reference strains sometimes do not match among studies. This can be related to reproducibility more than stability.

Typeability

- IS*900*-RFLP. *BstE* II= 92.3%, *Pst* I= 30.7%, *Bam*H I= 92.3%
- MIRU. 92.3% for MIRU-3, which could not deliver the profile from one isolate due to low quantity of DNA. This profile had to be established based on result of its homologue VNTR-1658. Remaining loci were 100% typeable.
- VNTR and MLSSR. 100% for all loci.

Discriminability

- The calculation of the Simpson's index of diversity (*D*) delivered variable results. Because of the lack of profiles from some MAP-isolates in the IS*900*-RFLP method, mainly due to the insufficient quantity and/or quality of cellular material and DNA, the calculation of the index for the IS*900*-RFLP method was profoundly affected (Table 13).

Table 13. Discriminatory index (*D*) obtained in the establishment and evaluation of MAP subtyping methods[a]

IS*900* RFLP Endonuclease	MIRU	VNTR	MLSSR	Discrimination index[b]	CI 95%
BstE II				0.77	0.53-1.01
Pst I				0.42	0.04-0.78
*Bam*H I				0.68	0.50-0.85
BstE II + *Pst* I + *Bam*H I				n.c.	n.c.
	2			0.69	0.58-0.80
	3			0.28	0.005-0.55
	2 + 3			0.78	0.59-0.97
		1658		0.28	0.005-0.55
			1	0.42	0.01-0.75
			2	0.87	0.81-0.94
			8	0.56	0.36-0.76
			1 + 2 + 8	0.91	0.83-0.99

[a]For this analysis, only loci that discriminate between MAP strains were taken into account.
[b]Calculated according to (Hunter and Gaston, 1988). *D* for VNTR method includes only the result of the unique locus (1658) showing polymorphisms among MAP.
n.c.: not calculated due to unavailability of some profiles in one or two endonucleases and in more than one isolate

The expression of the quantitative data of the Simpson´s index into a qualitative assessment of the discriminability of methods was:

- IS*900*-RFLP. Moderate to low, if all endonucleases are considered. Moderate if only *BstE* II results are taking into account.
- MIRU. Moderate to low
- VNTR. Low to very low
- MLSSR. High

Reproducibility

- IS*900*-RFLP. Very reproducible.
- MIRU-VNTR. Very reproducible.
- MLSSR. SSR-locus 2 presents troubles with long poly-g and poly-c repeats due to interruption during sequencing sometimes leading to variable interpretation of results. Strain ATCC 19698 has been reported having different SSR-2 profile in previous studies.

Concordance with the epidemiological picture

- IS*900*-RFLP and MIRU. Difficult to assess due to the non-epidemiological nature of the study and the low number of isolates analyzed.
- VNTR and MLSSR. Difficult to assess due to the non-epidemiological nature of the study and the low number of isolates analyzed.

In general, all methods for MAP subtyping used in this study (IS*900*-RFLP, MIRU, VNTR, and MLSSR) delivered discrimination in all MAP-isolates and strains. Although some loci posses only a limited ability or no ability at all to differentiate between MAP isolates, the majority of loci used in this study discriminate easily between MAP and MAH (Table 14).

Table 14. Results of 13 strains used for establishment and evaluation of MAP genotyping methods

Host species	Isolate / strain	IS*900*-RFLP profile[a]			MIRU (1-4) profile[b]	MIRU (4ARG-11UK) profile[c]	VNTR profile[d]	MLSSR profile[e]
		BstE II	*Pst* I	*Bam*H I				
Cattle	K-10	C1	1	1	1	1	I	A
	ATCC 19698	Cnew	*	1	1	1	I	B
	IPRK 297	C1	1	*	1	1	I	C
	RK 18	C1	1	11	2	1	I	C
	218/133	C1	3	11	3	1	II	D
	518	C18	1	11	4	1	I	E
	464	C1	1	11	3	1	II	D
	MW6	*	*	1	5	1	I	D
Sheep	JD 131	C17	1	1	4	1	I	C
Human	Pat 7 III A	C1	1	1	1	1	I	F
	Pat 30 III A	MAH[f]	MAH	MAH	6	2	III	G
	Hum 1	Cnew2	*	16	1	1	I	H
	Hum 2	C5	*	1	1	1	I	H
Profiles		**7**	**3**	**4**	**6**	**2**	**3**	**8**
***D* index[g]**		**0.77**	**0.42**	**0.68**	**0.78**	**0.15**	**0.43**	**0.91**
95% IC		**0.53-1.01**	**0.04-0.78**	**0.50-0.85**	**0.59-0.97**	**-0.09-0.4**	**0.13-0.74**	**0.83-0.99**

[a]According to (Pavlik et al., 1999) for *BstE* II, (Whipple et al., 1990) for *Pst* I, and (Cousins et al., 2000) for *Bam*H I.
[b]According to (Bull et al., 2003b).
[c]According to (Romano et al., 2005).
[d]According to (Overduin et al., 2004)
[e]According to (Amonsin et al., 2004)
[f]MAH: *Mycobacterium avium* subspecies *hominissuis*
[g]*D* index calculated according to (Hunter and Gaston, 1988).
* Profile not available due to low quantity of DNA or inactivation of endonuclease.

3.1.5.2. Convenience criteria

Flexibility

- All methods were very flexible for the two *M. avium* subspecies analyzed. The method was not assessed with other species because it was not the scope of the study.

Rapidity

- IS*900*-RFLP. Low. The whole process to get typing results always took several days.
- MIRU-VNTR. High. Simple electrophoresis was normally sufficient to estimate number of repeats, although precise estimation required sequencing.
- MLSSR. Moderate. Necessarily requires a sequencing step, with a duration of at least 24 hours.

Accessibility

- IS*900*-RFLP. Moderate. Required some special reagents and equipment.
- MIRU-VNTR. High.
- MLSSR. Moderate. The sequencing step required special equipment.

Ease of Use

- IS*900*-RFLP. Moderately difficult
- MIRU-VNTR and MLSSR. Easy

Cost

- IS*900*-RFLP. Moderately expensive due to reagents and equipment
- MIRU-VNTR. Cheap
- MLSSR. Moderately expensive due to sequencing step

Suitability for computerized analysis and storage of results

- IS*900*-RFLP. Suitable after digitalization of fingerprints with an specialized software.
- MIRU-VNTR. Suitable.
- MLSSR. Very suitable. Sequences are received in electronic format compatible with several software applications.

3.2. Diagnosis, genotyping and epidemiology of MAP in dairy cattle in Colombia

3.2.1. Determination of MAP infection (Screening)

3.2.1.1. ELISA–A

The ELISA–A test produced 31 out of 307 (10.1%) positive (95% CI; 7.0–14.0%), 268 (87.3%) negative and 8 (2.6%) doubtful results. Ten out of 14 (71%) of the herds were considered as positive (Table 15).

Table 15. Test results for detection of MAP in cattle from 14 dairy herds in Colombia, 2007

Test result		ELISA-A	ELISA-B[a]	Nested-IS*900*-PCR[b]	Real Time - PCR[b]		Culture[d]
					F57	IS*Mav2*	
Positive	Animals	31 (10.1%)	2 (5.1%)	6 (19.4%)	2 (6.5%)	1[c] (3.2%)	0
	Herds	10 (70%)	2 (14.3%)	4 (40%)	2 (20%)	1 (10%)	0
Negative	Animals	268	37	25	29	30	105
	Herds	4	8	6	8	9	4
Doubtful	Animals	8	0	0	0	0	0
Total	Animals	307	39	31	31	31	105
	Herds	14	10	10	10	10	4

[a]Performed only to serum samples from animals positive and doubtful by ELISA-A.

[b]Performed only to fecal samples from animals positive by ELISA-A.

[c]Sample simultaneously positive by nested-PCR and weakly-positive by real-time-PCR.

[d]Performed to all fecal samples of animals belonging to ELISA-A /PCR positive or real-time-PCR weakly-positive herds, regardless of individual ELISA result using HEYM, LJ, and MB7H11 media.

Information could not be collected from 53 animals sampled, which were removed from the descriptive analysis of age to avoid bias. Age of animals sampled ranged between 2.7 and 13 years (mean 5.7, SD 2.4), age mean in ELISA–A–positive was 6.4 (range 3–10.6, SD 2.13) and in ELISA–A–negative was 5.6 (2.7–13.9, SD 2.45). Analysis of age of animals vs. type of ELISA–A result revealed that group of 8.1–10.9 years was the group in which the highest proportion of ELISA–A–positive results (14.9%) were produced (Table 16). In the group of 5.1–8 years and 3–5 years, 13.2% and 6.8% of the samples produced positive results, respectively. In the older group (>11 years of age) no positive result by ELISA–A was produced. The highest proportion of doubtful results was found in the group of 5.1–8 years.

Table 16. ELISA-A results according to group of age of 307 animals from 14 dairy herds tested in Colombia, 2007

Group of age (years)	ELISA-A result			
	Positive (%)	Negative (%)	Doubtful (%)	Total (%)
3 – 5	6 (6,8)	81 (92,0)	1 (1,1)	88 (35.0)
5.1 – 8	14 (13,2)	89 (84,0)	3 (2,8)	106 (42.0)
8.1 – 10.9	7 (14,9)	39 (83,0)	1 (2,1)	47 (18.0)
>11	0 (0,0)	13 (100,0)	0 (0,0)	13 (5.0)

3.2.1.2. ELISA–B

From 39 serum samples (31 positive and 8 doubtful by ELISA–A), only 2 (5.1%, 95% C.I.; 0.6–17.3%), from two different herds were positive by ELISA–B, 37 (94.9%) were negative, and no sample was doubtful (Table 15). All serum samples that produced doubtful results by ELISA–A (n=8) produced negative results by ELISA–B. ELISA–B–positive serum samples (n=2) were from animals that were 6 and 4.2 years old at the moment of sampling and had never shown clinical signs of paratuberculosis. These ELISA–B–positive animals were from two different herds that belong to the same farmer. Between both herds cattle exchange was reported as a common practice.

3.2.1.3. Fecal PCR

The F57 and IS*Mav2*-real-time PCR produced 3 (9.7%) weakly–positive results out of 31 fecal samples from ELISA–A–positive animals belonging to 10 herds examined (Figure 16), while the IS*900*-conventional PCR produced 6 (19.4%) positive results (Figure 17). From the three F57–IS*Mav2*–real–time–PCR weakly–positive samples, 2 (6.5%) were positive only in the F57 and one (3.2%) only in the IS*Mav2* molecular target (Table 15). All PCR positive results were produced in fecal samples from animals belonging to herds 1, 2, 8, 9, and 10. Fecal samples from animals of remaining herds did not produce positive results by PCR. The two ELISA–B–positive animals produced negative results in both fecal PCR tests. Only in one fecal sample, both PCR tests produced a positive and a weakly–positive result simultaneously (Table 15). All fecal samples, except for one, were only positive (IS*900*-conventional PCR) or only weakly–positive (F57–IS*MaV2*–real–time–PCR) in one sample of the duplicate.

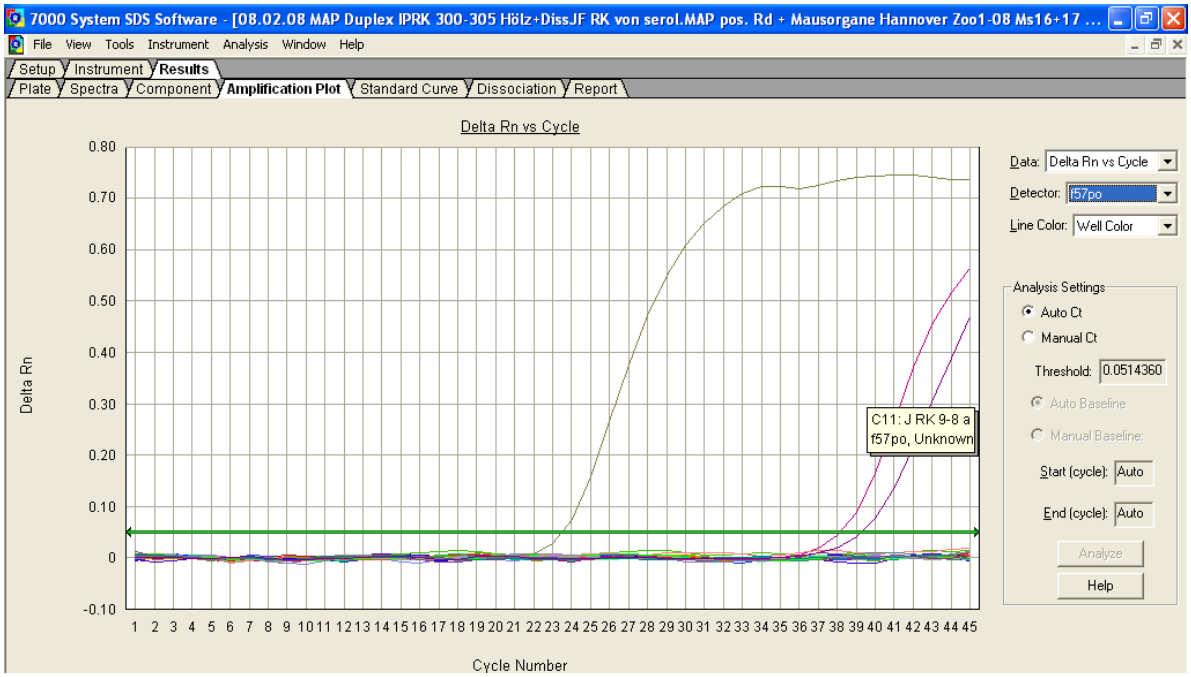


Figure 56. Representative amplification plot of F57 / IS*Mav2*-real-time PCR of fecal samples.
Leftmost curve: positive preparation control (Ct-value 23.48), middle curve: F57-weakly-positive result animal 8 of herd 9 (JRK 9-8a f57 po, Unknown; Ct-value 38.18), rightmost curve: unexpected weakly-positive result of the blank preparation control (Ct-value 39.30), flat curves: negative samples. Green line: thershold. Ct interpretation values: <37 positive, ≥40 negative, 37-30 weak-positive, positive control <28. Delta Rn (ΔRn) corresponds to the magnitude of the signal generated by the probe dyes VIC (F57) or FAM (IS*Mav2*) in the real-time PCR system.

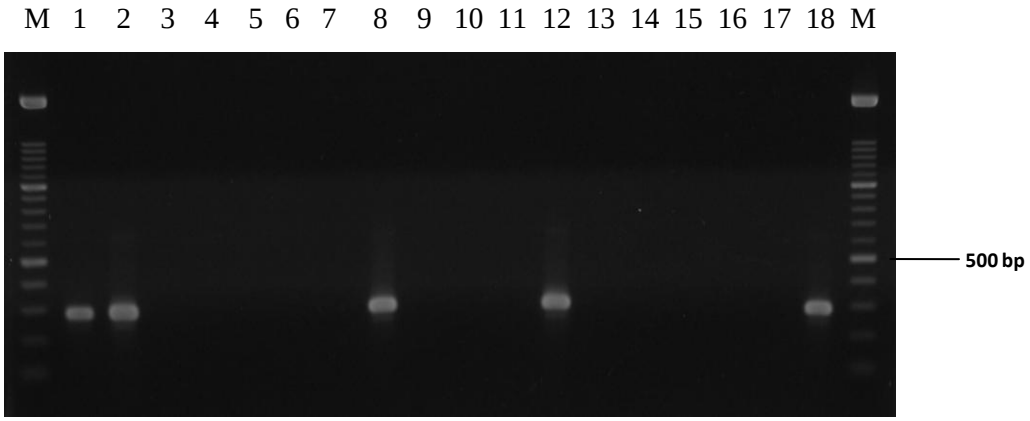


Figure 6. Representative IS*900* conventional PCR of fecal samples.
Lanes 1 and 2: animal 1 of herd 1, lane 8: animal 12 of herd 2, lane 12: animal 19 of herd 2, lane 18: positive control, lanes 3–7, 10–11 and 13–17: negative results, M: 100 bp DNA Ladder (Roche, Mannheim, Germany).

3.2.1.4. Fecal culture

Fecal culture of 105 fecal samples was negative for MAP on HEYM, LJ and MB7H11 media (Table 15). Fecal samples decontaminated with HPC and cultured on HEYM and on MB7H11 presented less contamination and less growth of atypical mycobacteria (AM) compared to

fecal samples decontaminated with NaOH – oxalic acid solution and cultured on LJ after 16 weeks of incubation. AM were isolated on approximately 50% of the LJ slants inoculated with fecal samples decontaminated with NaOH – oxalic acid. AM were rarely isolated on HEYM. AM isolates were confirmed as acid–fast rod–shape bacteria in the Ziehl–Neelsen stain of smears. They produced negative results by IS*900*-conventional PCR and F57–IS*MaV2*–real–time–PCR. These isolates were identified as *Mycobacterium engbackii* (*M. engbackii*) by sequencing of a fragment of the 16S rRNA gen. The comparison of the sequences of the 16S rRNA gene amplified showed 99.8% of similarity to the sequences on the RIDOM and NCBI databases used for species identification.

3.2.2. Factors associated with the serological status

3.2.2.1. Information of individual and general herd factors (interval-scaled variables)

General information regarding individual and general herd factors (interval-scaled variables) potentially associated with the ELISA status of dairy cows in Colombia is presented in Table 17.

Table 17. Individual and herd factors according to MAP serological status in Colombia (interval-scaled variables)

Group	Factor	ELISA result	Mean	Standard deviation	Smallest value	Largest value
Individual factors	Age (years)	Positive	6.3	2.1	3	10.6
		Negative	5.5	2.4	2.7	13.9
	Parity	Positive	4.0	1.6	2	7
		Negative	3.3	1.9	1	10
	Individual daily average milk production (liters)	Positive	19.9	4.4	10	28
		Negative	19.4	7.4	0	40
General information of herd	Herd size (hectares)	Positive	51.1	25.1	14	106
		Negative	44.8	32.4	6	114
	Herd daily average milk production	Positive	1089.7	464.3	600	2000
		Negative	899.9	444.9	329	2000
	Whole herd cattle population	Positive	133.0	56.9	77	274
		Negative	118.5	67	40	274

3.2.2.2. Univariable analysis of the individual and herd factors (categorical variables)

From the individual and herd factors (categorical variables), the factors *Treatment of symptomatic animals*, *Feed type of calves before weaning*, and *Manure spread on pastures* were significantly associated to the individual serological status at animal level in the univariable analysis. In the groups Individual Factors, General Information of Herd, and Factors Affecting Transmission between Herds, no factor was related to the serological status (Table 18).

Table 18. Individual and herd factors according to MAP serological status in Colombia (categorical variables)

Category	Factor	ELISA-A-positive		ELISA-A-negative		*P*-value
		N	%	N	%	
Individual factors	Cow born in farm					
	Yes	28	10.9	229	89.1	0.29 (a)
	No	3	6	47	94	
General information of herd	Access to veterinary assistance					
	Yes	25	10.4	6	9.1	0.75 (a)
	No	215	89.6	60	90.9	
Information about the presentation of disease in the herd	Currently presentation of symptomatic animals in herd					
	Yes	3	6.7	42	93.3	0.41 (a)
	No	28	10.7	234	89.3	
	Presentation of disease in the last 2 years					
	Yes	13	12.7	89	87.3	0.28 (a)
	No	18	8.8	187	91.2	
	Treatment of symptomatic animals					
	Not in herd	13	20.6	50	79.4	0.0018 (b)
	Still in herd	0	0	39	100	
	No symptomatic animals	18	8.8	187	91.2	
Factors affecting transmission between herds	Cattle purchase					
	Yes	19	10.5	162	89.5	0.78 (a)
	No	12	9.5	114	90.5	
	Own animals graze in foreign pastures					
	Yes	7	6.1	107	93.9	0.077 (a)
	No	24	12.4	169	87.6	
	Foreign animals graze in own pastures					
	Yes	4	5.5	69	94.5	0.13 (a)
	No	27	11.5	207	88.5	

Table 18. continued

Factors related to housing and hygiene	Existence of specific calving place					
	Yes	8	9.8	74	90.2	0.90 (a)
	No	23	10.2	202	89.8	
	Housing type of calves before weaning					
	Stall	0	0	19	100	0.1858 (b)
	Pasture	30	11.6	229	88.4	
	Other housing type	1	3.4	28	96.6	
	Feed type of calves before weaning					
	Colostrums own dam	25	14.5	148	85.5	0.004 (a)
	Colostrums' mix several cows	6	4.5	128	95.5	
	Manure spread on pastures					
	Yes	21	16.7	105	83.3	0.0014 (a)
	No	10	171	5.5	94.5	
	Birds sighting in feedstuff store					
	Yes	5	5.6	84	94.4	0.096 (a)
	No	26	11.9	192	88.1	

(a) Pearson Chi-square
(b) Fisher Freeman Halton test

3.2.2.3. Multivariable analysis of all factors

In the asymptotic stepwise and in the exact logistic regressions (multivariable analysis), only the factor *manure spread on pastures* was significantly associated to the serological response in the study. Strikingly, the factor *Feed type of calves before weaning with Colostrums' mix* was very near to the limit of significance (Table 19).

Table 19 Results of the logistic regression of individual and herd management factors according to MAP serological status in Colombia

Factor	**Coefficient**	**SE**	**EXP (Coef)**	**95% CI of EXP (Coef)**	***P*-value Asymptotic**	***P*-value Exact**
Manure spread on pastures	1.224	0.404	3.40	1.54-7.52	0.0015	0.0029
Feed type of calves before weaning						
Colostrums' mix	-0.8779	0.519	0.416	0.15-0.12	0.0860	n.c.

n.c.: not calculated

3.2.3. Determination of MAP infection (Confirmation)

3.2.3.1. ELISA-C

ELISA-C produced positive results in 1.8% (6/329, 95% C.I.; 0.7-3.9%), negative results in 97.5% (321/329), and doubtful results in 0.6% (2/329) of the serum samples examined, as well as positive results in 40% (2/5) of the herds. Of the six positive ELISA-C samples detected, two were detected in herd 1 and four were detected in herd 2 (Table 20 and 21). The true MAP-prevalence based on ELISA-C-apparent prevalence, sensitivity (42%) and specificity (99%) was 2.2%.

Table 20. ELISA-C, PCR and fecal culture positive results of five dairy herds examined for MAP in Colombia

Herd	District	Herd cattle population	Number of samples [a]	Serum ELISA-C	Fecal culture [c]	Fecal PCR [e]
1	Monterredondo	125	75	2	4[d]	2
2	El Yuyal	174	53[b]	4	0	0
3	El Yuyal	144	84	0	0	N.D.[f]
4	Santo Domingo	172	94	0	0	N.D.
5	Santa Bárbara	38	23	0	0	N.D.
Total		**653**	**329**	**6**	**4**	**2**

[a] Only cows over 2 years of age were sampled.

[b] In this herd 110 animals were sampled for feces, but only 53 of them were sampled for serum.

[c] Refers to fecal samples cultured individually.

[d] Positive results of one lymph node, one colon tissue, and two slurry samples are not included.

[e] Refers to individual fecal samples part of pooled fecal samples positive by culture, and to fecal samples of positive ELISA-C animals tested individually.

[f] N.D. not done

Table 21. ELISA-C, PCR and culture results of animals and individual slurry samples from positive pooled samples or ELISA-C positive animals of herd 1 and herd 2 in Colombia

Herd	Pool	Source	ELISA-C	PCR	Culture
1	1	C1	-	-	+
		C2	-	-	+
		C3	+	+	+
		C4	+	-	-
		C5	-	-	-
		C6	-	-	-
		C7	-	-	-
		C8	-	-	-
		C9	-	-	-
	2	C1	-	-	-
		C2	-	-	-
		C3	-	-	-
		C4	-	+	+
		C5	-	-	-
		C6	-	-	-
		C7	-	-	-
		C8	-	-	-
		C9	-	-	-
		B	-	-	-
	3	S1	N.A.	-	-
		S2	N.A.	-	-
		S3	N.A.	-	-
2	1	C1	+	-	-
	2	C2	+	-	-
	3	C3	+	-	-
	4	C4	+	-	-

C cow, B bull, S slurry pit, + positive result, - negative result, N.A. not applicable.

The age of the animals sampled ranged between 2.2 and 14 years (mean 5.9, SD 2.8). Analysis of age of animals vs. ELISA-C result (positive, negative, doubtful) revealed that the group of >11 years of age, was the group in which the highest proportion (6.3%, 1 out of 16) of ELISA-positive samples were produced (Table 22). However, it was in the group of 5.1-8

years of age in which the highest absolute number of ELISA-positive animals (n=3) of the whole study was found. In the group of the youngest cows (>2.2-2.9 years of age) no positive result by ELISA was produced. In the group of 3-5, 5.1-8 and 8.1-10.9 years, 0.8%, 2.9% and 1.9% of the samples produced positive results, respectively (Table 22).

Table 22. ELISA-C results according to group of age of 323 animals from five dairy herds in Colombia

Group of age	ELISA result			
	Positive (%)	Negative (%)	Doubtful (%)	Total (%)
2.2 - 2.9	0 (0.0)	32 (100)	0 (0.0)	32 (9.9)
3 - 5	1 (0.8)	116 (98.3)	1(0.8)	118 (36.5)
5.1 - 8	3 (2.9)	101 (97.1)	0 (0.0)	104 (32.2)
8.1 - 10.9	1 (1.9)	51 (96.2)	1 (1.9)	53 (16.4)
>11	1 (6.3)	15 (93.8)	0 (0.0)	16 (5)

3.2.3.2. Fecal culture and fecal PCR

The strategic pooling procedure for fecal samples from the five herds resulted in 36 pools, including the slurry pool prepared from herd 1, which had a slurry pit collecting liquid manure and wastewater from the herd's milking parlor. Two pools from herd 1 out of 36 pools analyzed produced positive results by culture after 5-6 weeks of incubation with >50 Colony Forming Units (CFU)/tube. The slurry pool produced positive results by culture after 17 weeks of incubation with <10 CFU/tube. Isolates obtained from pooled samples were confirmed as MAP by the real-time PCR method described above. Remaining pools of herds 2, 3, 4, and 5 did not show mycobacterial growth by culture in 20 weeks of incubation.

Fecal samples that were a part of the two positive pools of herd 1 (n=19) produced four positive results by individual culture (Table 21). All isolates showed no pigmentation and were confirmed as MAP by real-time PCR. Two cows (both ELISA-C-negative, PCR-negative, asymptomatic, 7.1 years old) were low shedders (<10 CFU/tube), one cow (ELISA-positive, PCR-positive, symptomatic, 6 years old) was a heavy shedder, and one cow (ELISA-negative, PCR-positive, asymptomatic, 9.5 years old) was a heavy shedder (>50 CFU/tube, Table 21). The cow 3 from pool 1 (herd 1) also produced positive results by mesenteric lymph node and colon tissue culture (Table 23). On HEYM-slants inoculated with mesenteric lymph node tissues, visible colonies grew before 16 weeks of incubation, while in those inoculated with colon tissues no visible MAP colonies grew in this period of time. Four fecal samples

from positive ELISA-C animals of herd 2 produced negative results by culture and PCR; these samples were all from different pooled fecal samples (Table 21). Surprisingly, although the pooled slurry sample produced positive results by culture and PCR, their individual samples (n=3) were negative by PCR, and by culture after 20 weeks of incubation (Table 21).

Table 23. Isolates of MAP recovered in a dairy herd in Colombia

Isolate number	Source	Isolated from	Genotype	
			MLSSR[a]	MIRU-VNTR[b]
1	Cow 1	Feces	A	1
2	Cow 2	Feces	A	1
3	Cow 3	Feces	A	1
4	Cow 3	Mesenteric lymph node	B	2
5	Cow 3	Colon tissue	A	1
6	Cow 4	Feces	A	1
7	Slurry pit	Slurry	A	1
8	Slurry pit	Slurry	B	2

[a] MLSSR-genotype A: 7g-10g-4ggt-5tgc and MLSSR-genotype B: 7g-10g-5ggt-4tgc.

[b] MIRU-VNTR genotype 1: 3951-42332228-2 (INMV 1) and MIRU-VNTR genotype 2: 3751-32332228-2 (INMV 2).

ELISA-C results were confirmed by culture in only one symptomatic animal (cow 3) of herd 1. Thus ELISA-C, culture and PCR only agreed on one single animal out of four animals that delivered positive culture results. According to these results, calculated agreement between ELISA-C and culture was poor (κ=0.19, 95% C.I. 0.09-0.29). ELISA-C did not detect MAP antibodies in three serum samples from asymptomatic animals of herd 1 (Cow 1 and Cow 2 from pool 1, and Cow 4 from pool 2) that produced positive results by fecal culture. In fecal samples from two of these three animals, MAP was also not detected by PCR. In one case, a cow (cow 4 of pool 1 from herd 1) produced negative results by ELISA-C, but positive results by PCR and culture (Table 21).

3.2.3.3. Comparison of results of the Screening and Confirmation

Based on results obtained in the screening and the confirmation parts of the study, it was determined that some animals (n=11) tested in the screening were tested again by ELISA, PCR and culture in the confirmation. None of these animals have presented symptoms of paratuberculosis before the screening or between the screening and the confirmation. Results

can be classified in five different categories, in which only positive findings are described, meaning that other tests produced negative results. In the category 1, one single animal produced positive results by ELISA-A and real-time PCR (molecular target F57) in the screening and ELISA C-positive results in the confirmation. In the category 2, one single animal produced positive results in ELISA A, PCR and real-time PCR (molecular target IS*Mav2*) in the screening. In the category 3, two animals produced ELISA A and ELISA B positive results in the screening. In the category 4, one single animal produced positive results by ELISA A and PCR in the screening. Finally, in the category 5, six animals produced positive results by ELISA A (Table 24).

Table 24. Comparison of diagnostic test results of single animals (n=11) tested for MAP in Colombia in 2007 and 2009

	2007 [a]					2009			
Category	**ELISA-A** [b]	**ELISA-B** [c]	**PCR** [d]	**Real-time PCR** [d]	**Culture**	**ELISA-C** [e]	**Culture** [f]	**PCR** [g]	**Real time - PCR** [g]
1	+	-	-	+ (F)	-	+	-	-	-
2	+	-	+	+ (I)	-	-	-	N.D	N.D
3	+	+	-	-	-	-	-	N.D	N.D
4	+	-	+	-	-	-	-	N.D	N.D
5	+	-	-	-	-	-	-	N.D	N.D

[a] According to the results of the study determination of MAP infection (screening, see 3.2.1).
[b] ELISA-A (Svanovir Para-TB Ab ELISA Kit, Svanova Biotech AB).
[c] ELISA B (ELISA paratuberculosis antibody verification, Institute Pourquier) performed only to positive and doubtful ELISA-A samples.
[d] PCR and real-time PCR performed only to fecal samples from positive animals by ELISA-A.
[e] ELISA-C (ID Screen Paratuberculosis Indiret, IDVET) according to the results of the study determination of MAP infection (confirmation, see 3.2.3).
[f] Culture performed initially from pooled fecal samples, and then individually if pooled sample was MAP positive
[g] PCR and real-time PCR performed only to individual fecal samples from culture positive pooled samples, and to fecal samples from ELISA C-positive animals.
+ positive, - negative, (F) positive real time - PCR in marker F57, (I) positive real time - PCR in marker IS*Mav2*, N.D.: not done.

3.2.3.4. Genotyping

In total eight MAP isolates were recovered. Four isolates were from fecal samples, one from mesenteric lymph node, one from colon tissue sample, and two from pooled slurry samples (Table 23). All isolates were confirmed as MAP by the F57/IS*Mav2* real-time PCR method described above. All isolates were obtained from samples from herd 1. All isolates grew

within 6-16 weeks of incubation, except from samples of slurry and colon tissue, which grew after the 16th week. According to this, MAP isolates recovered appeared to be of MAP-type C or type II. The four isolates obtained from fecal samples, the isolate obtained from colon tissue and one of the two isolates obtained from slurry sample were of the MLSSR genotype 7g-10g-4ggt-5tgc (hereafter MLSSR-genotype A). The isolates obtained from mesenteric lymph node and the remaining isolate obtained from slurry sample were of the MLSSR genotype 7g-10g-5ggt-4tgc (hereafter MLSSR-genotype B, Figure 18).

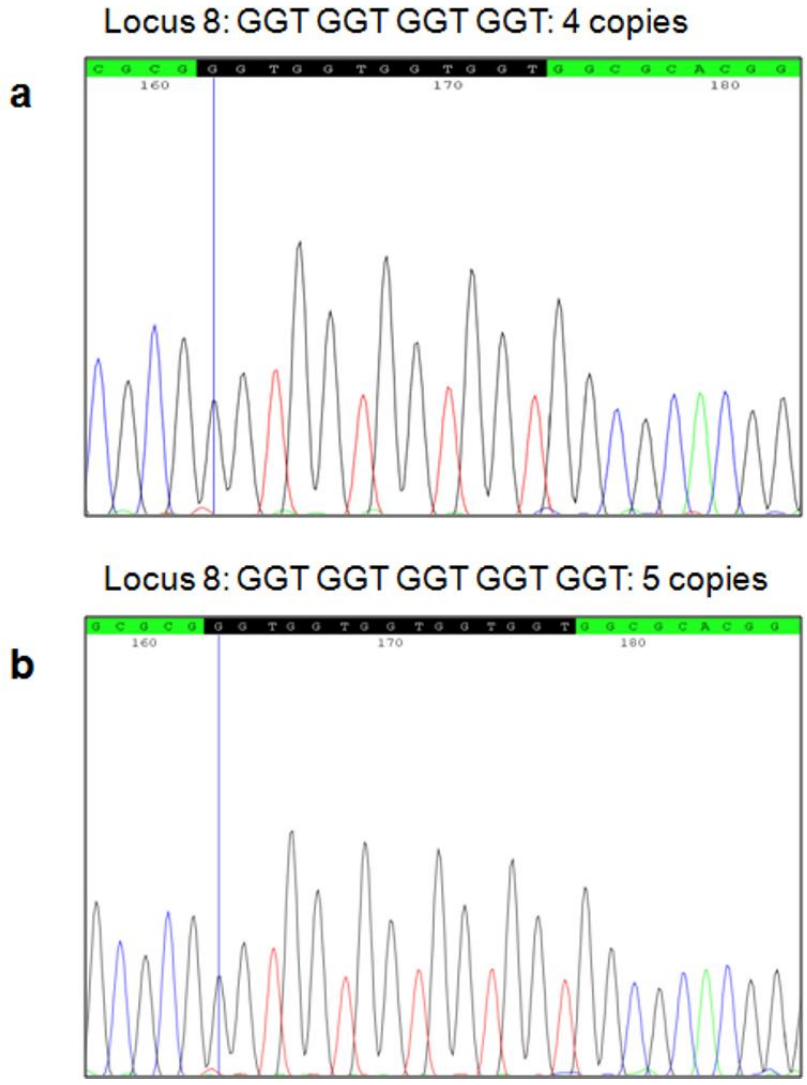
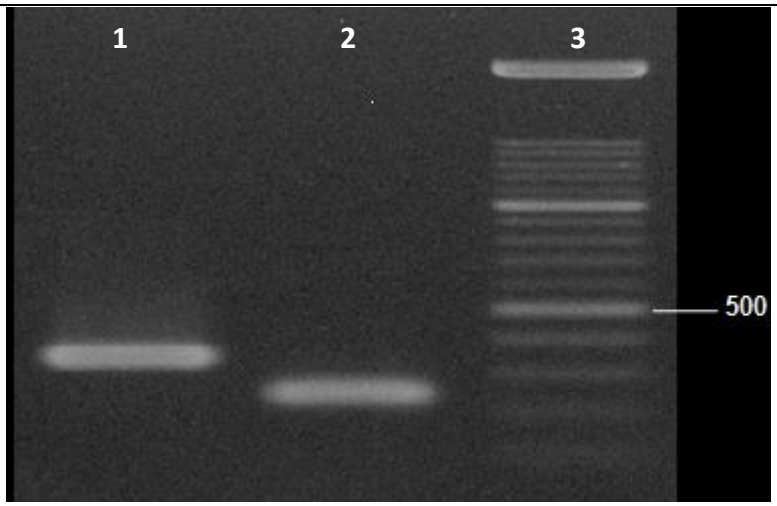


Figure 7. SSR-locus 8 alleles from MAP isolates from Colombia
a. Isolate 1 containing four copies of GGT (MLSSR-Type A). **b.** Isolate 5 containing five copies of GGT (MLSSR-Type B).

Similarly, the combination of MIRU-VNTR showed two different MIRU-VNTR genotypes, genotype 3951-42332228-2 (hereafter MIRU-VNTR genotype 1) and 3751-32332228-2 (hereafter MIRU-VNTR genotype 2, Figure 19). Interestingly, strain types A-1 and B-2 were both identified in cow 3, representing a case of double strain infection (Table 23).



a

9 repeats units

A2a-B2-A2i-B2-A2i-B2-A2i-B2-A2b

TGAACGAAGATCCTGGGACTGGACG
AGTTGGATGAGGAGCGCCCTAAGTG
ACACGCGCCGACGCGATGCAGAGCG
CAGCGATGAGGAGGAGCGGCGCAGA
TGACTCGCGCCGACGACGATGCAGA
GCGCAGCGATGAGGAGGAGCGGCGC
AGATGACTCGCGCCGACGACGATGC
AGAGCGCAGCGATGAGGAGGAGCGG
CGCAGATGACTCGCGCCGACGACGA
TGCAGAGCGCAGCGATGAGGAGGAG
CGGCGCAGATGACAGAAGCATTGCG
ACCGCCACGTGGGCCAAAGACCYTK
ATGCCCAAACGCTTTACGAGCTGCTC
AAGCTGCGGGTTGAGGTGTTCGTCG
TCGA

b

7 repeats units

A2a-B2-A2i-B2-A2i-B2-A2b

TGAACGAAGATCCTGGGACTGGACG
AGTTGGATGAGGAGCGCCCTAAGTG
ACACGCGCCGACGACGATGCAGAGC
GCAGCGATGAGGAGGAGCGGCGCAG
ATGACTCGCGCCGACGACGATGCAG
AGCGCAGCGATGAGGAGGAGCGGCG
CAGATGACTCGCGCCGACGACGATG
CAGAGCGCAGCGATGAGGAGGAGCG
GCGCAGATGACAGAAGCATTGCGAC
GCACGTGGGCCAAAGACCTTGATGC
CCAAACGCTTTACGAGCTGCWCAAG
CTGCGGGTTGAGGTGTTCGTCGTCG
A

Figure 8. MIRU-2 alleles of MAP isolates from Colombia

Above PCR profiles of representative MIRU-2. Lane 1: MIRU-2 of isolate 1 containing nine repeat units (MIRU-VNTR type 1), lane 2: MIRU-2 of isolate 5 contains seven repeat units (MIRU-VNTR type 2), lane 3: Molecular size marker (100 bp DNA Ladder, Roche, Mannheim, Germany).

Below corresponding sequences of the PCR MIRU profiles showing motif composition and distribution according to Bull et al. (2003). **a** Sequence from lane 1. **b.** Sequence from lane 2.

3.3. Genotyping of MAP from South American countries

3.3.1. MIRU–VNTR

So far, 7 different genotypes have been determined by MIRU–VNTR in MAP isolates from South American countries. From these 7 MIRU–VNTR genotypes, the genotype 1 (INMV1) was the most common (Table 25 and Table 26).

Table 25. Genotypes of MAP from South American countries obtained by MIRU–VNTR and MLSSR

Strain	Country[d]	Source[e]	MIRU–VNTR	INMV	MLSSR	MIRU–VNTR / MLSSR
258	CL	C	1	1	A	1–A
299	CL	C	7	33	A	7–A
300	CL	C	1	1	A	1–A
292	CL	C	1	1	A	1–A
134	CL	C	1	1	B	1–B
174	CL	C	1	1	A	1–A
175	CL	C	1	1	B	1–B
79	CL	H	1	1	B	1–B
18	CL	H	5	9	B	5–B
F2	CL	P	1	1	A	1–A
3208	CL	Sh	1	1	A	1–A
Cow 1[a]	CO	C	1	1	A	1–A
Cow 2[a]	CO	C	1	1	A	1–A
Cow 3 – A [a]	CO	C	1	1	A	1–A
Cow 3 – B [a]	CO	C	2	2	D	2–D
Cow 3 – C [a]	CO	C	1	1	A	1–A
Cow 4 [a]	CO	C	1	1	A	1–A
Slurry 1 [a]	CO	Sl	1	1	A	1–A
Slurry 2[a]	CO	Sl	2	2	D	2–D
222[b]	AR	C	1	1	B	1–B
224[b]	AR	C	1	1	C	1–C
226[b]	AR	C	6	11	F	6–F
227[b]	AR	C	1	1	C	1–C
229[b]	AR	D	1	1	A	1–A
269[b]	AR	C	1	1	A	1–A
291[b]	AR	D	1	1	B	1–B
304[b]	AR	D	4	8	E	4–I
225[c]	AR	C	1	1	N.A.	N.D.
280[c]	AR	C	1	1	N.A.	N.D.
299[c]	AR	C	1	1	N.A.	N.D.
300[c]	AR	C	1	1	N.A.	N.D.
301[c]	AR	C	1	1	N.A.	N.D.
309[c]	AR	C	1	1	N.A.	N.D.

Table 25. continued

234[c]	VE	C	3	3	N.A.	N.D.
284[b]	VE	C	3	3	G	3–K

[a] Genotypes taken from the publication of Fernandez–Silva et al. (2011).
[b] Genotypes taken from the publication of Thibault et al. (2008).
[c] Genotypes taken from the publication of Thibault et al. (2007).
[d] Country: CL Chile, CO Colombia, AR Argentina, VE Venezuela.
[e] Source: H hare, C cattle, Sl slurry, Sh sheep, P pudu, D deer.
N.A. information not available in the original publication.
N.D. information could not be determined due to absence of MLSSR genotypes in the original publication.

Table 26. MIRU–VNTR genotypes of MAP from South American countries

MIRU–VNTR genotype	**Number of repeats at MIRU–VNTR locus**											**Corresponding INMV Profile**
	1	**4**	**292**	**1658**	**25**	**47**	**3**	**7**	**10**	**32**	**259**	
1	3	1	4	2	3	3	2	2	2	8	2	1
2	3	1	3	2	3	3	2	2	2	8	2	2
3[a]	–	–	3	2	3	3	2	2	1	8	–	3
4[a]	–	–	3	2	3	3	2	3	2	8	–	8
5	3	1	2	1	3	3	2	2	2	8	2	9
6[a]	–	–	3	2	3	3	2	4	2	8	–	11
7	3	1	3	2	5	2	2	2	2	8	2	33

[a] Genotypes taken from the study of Thibault et al. (2007), in which loci MIRU–1, MIRU–4 and VNTR 259 were not analyzed.

3.3.2. MLSSR

On the other hand, 7 different genotypes have been determined by MLSSR, from which the genotype A (7G–10G–4GGT) was the most common (Table 25 and Table 27).

3.3.3. Combined analysis of MIRU–VNTR and MLSSR

Combined analysis of MIRU–VNTR and MLSSR revealed 9 different genotypes of MAP. The genotype with the combination 1–A was the most common (n=14), followed by the combined genotype 1–B (n=5, Table 25).

Table 27. MLSSR genotypes of MAP from South American countries

Genotype	Number of repeats at SSR locus		
	1 (G–repeat)	**2 (G–repeat)**	**8 (GGT–repeat)**
A	7	10	4
B	7	11	4
C	7	>11	4
D	7	10	5
E[a]	7	11	5
F[a]	>11	11	5
G[a]	11	10	5

[a] Genotypes taken from the publication of Thibault et al. (2008).

The genotype 1–B was detected in two MAP isolates (cattle isolate 175 and hare isolate 79) from one property in Chile. From 6 Argentinean and from 1 Venezuelan isolates a combined genotype could not be determined due to the absence of MLSSR results in previous studies (Table 25).

3.4. Molecular characterization of MAP in Rhineland–Palatinate (RP), Germany

3.4.1. MIRU–VNTR

MIRU–VNTR analysis produced 11 different genotypes among 91 isolates analyzed (Table 28).

Table 28. Results of genotyping of MAP from dairy cattle in Germany and Luxembourg using MLSSR and MIRU–VNTR

Counties[a]	MIRU–VNTR[b] Genotype	MLSSR[c] Genotype	Combined Genotype	Number of isolates	%
WIL, BIT, KUS, SIM, EMS, AC	1	A	1–A	8	8.8
AK, BIR, BIT, MYK, MZG, MZG, EMS, TR, TR	1	B	1–B	14	15.4
BIT	1	C	1–C	4	4,4
WIL, COC, BIT, GER, MZG	1	D	1–D	14	15.4
AK, KUS, NW, WW	2	A	2–A	4	4.4
KIB, BIT, SIM	2	B	2–B	6	6.6
BIT, LU, SIM, TR, DAU, WW	2	C	2–C	9	9.9
BIT	2	D	2–D	2	2.2
BIT, TR	2	E	2–E	3	3.3
TR	2	F	2–F	1	1.1
BIR, BIT	3	A	3–A	3	3.3
TR	3	B	3–B	1	1.1
BIT	3	C	3–C	3	3.3
AK, KUS, NW, PS	4	A	4–A	4	4.4
KIB, BIT	4	C	4–C	2	2.2
BIT, RÜD	5	A	5–A	3	3.3
COC	5	C	5–C	1	1.1
BIR, BIT	6	A	6–A	2	2.2
WIL	6	B	6–B	1	1.1
TR	6	C	6–C	1	1.1
PS	7	B	7–B	1	1.1
DAU	8	D	8–D	1	1.1
WW	9	D	9–D	1	1.1

Table 28. continued

NK	10	C	10–C	1	1.1
BIT	11	C	11–C	1	1.1
Total	**11**	**6**	**25**	**91**	**100**
Index of discrimination (*D*) [d]	**0.74**	**0.78**	**0.93**		
C.I. 95% for *D*[e]	**0.66–0.81**	**0.76–0.80**	**0.91–0.95**		

[a] Counties: **AC** Städteregion Aachen (North Rhine–Westphalia), **AK** Altenkirchen-Westerwald, **BIR** Birkenfeld, **BIT** Eifelkreis Bitburg–Prüm, **COC** Cochem–Zell, **DAU** Vulkaneifel, **EMS** Rhein–Lahn–Kreis, **GER** Germersheim, **KIB** Donnersbergkreis, **KUS** Kusel, **LU** Luxembourg, **MYK** Mayen–Koblenz, **MZG** Merzig–Wadern (Saarland), **NK** Neunkirchen (Saarland), **NW** Neuwied, **PS** Südwestpfalz, **RÜD** Rheingau–Taunus–Kreis (Hesse), **SIM** Rhein–Hunsrück–Kreis, **TR** Trier–Saarburg, **WIL** Bernkastel–Wittlich, **WW** Westerwaldkreis.

[b] Performed according to Bull et al. (2003), Overduin et al. (2004), Thibault et al. (2007), Castellanos et al. (2010).

[c] Performed according to Amonsin et al. (2004).

[d] Calculated for isolates without epidemiological relationship according to Hunter and Gaston (1988).

[e] Calculated according to Grundmann et al. (2001).

MIRU–VNTR genotypes were identified with numbers from 1 to 11 (Table 29). MIRU–VNTR analysis revealed that the most common genotypes detected in the panel of MAP isolates were genotype 1 (INMV1) and genotype 2 (INMV 2), representing 44% (40/91) and 27.5% (25/91) of all isolates, respectively (Table 29). The MIRU–VNTR genotypes 5, 10, and 11 have not been assigned the INMV codification system or had not been detected before (Table 29).

Table 29. MIRU–VNTR genotypes obtained from MAP isolates in RP (Germany)

MIRU–VNTR genotype [a]	No. of isolates	%	Number of copies of MIRU–VNTR									INMV Profile [b]
			1	292	1658	25	47	3	7	10	32	259	
1	40	44.0	3	4	2	3	3	2	2	2	8	2	1
2	25	27.5	3	3	2	3	3	2	2	2	8	2	2
3	7	7.7	3	3	2	5	2	2	2	2	8	2	33
4	6	6.6	3	4	2	3	3	2	1	2	8	2	19
5	4	4.4	3	4	3	3	3	2	1	2	8	2	Undet.
6	4	4.4	3	3	2	3	3	2	1	2	8	2	6
7	1	1.1	3	4	2	3	3	2	2	1	8	2	5
8	1	1.1	3	3	2	3	3	2	2	2	9	2	4
9	1	1.1	3	3	2	3	3	2	2	1	8	2	3
10	1	1.1	3	2	2	5	2	2	1	2	8	2	Undet.
11	1	1.1	3	2	2	5	2	2	2	2	6	2	Undet.

[a] Performed according to Bull et al. (2003); Overduin et al. (2004); Thibault et al. (2007); Castellanos et al., (2010).
[b] INMV profile based on loci MIRU–VNTR 292–1658 (alias X3)–25–47–3–7–10–32 (shaded area) according to Thibault et al. (2007)
Undet. Undetermined

The calculated numerical index of discrimination (*D*) for the MIRU–VNTR was 0.74 (95% CI: 0.66–0.81, Table 28). Seven MIRU–VNTR loci (292, 1658, 25, 47, 7, 10, and 32) out of 10 loci analyzed presented allelic variation. No isolate produced allelic variation by MIRU–1, MIRU–VNTR 3 and MIRU–VNTR 259. The calculation of the allelic diversity (*h*) for individual MIRU–VNTR loci revealed differences ranging from 0.03 (MIRU–VNTR 32) to 0.50 (MIRU–VNTR 292, Table 31).

3.4.2. MLSSR

MLSSR were analyzed in the same panel of isolates genotyped by MIRU–VNTR. MLSSR analysis detected six different genotypes among the 91 isolates analyzed (Table 27). MLSSR genotypes were identified with letters from A to F (Table 30). The genotype A (>11g–4ggt) was the most common MLSSR genotype found, representing 26.4% (24/91) of all MAP isolates (Table 30).

Table 30. MLSSR genotypes obtained from MAP isolates in RP (Germany)

MLSSR Genotype[a]	No. of isolates	%	Number of copies of SSR Loci	
			2 (g)	8 (ggt)
A	24	26.4	>11	4
B	23	25.3	11	4
C	22	24.2	10	4
D	18	19.8	9	4
E	3	3.3	10	5
F	1	1.1	9	5

[a] Performed according to Amonsin et al. (2004).

The calculated numerical index of discrimination (*D*) for MLSSR was 0.78 (95% CI: 0.76–0.80, Table 28). The two SSR–loci analyzed produced allelic variation. Locus 2 presented the highest allelic variation with four alleles, while locus 8 presented two alleles. The allelic diversity (*h*) was 0.03 for locus 8 and 0.75 for locus 2 (Table 31).

Table 31. MIRU–VNTR and MLSSR allelic distribution of MAP in RP (Germany)

Locus		No. of isolates with the allele												Allelic
		0	1	2	3	4	5	6	7	8	9	10	11	>11	diversity (*h*)[a]
MIRU–VNTR	292			2	38	51									0.50
	7		15	76											0.29
	47			9	82										0.11
	10		2	89											0.11
	25				82		9								0.10
	1658			87	4										0.08
	32							1		89	1				0.03
MLSSR	2										19	25	23	24	0.75
	8					87	4								0.03

[a] Calculated only for isolates without epidemiological relationship according to Selander et al. (1986).

3.4.3. Subdivision of MIRU–VNTR and MLSSR genotypes

Six MIRU–VNTR genotypes (genotypes 1 to 6) were subdivided by MLSSR. Conversely four MLSSR genotypes (A, B, C, and D) were subdivided by MIRU–VNTR (Table 32). The most common MIRU–VNTR genotypes 1 (INMV 1) and 2 (INMV 2) were subdivided by

MLSSR into four and six different MLSSR–genotypes, respectively. The most common MLSSR genotype A was divided by MIRU–VNTR into six different genotypes, while MLSSR genotype B, C, and D were subdivided into five, eight, and four MIRU–VNTR genotypes, respectively (Table 32). Adjusted Rand index was 0.054. The Wallace coefficient (W) for MIRU–VNTR → MLSSR, and for MLSSR →MIRU–VNTR was 0.261 (95% C.I. 0.20–0.312) and 0.321 (95% C.I. 0.239–0.403), respectively. The expected W value if the classifications are independent (Wi) was 0.275 and 0.224 for MIRU–VNTR and MLSSR, respectively (Table 32).

Table 32. Subdivision of genotypes obtained by MIRU–VNTR and MLSSR in RP (Germany)

Genotype	No of isolates	Subdivided by	
		MIRU–VNTR	**MLSSR**
1	40		A, B, C, D
2	25		A, B, C, D, E, F
3	5		A, B, C
4	6		A, C
5	4		A, C
6	4		A, B, C
Wi[a]		0.275	
W[a]			0.261
C.I. 95% of W[a]			0.20–0.312
A	24	1, 2, 3, 4, 5, 6	
B	23	1, 2, 3, 6, 7	
C	22	1, 2, 3, 4, 5, 6, 10, 11	
D	18	1, 2, 8, 9	
Wi			0.224
W		0.321	
C.I. 95% of W		0.239–0.403	

[a] The Wallace coefficient (W), the 95% C.I., and the expected W value if the classifications are independent (Wi) were calculated according to Pinto et al. (2008)

3.4.4. Combined analysis of MIRU–VNTR and MLSSR

Combined analysis of MIRU–VNTR and MLSSR produced 25 genotypes among the 91 isolates analyzed (Table 28). The combined genotypes of every isolate were expressed as the combination of the numbers and letters of MIRU–VNTR and MLSSR results, respectively. The calculated numerical index of discrimination (*D*) for the combined methods was 0.93

(95% CI: 0.91–0.95, Table 28). Combined genotypes 1–B and 1–D were detected in 14 out of 91 isolates (15.4%) each, being the most detected genotypes among the isolates analyzed. Genotypes 2–C (9.9%) and 1–A (8.8%) were detected in nine and eight isolates, respectively Remaining isolates were found in six or fewer isolates (Table 28).

3.4.5. Molecular epidemiology of MAP in Rhineland–Palatinate

Results of combined MIRU–VNTR and MLSSR were used to analyze the molecular epidemiology of MAP in Rhineland–Palatinate, as only both methods combined achieved a minimum discriminatory ability needed for epidemiological studies ($D \geq 0.90$) but not reached by application of each method independently. Diverse combined genotypes were widely distributed on the counties of the federal state of Rhineland–Palatinate, Luxembourg, and in isolates of the neighboring German federal states of Saarland, Nordrhein–Westphalia and Hesse (Table 28). Although in some counties of Rhineland–Palatinate fewer isolates were obtained, probably related to a lower concentration of cattle reporting clinical cases of paratuberculosis along time, diverse combined genotypes were also detected (Table 28). In RP, all genotypes excepting genotype 10-C were found in isolates of RP (Table 28).

The distribution of MAP genotypes in RP correlates with the cattle population density in the counties (Anonymous, 2011b). The three counties with the highest cattle population (BIT, DAU, and WILL) concentrate 64% of all genotypes detected in the whole study. However, some genotypes detected in low–populated counties were not detected in the most populated. For example, genotypes 2–A and 4–A were detected in the counties Altenkirchen (Westerwald, AK), Neuwied (NW), Kusel (KUS), Westerwaldkreis (WW) and Südwestpfalz (PS), but were not detected in Eifelkreis Bitburg–Prüm (BIT, Table 33).

Table 33. Distribution of MAP genotypes in the counties of RP, Germany

County[a]	Cattle population	Communes	Herds	Isolates	Genotypes
BIT	99.162	27	32	42	1-A, 1-B, 1-C, 1-D, 2-B, 2-C, 2-D, 2-E, 3-A, 3-C, 4-C, 5-A, 5-A, 6-A, 8-D, 11-C
DAU	31.366	1	1	1	2-C
WIL	24.474	3	4	4	1-A, 6-B, 1-D
WW	24.154	3	3	3	9-D, 2-C, 2-A
TR	21.080	9	9	9	2-E, 1-B, 6-C, 2-F, 2-C, 3-B
AK	19.115	3	3	3	4-A, 1-B, 2-A
SIM	16.777	3	3	3	2-C, 2-B, 1-A
PS	15.135	2	2	2	7-B, 4-A
NW	13.497	2	2	2	2-A, 4-A
BIR	13.383	3	3	4	3-A, 1-B, 6-A
KUS	12.769	3	3	3	2-A, 1-A, 4-A
EMS	11.950	2	2	2	1-A, 1-B
MYK	9.439	1	1	1	1-B
COC	8.202	1	1	1	1-D
KIB	7.035	2	2	2	4-C, 2-B
Total	**329.182**	**65**	**71**	**82**	**25**

[a] Counties: **AK** Altenkirchen-Westerwald, **BIR** Birkenfeld, **BIT** Eifelkreis Bitburg–Prüm, **COC** Cochem–Zell, **DAU** Vulkaneifel, **EMS** Rhein–Lahn–Kreis, **GER** Germersheim, **KIB** Donnersbergkreis, **KUS** Kusel, **MYK** Mayen–Koblenz, **NW** Neuwied, **PS** Südwestpfalz, **SIM** Rhein–Hunsrück–Kreis, **TR** Trier–Saarburg, **WIL** Bernkastel–Wittlich, **WW** Westerwaldkreis.

Eight isolates from Saarland, North Rhine–Westphalia, and Luxembourg produced the common genotypes 1–A, 1–B, 1–D and 2–C, while two isolates from Hesse (county Rheingau–Taunus–Kreis, RÜD) and Saarland (county Neunkirchen, NK) produced the two uncommon genotypes 5–A and 10–C, respectively. Genotypes 5–A was also found in other herds of Rhineland–Palatinate, while genotype 10–C was only found in the isolate from Saarland (Table 28).

4. DISCUSSION

4.1. Establishment and evaluation of MAP genotyping methods

In this study two MAP reference strains, 10 MAP field isolates, and one MAH isolate from different host species and clinical samples were analyzed using the genotyping methods IS*900*-RFLP, MIRU, VNTR, and MLSSR. The purpose of the study was the establishment and evaluation of methods, taking into account that the number and arbitrary selection of isolates does not allow to draw epidemiological conclusions.

Within the methods for MAP genotyping based on insertion elements, the IS*900*-RFLP using different endonucleases has been widely used to determine the diversity of MAP isolates in many regions of the world and has been consider an useful and standard method for MAP genotyping in further studies (Pavlik et al., 1999; Mobius et al., 2008b). The IS*900*-RFLP results, taking into account the low number of strains used, showed an adequate capacity to differentiate between MAP and MAH, and between MAP strains. However, coinciding with other authors the results are extremely dependent on the endonuclease used (Pavlik et al., 1999; Mobius et al., 2008b). In contrast to reports of other authors, the *BstE* II endonuclease and not *Bam*H I or *Pst* I delivered the highest discriminatory index in the present study (Cousins et al., 2000; Mobius et al., 2008b). Nevertheless, this finding could be random, due basically to the low number of isolates and strains used. Results also agree with previous reports about the increment of the discrimination ability when different endonucleases are used (Mobius et al., 2008b). In the same way, results agree with a recent study, in which *Pst* I profiles from several MAP isolates were difficult to obtained (Stevenson et al., 2009) and *Pst* I was the endonuclease that delivered the lowest number of profiles (Cousins et al., 2000; Mobius et al., 2008b). The calculation of the Simpson's index of diversity of IS*900*-RFLP was affected by the unavailability of profiles from one or more endonucleases in several MAP-isolates, which produced a significant reduction of the index. IS*900*-RFLP profiles using *BstE* II from the reference strain K-10 agrees with the profile reported previously (Cousins et al., 2000).

The analysis of MIRU was first proposed for its application to differentiate MAP from other species of the MAC (Bull et al., 2003b) and has been further used to this purpose and to differentiate between diverse MAP and MAH isolates (Overduin et al., 2004; Romano et al., 2005). The present results agree with previous publications in which the PCR-based method is

easy to perform, but has shown a relative limited capacity to differentiate between MAP strains, except for MIRU-2 and MIRU-3 (Bull et al., 2003b; Mobius et al., 2008b; El Sayed et al., 2009). Nonetheless, MIRU-2 and MIRU-3 were not 100% effective to differentiate MAP from MAH in the present study. As expected MIRU-1 showed no variability among MAP isolates, although in a previous study in Germany two MAP-alleles in this locus were reported (Mobius et al., 2008b). The MIRU-VNTR 4ARG to 11UK were completely ineffective to differentiate between MAP isolates, but very efficient to differentiate MAP from MAH. This agrees with the original report of (Romano et al., 2005) and with a recent application to field isolates in Germany (El Sayed et al., 2009). This latter application to field isolates of MAP is striking because these loci were clearly reported by Romano et al. (2005) as ineffective for MAP differentiation purposes.

Similar to the case of MIRUs, the majority of VNTR-loci were able to differentiate between MAP and MAH isolates, but poorly between MAP isolates. The only exception was locus 1658, which could differentiate between MAP, but not between MAP and MAH isolates. Several VNTR-loci used in this study have been reported as targeting the same genome regions as MIRU (Romano et al., 2005; Castellanos et al., 2010b). Therefore is not surprising that the results obtained with VNTR-1658 delivered the same information in terms of discriminatory index as MIRU-3. Based on the results of the index, only MIRU-2 and MIRU-3 (or its homologous VNTR-1658) can be considered as appropriate for further MAP differentiation. The remaining loci could be useful for differentiation between members of the MAC, especially between MAP, MAA/MAS, and MAH, but not for MAP strain differentiation. In addition to VNTR 1658 and MIRU-3, MIRU-7 ARG (Romano et al., 2005) and VNTR-3527 (Overduin et al., 2004); and MIRU-6 ARG (Romano et al., 2005) and VNTR-1605 (Overduin et al., 2004) target the same MAP-genomic regions (Castellanos et al., 2010b).

The MLSSR method using three loci delivered the highest discriminatory index of all methods applied in the present study, as reported in previous studies using more than one genotyping method (El Sayed et al., 2009; Douarre et al., 2011). This high discriminatory index was predominately determined by the high discriminatory ability of locus 2, as reported also previously (El Sayed et al., 2009; Douarre et al., 2011). However, this locus has been also reported as having lower discriminatory indexes compared to indexes of loci 1 and 8 (Amonsin et al., 2004) revealing the influence of the MAP population under study on the

prevalence of some genotypes and on the discriminatory index (Stevenson et al., 2009). The reference strain K-10 showed the profile previously reported in several studies (Amonsin et al., 2004; Thibault et al., 2008). However, this profile has been reported as >14 and >11 G-repeats for simplicity and to circumvent some problems in the sequencing of large G-repeats.

The performance evaluation of the genotyping methods applied included all aspects previously suggested for this purpose by (van Belkum et al., 2007). Logically the estimation of the discriminatory ability using the Simpson's index of diversity (*D*) represents the most important aspect and was decisive to achieved practical conclusions. Stability of methods used appeared to be high according to this study, in contrast to the extreme frequent changes reported from other species (Collins, 2010). Typeability was affected in MIRU-3 due to some difficulties in the establishment of the PCR technique for this locus, fortunately this locus possesses more than one homologue allowing the effective analysis of isolates (Castellanos et al., 2011)

In general, MLSSR was the method with the highest index followed by IS9*00*-RFLP (endonuclease BstEII), which delivered a slightly lower index but more profiles. However, results of IS*900*-RFLP have to be analyzed carefully due to the unavailability of some profiles caused by low DNA quality and/or endonuclease inactivity. Nonetheless, results of BstEII-IS9*00*-RFLP are acceptable as only one isolate lacked of a profile, demonstrating the relative high discriminatory ability of this method. Regarding stability of typing methods, although the study did not include specific methods to evaluate this aspect, concordance to previous results, as well as the previous report of one study in the United States (Harris et al., 2006), suggest adequate stability of the methods for further use. Typeability of IS*900*-RFLP was not 100%, which compared to the 100% typeability of the PCR-based methods targeted to repetitive sequences (MIRU-VNTR and MLSSR) represents a clear advantage of the latter over the former.

Reproducibility was analyzed within the study and between studies revealing surprising results, especially in IS*900*-RFLP (endonuclease *BstE* II) and MLSSR (locus SSR-2). In the first, the reference strain ATCC 19698 has been reported having more than one profile according to previous reports (Stevenson et al., 2002); (Borrmann et al., 2011). These authors reported the profiles C1 and C5 for this reference and type strain, respectively. This striking variability of a reference strain profile is difficult to explain and states interrogates regarding

the reproducibility of the method, subjective interpretation of results, and the purity of the strain. Epidemiological concordance of the methods was not systemically evaluated along this study, but there are previous evidence supporting adequate concordance with the epidemiologic picture of Germany, source of the majority of isolates used (Mobius et al., 2008b). This was also confirmed in following studies aiming to solve epidemiological questions.

Concerning convenience criteria, methods based on PCR (MIRU and VNTR) are clearly superior regarding rapidity, accessibility, ease of use, and cost. MLSSR method was also acceptable in the same aspects, but it is 100% dependent on the sequencing step, which demands time and reduces rapidity, accessibility, and ease of use, and increases cost. Finally, the IS*900*-RFLP is the slowest method to deliver results needing several days to report final profiles, its accessibility was high in the present study, but it was relatively complicated to use, and its cost was high. All methods were amenable for computerized analysis, with a clear advantage of sequence information obtained by MLSSR, which can be processed directly after electronic reception. One clear disadvantage of IS*900*-RFLP is the absence of amplification of DNA in frame of its method, which demands the availability of high quantities of bacteria not always possible with some MAP isolates and strains. Almost all of these methodological features of IS*900*-RFLP, MIRU-VNTR, and MLSSR have been reported previously by several authors (Motiwala et al., 2006; Collins, 2010).

Conclusion. From the methods used in the present study, the PCR-based methods (MIRU-VNTR and MLSSR) were superior compared to IS*900*-RFLP in terms of performance (stability, typeability, and discriminatory ability) and convenience (rapidity, accessibility, ease of use, cost, and amenability to computerized analysis). Therefore, MIRU-VNTR and MLSSR methods were found to be the primary option for further genotyping or subtyping of MAP along this dissertation. However, from the MIRU-VNTR loci tested, only loci MIRU-1, MIRU-2, and MIRU-4, as well as the locus VNTR-1658 (alias MIRU-3) were further applied based on the evaluation performed. Consequently, the MIRU-VNTR loci VNTR-292, X3 (alias VNTR-1658), VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32 proposed by (Thibault et al., 2007), and the locus VNTR-259 (Castellanos et al., 2010b) were also established and used in addition to MIRU-VNTR 1, 2, 1658 and 4 in other studies of this dissertation that included subtyping of MAP isolates.

4.2. Diagnosis, genotyping and epidemiology of MAP in dairy cattle in Colombia

In this study a combination of direct and indirect diagnostic methods was applied to detect MAP infection in cattle of dairy herds in Colombia. This is the first report of isolation and molecular characterization of Colombian MAP-strains from dairy herds. Despite the importance of the cattle production in Colombia, paratuberculosis has remained relatively uninvestigated, and very limited epidemiological information and data on molecular characterization of MAP were available. For about 60 years, some studies have tried to widen the clinical and epidemiological information of paratuberculosis in the country through research on diagnosis, treatment, epidemiology and molecular biology. Now a consistent study including a significant cattle population, using different diagnostic tests, and including the molecular characterization of the circulating causal agent is presented. As previously suggested in some studies, ELISA, PCR and culture were used to increase sensitivity of MAP detection, in order to confirm whether herds with or without history of Johne´s disease or MAP diagnosis were truly infected (Pinedo et al., 2008). However, it was expected to find a higher proportion of MAP–positive animals in all herds examined based on the observation of some inappropriate herd management practices known to be associated with an increment of the risk of paratuberculosis transmission.

Screening. The proportion of ELISA–A positive results obtained in the screening step (10.1%) falls in the range of the apparent animal level prevalences reported for cattle in the world. In the same way, the proportion of ELISA–A positive herds (70%) agrees with the apparent herd prevalences from other countries using both unabsorbed and absorbed ELISA tests. Results of the distribution of age according with ELISA–A positive results disagrees with the finding of a highest probability of testing positive with an ELISA test between 2.5 and 5.5 years in infected animals (Nielsen and Ersboll, 2006).

The ELISA–B results were surprising because a higher concordance to ELISA–A results was expected. Studies with similar results by ELISA–A and ELISA–B have shown that the difference in antigens (LAM vs. protoplasmic antigen) is responsible for the poor concordance between both tests. In addition, the pre–absorption step with *M. phlei* applied in ELISA–B reduces the number of false positives, but could also reduce the sensitivity to detect true positives making an unabsorbed test more sensitive but less specific (Kohler et al., 2008). In addition, the animals evaluated in the study, except from one herd, were animals from

herds without previous diagnosis of MAP and were asymptomatic for Johne´s disease. This subclinical status could be responsible for a diminished sensitivity and specificity of tests, compared to the sensitivity and specificity if they have been applied to affected or to infectious animals (Kohler et al., 2008; Nielsen and Toft, 2008).

On the other hand, it is possible that other mycobacteria could have played an important role in the positive results of unabsorbed ELISA–A, according to previous reports (Osterstock et al., 2007). In the present study atypical mycobacteria (AM) were isolated by fecal culture of feces of ELISA–A–positive herds. Therefore some of the ELISA–A positive results could be false positive, especially in those animals in which ELISA–B (absorbed test) and PCR produced negative results. Likewise it is possible that some of the positive ELISA results have been produced due to the interference with tuberculin from intradermal test applied occasionally to some animals, in order to declare herds as free from bovine tuberculosis (Varges et al., 2008).

PCR results confirmed the results of Zapata et al. (2010) who found 3 out of 15 positive fecal samples from the herd number 1 of the study by IS*900*–real–time–PCR. Nevertheless the results suggest that some asymptomatic animals of ELISA–A–positive herds were probably light shedders, which could not be easily detected by the PCRs used and probably by any other currently PCR system available, even in herds with previous history of paratuberculosis (Dieguez et al., 2009). In contrast, some studies have found a higher sensitivity by IS*900* PCR when this method is applied to fecal samples from clinically suspected cases of Johne´s disease (Soumya et al., 2009).

Low concordance between ELISA and PCR results seems to be explained by the fact, that different target regions with different characteristics have been chosen to develop the PCR systems used. The molecular target IS*900* has been implicated since more than a decade in producing false positive results in MAP diagnosis (Cousins et al., 1999). Moreover, the IS*Mav2* marker and the nested–PCR procedure have been implicated in producing false positive results and disturbance due to contamination compared to other markers and to single–round PCRs (Mobius et al., 2008a). However, the PCR systems used in the study are very reliable. F57 and IS*MaV2* real–time PCR has been strength tested for specificity and includes an internal amplification control, which makes the system very improbable to produced false negative results. IS*900*–nested–PCR and F57 and IS*MaV2* real–time PCR are

carried out using multiple reaction controls, which avoid the misinterpretations of results due to disturbed contamination (Schonenbrucher et al., 2008).

Based on the lack of information of which MAP strains could be found in Colombian fecal samples, three different culture media and two different decontamination procedures were used in order to increase sensitivity by meeting growth requirements of diverse MAP strains. However, no isolation of MAP was obtained even in herds with history of paratuberculosis. A possible explanation of this is that the sampling plan did not consider the collection of fecal samples of all adult cattle in every herd or a serial testing, which could have lead to some MAP–shedders not to be included in the sample, while non–infectious animals could have been randomly sampled. It is also possible that conservation of the serum and fecal samples at −20°C for several weeks or months could have affected the ELISA–A and the culture results, as previously reported (Khare et al., 2008; Alinovi et al., 2009).

On the other hand, the isolation of AM and the higher proportion of contaminated slants obtained with a NaOH – oxalic acid solution and cultivation on LJ agrees with previous studies (Glanemann et al., 2004; Nielsen et al., 2004). Characteristics of *M. engbaekii* on LJ slants inoculated with fecal samples decontaminated with 4% NaOH and 5% oxalic acid agree with the description of mycobacteria with pink–colored colonies isolated from cattle feces, as previously described (Korsak and Boisvert, 1972). This mycobacterium has been also isolated from water samples collected from the drinking troughs of buffaloes in Africa (Michel et al., 2007). Acid–fast Mycobacteria testing negative for MAP–PCR and sharing the distinct phenotypical characteristics of *M. engbaekii* were not further tested for similarity–based species identification and were assumed to be an isolate of these mycobacteria.

The low concordance between ELISA and fecal culture results has been also reported before (Muskens et al., 2003b; Glanemann et al., 2004; Dreier et al., 2006) and could be explained in the fact that ELISA negative or ELISA false positive results have a low probability of delivering a positive culture result if just a single sampling is planned as normally done in a cross–sectional study (Sweeney et al., 2006b).

Risk factors. Individual and management herd factors were associated to the individual serological response to paratuberculosis in dairy cattle for the first time in Colombia. Surprisingly, some factors expected to be associated with the serological response were non-

significant both in the univariable analysis (two-way frequency tables) and in the multivariable analysis (logistic regression) with the information collected for the present study.

The factor *treatment of symptomatic animals* from the group of factors regarding the presentation of disease in the herd has been reported as risk factor in previous studies (Obasanjo et al., 1997; Muskens et al., 2003a). This factor relates to the actions taken by the farmer when animals with symptoms compatible with paratuberculosis are detected in the herd. In the control of paratuberculosis, the restrictions to entrance of infected animals and the prompt culling of affected, infected (Nielsen and Toft, 2011), and infectious animals has a determining roll in the spread of the disease in the herd. In the study, this factor was not significant in the stepwise asymptotic logistic regression analysis, but its result in the univariable analysis revealed an association to the individual ELISA response that should not be ignored.

In the group of factors related to housing and hygiene, the *feed type of calves before weaning* was significantly associated to the individual ELISA response in the univariable analysis, but not in the multivariable analysis (stepwise asymptotic logistic regression). However, *p*-value was not very far from the minimum *p*-value established for significance, which could suggest a trend to association between the factor and the individual serological response. If the former would be true, animals from herds in which calves are feed with colostrums' mix from several cows before weaning would have an odds ratio of 0.42 of being ELISA positive compared to animals from herds in which calves are feed with colostrum from the own dam. MAP has been isolated from colostrum of subclinically infected cows (Streeter et al., 1995) and has been identified as a risk factor for MAP in herds in which calves are fed colostrum collected from known MAP infected cows (Dieguez et al., 2008), and from herds in which cows fed colostrum from multiple cows or sources compared to cows fed colostrum collected only from their own dams (Nielsen et al., 2008). Therefore, the results although non-significant, disagree with previous evidence mentioned and should be interpreted carefully. It is also possible that MAP contaminated teats (Pithua et al., 2011) increase the likelihood of becoming infected by suckling directly from an infected dam compared to feeding from a colostrum mix of MAP free cows. In the study the form how colostrum was exactly administered to calves was not asked, therefore this hypothesis cannot be confirmed. In Colombia, the practice of feeding calves with colostrum from different cows when the own

dam is unable to suckle her own calf or simply as a management practice to guarantee an equal colostrum offer to all calves in the farm is relatively common. The hygienic conditions in which this colostrum is offered to calves remain most times unattended. It is also a common practice to feed other animals species with colostrum or even to give it to human consumption, which represent an increased risk of perpetuation of disease in the herd and potential health risk to humans.

The factor *manure spread on pastures* was already identified as a factor associated with the MAP culture results in the univariable, but not in the multivariable logistic regression analysis in a previous study in the United States (Obasanjo et al., 1997). In the present study, this factor was the only factor significantly associated with the serological response in the univariable and in the multivariable analysis (stepwise asymptotic logistic regression and exact logistic regression). Animals from herds that spread manure on pastures had 3.40 the odds of being ELISA positive. In Colombia is a common practice of some dairy farms to spread slurry (cattle feces alone or in combination with pig feces) as manure for pasture. This type of mix has already been identified as a potential source of MAP for cattle due to its survival capacity for long periods of time (up to 252 days at 5°C) in cattle, in pig, and in cattle-pig combined slurry (Jorgensen, 1977), and due to its persistence during simulated composting, manure packing and liquid storage of dairy manure (Grewal et al., 2006).

Other factors like to *born in a foreign herd* (Wells and Wagner, 2000), *commingle with foreign cattle* (Fredriksen et al., 2004) and *high parity* were previously associated to the paratuberculosis status of herds. However, they were non-significant in relation to the individual ELISA response in the present study. In Colombian dairy production systems, the purchase and exchange of animals between herds is relatively common. Animals regularly purchased for replacement or for fattening are kept in the herd of origin until weaning and then transported to their final destination, which increases the risk of transmission to free herds due to introduction of young subclinical infected animals (Manning and Collins, 2010). In addition, it is still common to find several dairy farms sharing one single bull for reproduction by natural breeding running the risk of introducing MAP in an uninfected herd (Sweeney, 1996). Similarly, the factor parity was not associated to the paratuberculosis serological response, though animals up to 10 calving were included disagreeing with a previous study in which high parity (≥5, (Jakobsen et al., 2000) was associated to the probability of a positive ELISA test-result and with a previous study in which the probability

of a positive ELISA test-result was two to three times lower for cows in parity 1 relative to cows in other parities (Nielsen et al., 2002).

The first report of epidemiological factors associated to the paratuberculosis status in dairy cattle in Colombia is the main strength of this study of exploratory nature. The inclusion of all variables in the logistic regression analysis to avoid lost of valuable information were done to increase the validity of the information generated. However, the low number of cattle sampled, as well as the use of the individual animal as the unit of statistical analysis instead the herd assuming a contiguous population are the main weakness of the study. Another disadvantage is the use of the ELISA test result as outcome or dependent variable. ELISA is a very useful and economic tool to determine paratuberculosis infection status of animals, but it is considered that ELISA has a low sensitivity when use to detect antibodies in asymptomatic adult cattle (Nielsen and Toft, 2008).

Paratuberculosis infection of animals in this study, determined by an unabsorbed ELISA, could not be confirmed by using of an absorbed ELISA test and bacteriological culture, while only some of the positive unabsorbed ELISA results were confirmed by fecal PCR (see Study 2: Diagnosis of MAP from dairy cattle in Colombia, 2007). In the same way, only two herds included in the present study were found ELISA positive two years later (see 3.2.3 Determination of MAP infection- confirmation). These findings raise the question regarding the reliability of the data produced by a high number of studies reporting the determination of risk factors for paratuberculosis based only on ELISA tests, most of them lacking of sufficient sensitivity and concordance with other diagnostic results. For this reasons the paratuberculosis status definition of animals should also be based in more than one single test and serial testing (Stevenson, 2010b) to avoid overestimations of paratuberculosis prevalences and to appropriately identify influence factors.

Confirmation. Concerning the results of the tests used for confirmation of MAP detection two years later, the lower proportion of the current ELISA-C positive results (1.8%) compared to the previous ELISA-A positive results (10.1%) in four of the five herds examined was surprising at first sight, but it is explained by the characteristics of the ELISA tests used in both studies. ELISA-C is an absorbed test using purified MAP extract, IgG-conjugate and pre-incubation with *M. phlei*, which are characteristics that have been considered of critical influence on the increment of specificity for the serological diagnosis of

paratuberculosis (Yokomizo et al., 1985; Jark et al., 1997; Sugden et al., 1997; Kalis et al., 2002). Therefore, the use of an absorbed test (ELISA-C) has produced negative or a lower proportion of positive results in herds with previous MAP diagnosis (ELISA-A and PCR), or even with previous history of clinical cases of paratuberculosis (e.g. herd 1), compared to the screening study of 2007. In screening study, herds of a dairy region mostly without previous diagnosis of paratuberculosis were tested using an unabsorbed test that used LAM as MAP antigen (ELISA-A), which produced a higher proportion of seropositives confirmed only in two animals by an absorbed ELISA (ELISA-B). Interestingly, the results of both absorbed tests, ELISA-B in 2007 and ELISA-C in 2009, produced closer results (5.1% vs. 1.8%) than those obtained with the unabsorbed ELISA-A (10.1%) in 2007. This suggests that the characteristics of the tests used were determinant in the different proportions of seropositives obtained in both studies.

Furthermore, the absence of reliable preliminary epidemiological information on the disease makes also plausible that dairy herds in the region of study were of a very low prevalence or even negative for MAP, or at least undetectable with the current diagnostic tests, if only cross-sectional studies instead of a longitudinal study or serial testing is carried out. In any case, these studies are the first step of the systematic epidemiological study of paratuberculosis in Colombia, and therefore further studies have to be conducted to elucidate the situation of the disease in the country.

The case of herd number 2, in which clinical paratuberculosis has never been reported, but some animals were positive by ELISA-A and PCR in 2007, and again in 2009 (four positive ELISA-C, but negative results by culture) is striking and difficult to explain. In this case, it could be possible that other mycobacteria could influence the positive results of the unabsorbed ELISA-A in 2007 and of the ELISA-C positive in 2009, making the proportion of positive animals higher than it really is (Osterstock et al., 2007). In the study of 2007, AM (*M. engbaekii*) were isolated. However, the lack of testing of the half of the adult cattle population of this herd for ELISA-C, limits any definitive conclusion about the current negative PCR and culture results obtained from seropositive animals, taking into account the high quality of the tests employed. Additionally, the four fecal samples from the positive ELISA-C animals were cultured individually, which could have been reduced the chances of a probable concentration of low quantities of MAP, if these animals were really shedders. Likewise, it is possible that the positive ELISA results, not only in herd 2, but all herds of both studies have been

produced due to the interference with tuberculin from intradermal tests (caudal fold tuberculin test, (Varges et al., 2008) applied occasionally in order to be certified as free herd from bovine tuberculosis in frame of the national program for eradication of tuberculosis. In conclusion, some of these results of ELISA-A and even ELISA-C could have been simply false positives in 2007 and in 2009, respectively.

The low apparent prevalence and the true prevalence obtained (1.8 vs. 2.2%) was probably related to the high specificity of test used (99%), as previously reported for studies with these characteristics (Martin, 1984). Although the study was biased for prevalence determination, due to analysis of herds with previous history or diagnosis of paratuberculosis, animal level apparent prevalence calculated appeared to be lower compared to prevalences obtained in European countries (Nielsen and Toft, 2009). However, no similar studies were found aiming the determination of prevalence of MAP infection by using the ELISA-C, which made impossible a better comparison of results.

In this study only 0.8% of ELISA-C-positive cows were detected in the group of 3-5 years and the only symptomatic animal found was a 6 year old cow. This results slightly disagree with a report of a higher probability of testing positive by ELISA between 2.5 and 5.5 years in infected animals (Nielsen and Ersboll, 2006) and with the knowledge that most clinical cases of paratuberculosis occur between 3-5 years (Chiodini et al., 1984). Cattle in Colombia tend to be kept longer in production and to be culled latter compared to North American or European countries. In this manner, cows can live long enough to be tested and detected by ELISA or fecal culture, or to show symptoms of paratuberculosis out of the age limits reported for other countries.

As reported before, culture of pooled fecal samples of 8-12 animals per pool permitted the examination of a high number of fecal samples by culture at low cost and with acceptable sensitivity (Kalis et al., 2000; Tavornpanich et al., 2004; van Schaik et al., 2007). The option of pooling 5 fecal samples instead of 10 was not considered due to economic reasons. Furthermore, precise information about MAP within-herd prevalence was absence to take a better decision of the best pool size according to a previous modeling study (van Schaik et al., 2003). This option was also discarded because of the reported insignificant difference in sensitivity between pooling 5 or pooling 10 cows in a previous study in a comparable South American cattle production system, in which it was concluded that the sensitivity of the pool

is related more to the prevalence of the herd and to the infection status of the cows as with the size of the pool (van Schaik et al., 2007). In the same way, a study in the United States reported acceptable sensitivity with 10 samples per pool (35%), compared to pooled samples of 5 animals (44%), leading to the conclusion that in herds with at least one high fecal shedder, pools of more than 5 samples might also detect MAP (Wells et al., 2002). Although, some studies have concluded a better sensitivity of pooling five animals instead of 10 or more, these studies have been based on the detection of MAP using radiometric fecal cultures to reliably detect low-shedders, which could be not comparable to classical bacteriological methods (Eamens et al., 2007). Other studies refer more to a theoretical calculation than to a sensitivity estimation, difficult to extrapolate to South American field conditions (van Schaik et al., 2003); or reported the use of 5 samples per pool focusing on the determination of the sensitivity of culture of pooled fecal samples compared with culture of individual fecal samples, with special attention to the number of pooled fecal samples per herd, rather than to the number of animals to be included in the pooled fecal samples (Wells et al., 2002).

The detection of a positive pooled slurry sample by culture from a positive ELISA-C herd (herd 1) agrees with the knowledge of the correlation of this finding with seropositive results, and with the higher probability of isolation from lagoon samples compared to other environmental samples (Berghaus et al., 2006). The result of the single slurry samples producing negative results by individual culture and PCR has been reported and has been attributed to uneven distribution of MAP in the fecal sample (van Schaik et al., 2007), to the lack of homogeneity in the fecal sample or to different sensitivities of individual fecal culture procedure between laboratories (Tavornpanich et al., 2004), to the presence of MAP in the feces of at least one animal within the pooled fecal sample, although this animal was not detected by bacteriological culture of individual fecal samples (Wells et al., 2003), or to unclear reasons (Wells et al., 2002). In any case, Kalis *et al.* (2000) properly concluded that there is an element of chance apart from the element of dilution related to the detection of MAP in feces, particularly when samples contain low numbers of the organism and the bacteria are not uniformly distributed in the fecal samples. Thus, although a complete homogenization of the pooled slurry sample was achieved and the PCR systems used are very reliable, it is possible that the 3g or 1.5g of slurry samples taken to test by individual culture and by PCR, respectively, lacked of enough MAP cells to be detected by bacteriological culture in a 20 weeks period of incubation, and in two PCR systems (F57-IS*Mav2* real-time and conventional IS*900* PCR) carried out in duplicate. The PCR systems used, specially the

real-time PCR, are strict tested for specificity. It also included an IAC and use multiple reaction controls, which avoid the misinterpretations of results due to disturbed contamination or very improbable false positive results (Schonenbrucher et al., 2008).

Close analysis of the individual results obtained in herd 1 revealed that one single symptomatic animal producing positive results by ELISA-C and PCR, confirmed that regardless of the ELISA or PCR type used sensitivity is higher for detection of symptomatic animals and fecal high shedders (Bogli-Stuber et al., 2005; Kohler et al., 2008). The results of three asymptomatic ELISA-C-negative cows that produced positive results by culture could be: two cases (MAP low-shedders and PCR-negative) of the known "passing through" phenomenon previously described (Sweeney et al., 1992), and one case (MAP high-shedder and PCR-positive) of a positive animal with undetectable antibodies. On the other hand, the results of one ELISA-C positive-animal of herd 1, that produced negative results by culture, does not necessarily mean that the animal was not really infected, but that the shedding phase has probably not yet started (infected animal in a non-infectious phase) or was absent at the moment of fecal sampling (intermittency). Another possibility is that in this animal MAP-antibodies have been detected prior to the start of bacterial shedding, which could begin later and could be then detected by PCR or culture (Nielsen, 2008). MAP is shed in feces of infected animals at all stages but at different levels and sporadically, which demands repeated testing to detect animals shedding very low number of MAP, which could anyway go undetected (Stevenson, 2010b).

One ELISA C-negative animal was positive by PCR, real-time PCR and culture. On the contrary, one ELISA C-positive animal in the same herd showed negative results by PCR, real-time PCR and culture. In herd 2 four ELISA C-positive animals produced negative results by fecal PCR and fecal real-time PCR, as well as negative results by individual fecal culture. Muskens *et al.* (2003b) found a low percentage of ELISA-positive cattle testing fecal culture-positive for all age groups included. Among their arguments they stated a possible limited sensitivity of the fecal culture and/or false-positive ELISA test results and a non-homogeneous distribution of MAP in feces especially for low shedders. In general, explanations for the poor concordance of diagnostic tests could be attributed to false-positive ELISA results, to non-homogeneous distribution of MAP in feces (especially for low shedders), to relatively low prevalence of MAP infection, and very low positive predictive value of ELISAs applied. In addition, it has to be taken into account that not only the

combination of different tests, but repeated sampling is necessary to achieve the identification of individual animals (Stevenson, 2010b).

Although many animals sampled in a previous study (2007) were no longer in the herds at the time of the second sampling (2009), it was an interesting finding to compare the diagnostic results of animals sampled in 2007 with those results obtained from the same animals in 2009, simulating a longitudinal study or repeating testing for these animals. Changes in diagnostic test results between 2007 and 2009 agree with studies that report fluctuations of serum ELISA, PCR and culture results over time (Hirst et al., 2002; Sweeney et al., 2006b; Geisbauer et al., 2007). Many test factors (sensitivity, specificity, within-herd prevalence of herd) in every diagnostic procedure influence the variability of results, when the same animals are tested more than once over time. Particularly for ELISA, fluctuations in test results have been attributable to false-positive results on the first or on the second test, fluctuation in antibody production by the cow, application of tests to low prevalence herds, in which the positive predictive value of tests is lower, or to analytic error. Analytic error occurs when samples were not tested in duplicate as suggested by manufacturers, and repeat analysis give negative results (Sweeney et al., 2006b). Nevertheless, multiple testing over time increases the chance of detection of an infected animal, this would also increase the chances of a false-positive result (Sweeney et al., 2006b). Therefore ELISA results have to be analyzed carefully when this test is applied for individual animal diagnosis (McKenna et al., 2006; Kohler et al., 2008). However this is not an uniform process because as it has been reported, cows with negative results are less likely to change ELISA status than cows with positive results, regardless of within-herd prevalence (Hirst et al., 2002).

Phenotypic characteristics of fast growth, mycobactin dependency and no pigmentation of Colombian MAP isolates coincide with the description of type II (or cattle type) strains described in previous studies (Stevenson et al., 2002). The combination of MIRU-VNTR and MLSSR, as done previously (Thibault et al., 2008) made possible the reliable differentiation for the first time of two MAP genotypes among eight different MAP isolates of one herd in Colombia. These methods were applied combined to increase the minimum discriminatory ability needed and not reached if one single method had been used, as reported before (Stevenson et al., 2009). According to MLSSR, the types isolated in the study are commonly found in cattle and other species in different countries (Ghadiali et al., 2004; Corn et al., 2005; Thibault et al., 2008; El Sayed et al., 2009). Interestingly, a bovine isolate from Colombia's

neighbor country Venezuela has shown a different genotype (11g-10g-5ggt-5ggt), suggesting strain diversity in the northern part of the subcontinent (Thibault et al., 2008).

Although comparison with other studies is very difficult because of the use of different loci for analysis, genotype 1 (INMV1) and genotype 2 (INMV 2) were previously reported as the most common genotypes found in isolates from Argentina and Venezuela (Thibault et al., 2007), and in European isolates (Stevenson et al., 2009; Douarre et al., 2011). Cases of double strain infection has been also reported at herd level in United States (Harris et al., 2006), Germany (Mobius et al., 2008b) and the Netherlands (van Hulzen et al., 2010), while cases of double strain infection at animal level have been reported in Germany (El Sayed et al., 2009).

The finding of two strain types among eight isolates recovered from herd 1, including isolates from four cows all born in the herd, but unrelated each other, and isolates of slurry samples of the slurry pit collecting liquid manure and wastewater from the herd's milking parlor, suggests the circulation of MAP from and to the environment, and among different animals in the herd. In the same way, the isolation of two different types in one single animal, types that were also isolated in slurry samples, supports the idea of a highly MAP contaminated environment, which leads to the infection with more than one different strain genotype in the herd. In herd 1, animal feces are used as fertilizer on the pastures and no paratuberculosis control program is carried out. It has been presenting sporadic cases of animals with symptoms of paratuberculosis confirmed by histopathology (unpublished data) and MAP has been detected by PCR and serology (Zapata R. et al., 2010). Shedding cows were relatively old cows (≥6 years) at the time of sampling suggesting that these animals have been contaminating the environment with MAP until they are removed from the herd, contributing to the perpetuation of MAP and the presentation of new infections, if no control program is established.

Regarding technical considerations of the genotyping methods, MLSSR could be less accessible and more expensive than MIRU-VNTR due to the sequencing step required (Thibault et al., 2008). This aspect could represent a limitation in some developing countries (e.g. Colombia) in which sometimes sequencing has to be carried out abroad incrementing even more the costs of application of MLSSR method. However, MLSSR analysis is an excellent MAP molecular characterization method in terms of *in vitro* stability and

discriminatory index (Harris et al., 2006), which could justify the cost of the sequencing step needed.

Conclusion. The results confirmed the presence of MAP in dairy herds in Colombia, and the limitations of serum ELISA, fecal PCR and fecal culture for the detection of this microorganism in asymptomatic dairy cattle from herds with and without history of Johne's disease and the usefulness of pooled fecal samples and environmental sampling to screen herds for MAP. The results also confirmed the circulation and transmission of at least 2 different MAP genotypes between individuals of the infected herd. On the other hand, the factor *Manure spread on pastures* appeared to be a herd practice influencing MAP infection in dairy cattle from the dairy herds analyzed. Further epidemiological studies including more dairy animals and herds are necessary to increase epidemiological knowledge of paratuberculosis in Colombia.

4.3. Genotypes of MAP from South American countries

Paratuberculosis has been reported unequally in the countries of origin of isolates analyzed. The predominance of one genotype in the South American isolates of MAP could be the result of higher infection ability (Gollnick et al., 2007) or better culturability of this genotype on the media used for isolation (Cernicchiaro et al., 2008). In Europe the MIRU–VNTR genotype 1 (INMV1) and in the United States the MLSSR genotype A are commonly detected (Harris et al., 2006; Stevenson et al., 2009). This could suggest that this predominant MAP genotype was introduced from Europe or North America during the colonization or more recently through cattle importation. However, one previous study found differences in the predominant MAP genotypes between Argentina and Europe, based on the results of the typing method IS*900*–RFLP (Moreira et al., 1999).

MAP genetic adaptations to hosts in local wildlife and tropical or subtropical environments has been only recently addressed (Singh et al., 2010) and could be an additional source of genomic variation also in South America. The detection of MAP in more than one host species of the same property has been associated to interspecies transmission of MAP in herds of South America and Europe (Moreira et al., 1999; Stevenson et al., 2009). However, it was recently reported that hares in Chile appear to be potential mechanical vectors but no reservoirs for MAP in dairy herds (Salgado et al., 2011b).

The main strength of present study is the comparative analysis of MAP isolates from different hosts and different countries of South America using methods of high discriminatory ability. The main weakness of the study is the relatively reduced number and the restricted areas of origin of some of the isolates analyzed. Nevertheless, taking into account the size of livestock populations of some countries included and that MAP isolation is not an easy task under Latin-American conditions, the information presented in this study is valuable to contribute to the knowledge of global epidemiology of MAP. It is necessary to conduct further studies including more local MAP isolates, actually more isolates from South American countries were asked, but no answers or negative answers due to different reasons from colleges in Brazil, Argentina, Venezuela, Peru, and Ecuador were received. In general, collaborative studies between South American countries, or between countries of the north and the south regarding global animal pathogens are relative infrequent. This is probably due to the different epidemiological profiles and disease interests.

Further studies on MAP molecular epidemiology, based on highly discriminatory methods (e.g. MIRU–VNTR and MLSSR) should be carried out to increase the knowledge of global epidemiology of MAP. In these studies, the influence of culture media, the role played by the local wild life, the diversity of agro–ecosystems, and the cross–breeding of imported and indigenous animals must be taken into account in the analysis as possible sources of genomic diversity of MAP.

4.4. Molecular characterization of MAP in Rhineland–Palatinate, Germany

In this study MIRU–VNTR and MLSSR were applied, evaluated and used for epidemiological analysis of MAP in RP, Germany. Genotyping of MAP at a regional scale in areas in which control actions or programs are being applied routinely or experimentally is relatively rare. In Germany, one study genotyped MAP isolates from herds of Lower Saxony and North Rhine–Westphalia, in which voluntary control programs against paratuberculosis are currently run (Schulze, 2009). These studies are useful to increase the knowledge about MAP epidemiology in order to rationally evaluate, improve, or redirect undertaken control measurements or programs.

Previous European studies have reported low or no allelic diversity in loci MIRU 1, VNTR 3 and VNTR–259 of MAP (Stevenson et al., 2009; Castellanos et al., 2010b; Douarre et al., 2011; van Hulzen et al., 2011). Nonetheless, MIRU 1 was analyzed due to previous reports of allelic diversity in this locus in one German and in one Dutch isolate (Mobius et al., 2008b; van Hulzen et al., 2011). The results of locus VNTR–259 suggest that MAP isolates of the present study (not tested genetically for MAP type differentiation) are all of the MAP type II according to the results of (Castellanos et al., 2010b). In this study, MAP type II strains always produced two repeats in VNTR–259, while MAP type III strains always produced one repeat. Nonetheless, VNTR–259 is not a specific biomarker for MAP type identification and their results only imply that these strains could be of the Type II. On the other hand, MIRU–VNTR genotypes 1 (INMV1) and 2 (INMV 2) were the most common MIRU–VNTR in previous European studies (Stevenson et al., 2009; Douarre et al., 2011). The remaining MIRU–VNTR (and INMV) genotypes are either reported for the first time in this study or have been detected previously in isolates from cattle, rabbit, goat and human in different European countries.

MLSSR genotypes A, B, and C have been detected in isolates from different European countries including Germany (Thibault et al., 2008; El Sayed et al., 2009; Douarre et al., 2011). Interpreting the results and assigning alleles from locus 2 was especially difficult due to the described strand slippage during PCR and some troubles during sequencing. The suggestions of researchers to circumvent this limitation were strategically applied. Nonetheless, it cannot be fully ruled out that some isolates could have been unintentionally misclassified due to these technical limitations of PCR and sequencing.

The low global congruence obtained between MIRU–VNTR and MLSSR (Adjusted Rand´s coefficient = 0.054) was expected, due to the use of only two loci (SSR–loci 2 and 8) for MLSSR. This produced a mutual refinement of the groups formed by each method and vice versa. If more SSR–loci had been included, the refinement of MIRU–VNTR by MLSSR should have been higher as reported previously (Douarre et al., 2011). Results of the Wallace coefficient (W) for MIRU–VNTR → MLSSR and for MLSSR → MIRU–VNTR revealed low predictability between MIRU–VNTR and MLSSR in the form used in the present study, and *vice versa*. The result of the expected W value under independence (Wi) confirmed the need to combine them when used in the form of this study, or that the use of the two methods was not redundant to obtain acceptable results in terms of discriminatory ability. MLSSR was slightly more discriminatory in the study than MIRU–VNTR. However, the hypothesis that both methods as used in this study have similar discriminatory powers at a 95% confidence level cannot be excluded due to the overlapping CI 95% of *D*.

The analyzed clinical cases of Johne´s disease in dairy herds in RP are caused by several MAP genotypes with the dominance of genotypes 1–B and 1–D. The dominance of specific MAP genotypes could be the cause of specific characteristics that make these strains to transmit more efficiently between animals or that cause disease in animals in contrast to other genotypes. It is striking that isolates showing 4ggt or 5 ggt repeats at locus 8, as those found in the present study, were able to survive more efficiently in macrophages than MAP strains with a lower number of ggt–repeats (Gollnick et al., 2007).

Another possibility is that MAP strains with genotypes 1–B and 1–D could be of better culturability on HEYM routinely used for MAP primary isolation in RP and for subcultivation or propagation in the present study. It is striking that 13 MAP strains originally isolated on HEYM and lyophilized in RP could not be recovered on HEYM in the laboratory and could not be included in this study. HEYM can influence the selection of a less genetically diverse subtype population of MAP (Cernicchiaro et al., 2008). Nonetheless, a recent study found no correlation between genotype and overall culturability of MAP after initial isolation (Whittington et al., 2011), which suggests that the relation MAP subtype and culturability requires further study.

The highly prevalent strains of MAP could be candidate MAP types for locally based vaccine studies in cattle populations in RP. Previous results support the higher efficacy of vaccination with a field strain of MAP (Uzonna et al., 2003; Singh et al., 2007). Few isolates analyzed from the neighboring federal states of North Rhine–Westphalia and Luxembourg delivered genotypes widely spread in RP. It has been reported that MAP in North Rhine–Westphalia posses a relative degree of genetic heterogeneity based on results of 11 MAP isolates analyzed using IS*900*–RFLP and MPIL in a previous study (Schulze, 2009). The rare combined genotype 5–A of one single isolate from Hesse correspond to a genotype detected in at least two isolates in a previous study that analyzed 34 isolates of this federal state using MLSSR and MIRU–VNTR (El Sayed et al., 2009).

Conclusion. MAP isolated from symptomatic dairy cattle in RP is genetically diverse with dominant genotypes causing Johne´s disease. The dominant genotypes of this study are similar to those previously determined in other parts of Europe. Regional control programs in RP should consider both the high genetic diversity and the dominance of some genotypes to strategically improve control interventions. MIRU–VNTR and MLSSR are useful methods for epidemiological studies at regional scale.

5. GENERAL CONCLUSION

Based on the main results of the present dissertation the following general conclusions can be formulated:

- All diagnostic and genotyping methods for MAP used in this work have advantages and disadvantages regarding accuracy, concordance with other methods, availability, cost, sensitivity, specificity and discriminability. Therefore the combination of test and methods is the best option to increase performance according to the aim of the study and the circumstances of diagnostic or subtyping.
- Diagnostic tests for paratuberculosis were limited to accurate detect MAP in subclinical animals. In general, the correlation between diagnostic test results was low. Testing all animals with more than one diagnostic test and the strategic use of environmental and pooled sampling increased the probability of MAP detection or confirmation in dairy cattle.
- Genotyping methods of MAP evaluated and applied in this work were useful to answer the epidemiology questions formulated. Although they demanded reasonable efforts to the establishment and application.
- In the studies carried out in this dissertation contributions to the knowledge of paratuberculosis in Colombia and Germany were done. In Colombia, the situation of paratuberculosis appears to be of a lower proportion of MAP-positives as initially expected and is in the range determined of previous studies. In addition, two different subtypes and one risk factor for paratuberculosis were determined for the first time in this country. This information will be useful as a baseline for future studies on paratuberculosis in dairy cattle in Colombia. In Germany, analysis of a representative number of isolates obtained from dairy cattle in a region contributed to the increase the knowledge of the molecular epidemiology picture of MAP in the country and will help to improve future interventions to control this disease.
- Exploration of molecular epidemiology of MAP in South American by comparing genotypes obtained in different countries revealed similarities. In addition, these results revealed very low number of MAP isolates of South America obtained using genotyping methods fulfilling performance and convenience criteria for accurate MAP strain differentiation. More isolates should be analyzed to be able to draw better conclusions on distribution of MAP genotypes between continents and subcontinents.

SUMMARY

In the present dissertation four studies related to the diagnosis, genotyping, and epidemiology of MAP in dairy cattle were carried out.

In the **first study**, IS*900*-RFLP, MIRU-VNTR, and MLSSR genotyping methods were evaluated for further application. From these methods, MIRU-VNTR and MLSSR were superior compared to RFLP-IS*900* in terms of performance and convenience and were chosen as the primary option for further genotyping of MAP along this dissertation. However, some MIRU-VNTR loci showed no discriminability in MAP strains and were discarded and replaced with the MIRU-VNTR loci 1658, 292, 25, 47, 3, 7, 10, 32, and 259.

In the **second study**, serum and fecal samples from asymptomatic cows (n=307) of 14 dairy herds from Colombia were screened for MAP by serum ELISA, fecal PCR and fecal culture. Fecal samples from animals of herds positive by ELISA and PCR (n=105) were inoculated onto 3 different culture media. ELISA–A produced positive results in 10.1% of the serum samples and 71% of the herds. ELISA–B and PCR results were positive in 2 and 6 serum and fecal samples from positive ELISA–A–animals, respectively. Fecal samples were negative for MAP on all culture media. Individual ELISA-A test results and collected information on individual animal features and management herd practices were analyzed for risk factors determination. The herd management factors *Measures taken in the past with symptomatic animals*, *Feed type of calves before weaning*, and *Manure spread on pastures* were significantly associated with the individual serum ELISA response on the univariate analysis. In the logistic regression, only the factor *Manure spread on pastures* was significantly associated with the individual serum ELISA response. In the second part of this study, serum and feces from animals of five suspicions dairy herds of the first part (i. e. simultaneously positive by ELISA and PCR) were tested by a different ELISA, pooled fecal culture, and PCR. In one herd, slurry and tissue samples from one animal were also taken and tested by PCR and culture. MAP isolates were genotyped by analysis of the MLSSR and the MIRU-VNTR methods. ELISA produced positive results in 1.8% (6/329) of the animals and 40% (2/5) of the herds. Four fecal, two tissue, and two slurry samples from a herd were positive for MAP by culture and PCR. Genotyping revealed two different strain profiles among the eight MAP isolates recovered.

In the **third study,** MIRU–VNTR and MLSSR genotypes of MAP isolated from different hosts in Chile, Colombia, Argentina, and Venezuela were compared. So far, seven different MAP genotypes were produced by MIRU–VNTR and MLSSR in South American isolates,

respectively. The combination of both methods produced 9 genotypes. Results revealed a predominant combined MIRU–VNTR and MLSSR profile, little differences in MAP genotypes among countries, and a similar MAP-genotype in livestock and in wild animals in one country.

In the **forth study**, 91 MAP isolates from 71 dairy herds of Rhineland-Palatinate were genotyped by MIRU–VNTR and MLSSR. The combined analysis of both methods produced 25 genotypes with an index of discrimination (*D*) of 0.93 and the dominance of 2 genotypes. The results revealed the usefulness of genotyping methods in studies at regional scale, the high genetic diversity of MAP from cattle in Rhineland–Palatinate, and provided additional information for control programs currently carried out in the region.

The main conclusion of the dissertation is that tools for diagnosis and genotyping of MAP were very useful to increase the knowledge of paratuberculosis in Colombia and Germany. In addition, all methods used in the present dissertation can be considered more or less imperfect and required strategic use and combination with other methods to increase accuracy.

ZUSAMMENFASSUNG

In der vorliegenden Dissertation werden vier Studien zur Diagnose, Genotypisierung und Epidemiologie von MAP in Milchkühen vorgestellt.

Die **erste Studie** diente der Evaluierung der Genotypisierungsverfahren IS*900*-RFLP, MIRU-VNTR und MLSSR. MIRU-VNTR und MLSSR zeigten hierbei deutliche Vorteile gegenüber der IS*900*-RFLP bezüglich Durchführbarkeit und Anwendungsfreundlichkeit auf und wurden somit für die nachfolgenden Genotypisierungsarbeiten der Dissertation gewählt. Einige MIRU-VNTR Loci erwiesen sich allerdings als problematisch in der Diskriminierung von MAP-Stämmen. Diese wurde daher verworfen und in späteren Versuchsreihen durch die MIRU-VNTR Loci 1658, 292, 25, 47, 3, 7, 10, 32 und 259 ersetzt.

Im ersten Teil der **zweiten Studie** wurden in Kolumbien gewonnene Serum- und Kotproben klinisch gesunder Kühe (n = 307) aus 14 Milchherden mittels Serum-ELISA-A, Kot-PCR und Kot-Kultur auf MAP gescreent. Zur Erhöhung der Treffsicherheit erfolgte die Kultivierung bei den Tieren aus ELISA-A- und PCR-positiven Betrieben (n = 105) mit 3 unterschiedlichen Nährmedien. Mit dem eingesetzten ELISA-A erwiesen sich 10.1 % der Einzeltiere und 71 % der Herden als positiv. Zwei Tiere, die mittels ELISA-A als positiv befundet wurden, erwiesen sich ebenfalls mit dem ELISA-B als positiv, 6 Tiere konnten mittels PCR als positiv befundet werden. Die Anzüchtung des Erregers gelang zu keinem Zeitpunkt. Risikofaktoren anhand von im ersten Teil der Studie 2 erhobenen ELISA-A Ergebnissen sowie Informationen über Einzeltiermerkmale und Herdenmanagement-Praktiken wurden analysiert. Die Herdenmanagementfaktoren *ergriffene Maßnahmen bei klinischen Paratuberkulose-Fälle in Vergangenheit, Fütterung der Kälber vor der Entwöhnung* und die *Ausbringung der Gülle auf Weiden* korrelierten in der univariaten Analyse signifikant mit den erhobenen ELISA-Einzeltierergebnissen. Im Logit-Modell erwies sich hingegen nur der Faktor *Ausbringung der Gülle* als signifikanter Risikofaktor. In dem zweiten Teil der zweiten Studie wurden Kot- und Serumproben von 5 Betrieben, die sich in der Screening-Studie als Paratuberkulose-verdächtig erwiesen hatten, einbezogen. Zum Einsatz kamen ELISA-C, Kot-Kultur mit gepooltem Probenmaterial und PCR. In einer Herde wurden zusätzlich Umgebungsproben sowie Gewebe einer Kuh gewonnen und mittels PCR und Kultur untersucht. Die gewonnen MAP-Isolate wurden unter Einsatz von MLSSR und MIRU-VNTR genotypisiert. 1,8 % (6/329) der untersuchten Tiere sowie 40 % (2/5) der untersuchen Herden erwiesen sich im ELISA-C als positiv. In einer der Herden wurden 4 Kotproben, 2 Gewebeproben und 2 Umgebungsproben sowohl in der Kultur als auch mit dem PCR-Verfahren als positiv

befundet. Insgesamt konnten 8 Isolate gewonnen werden, die 2 verschiedene Stammprofile aufwiesen.

In der **dritten Studie** wurden MAP-Genotypen verschiedener Wirte aus Chile, Kolumbien, Argentinien und Venezuela mittels MIRU-VNTR und MLSSR verglichen. Mit dem MIRU-VNTR und MLSSR-Verfahren wurden bisher 7 Genotypen ermittelt. Wurden die beiden Methoden miteinander kombiniert, wurden 9 verschiedene Genotypen ermittelt. Die erhobenen Ergebnisse zeigten ein dominantes kombinierte MIRU–VNTR und MLSSR Profil, kleine Unterschiede zwischen den MAP-Genotypen der verschiedenen Länder und genotypische Ähnlichkeiten zwischen MAP-Genotypen von Haus- und Wildtieren auf.

In der **vierten Studie** wurden 91 MAP-Isolate aus 71 Milchviehherden aus Rheinland–Pfalz mittels MIRU-VNTR und MLSSR genotypisiert. In Kombination dieser beiden eingesetzten Verfahren konnten 25 unterschiedliche Genotypen mit einem Diskriminierungsindex (*D*) von 0,93 sowie eine Prädominanz zweier Genotypen ermittelt werden. Die Ergebnisse zeigen, dass der Einsatz von Genotypisierungsverfahren in regional begrenzten Studien zu empfehlen ist. Die in Rheinland–Pfalz isolierten MAP-Stämme wiesen in den eigenen Untersuchungen eine hohe genetische Vielfalt auf, was für regionale Paratuberkulose-Kontrollprogramme durchaus von Bedeutung ist.

Die Dissertation zeigt, dass Methoden zur Diagnose und Genotypisierung sehr hilfreich für das Verständnis der Paratuberkulose in Kolumbien und Deutschland sind. Es kann geschlussfolgert werden, dass keine der in der Dissertation geprüften Methoden im für den alleinigen Einsatz als optimal anzusehen ist. Die Wahl der jeweiligen Methode sollte daher strategisch in Abhängigkeit der Fragestellung erfolgen; zur Erhöhung der Treffsicherheit wird der kombinierte Einsatz mehrerer Methoden empfohlen.

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DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Jorge A. Fernández-Silva

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ANNEXES

Annex 1

Questionnaire for the determination of individual risk factors for paratuberculosis in dairy herds in Colombia

Date:_____
Name of herd: _____
District: _____

Number of animals sampled:
Whole herd cattle population:

	Identification number	Name	Date of birth or age (in years)	Born in farm? Yes / No	Parity	Daily average milk production (liters)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						

Remarks: _____
_____

Annex 2

Questionnaire for the determination of herd management risk factors for paratuberculosis in dairy herds in Colombia	
General information of herd	
Questionnaire number (consecutive)	
Date	
Name of herd	
Name of owner	
Telephone of owner	
District	
Area of herd (in hectares)	
Access to veterinary assistance?	Yes No
Herd daily average milk production (liters)	
Cattle population Whole herd cattle population	Calves Heifers Milking cows / Dry cows Bulls
Information about the presentation of disease in the herd	
Are there currently symptomatic animals compatible with paratuberculosis in herd?	Yes No
There have been symptomatic animals compatible with paratuberculosis in herd in the last 2 years?	Yes No
What has been done (treatment) with these symptomatic animals?	Not in herd Still in herd Other measure? _____ No symptomatic animals
Factors affecting transmission between herds	
Do you purchase cattle for the herd?	Yes No
Do own animals graze on foreign pastures?	Yes No
Do foreign animals graze on own pastures?	Yes No
Factors related to housing and hygiene	
Is there an specific calving place?	Yes No
How is the housing type of calves before weaning?	Stall Pasture Other housing type? _____
How is the feed type of calves before weaning?	Colostrums own dam Colostrums' mix several cows
Do you spread manure on pastures as fertilizer?	Yes No
Have you seen birds in feedstuff storeroom?	Yes No

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