Faculteit Farmaceutische Wetenschappen
Laboratorium voor Farmaceutische Microbiologie

Instituut voor Tropische Geneeskunde
Departement Diergeneeskunde

Johne's disease in zoo animals: development of molecular tools for the detection and characterisation of *Mycobacterium avium* subspecies *paratuberculosis*

De ziekte van Johne in zoo-dieren: ontwikkeling van moleculaire methoden voor de detectie en karakterisatie van *Mycobacterium avium* subspecies *paratuberculosis*

Elke Vansnick
Lic. Biomedische Wetenschappen

Thesis submitted in fulfilment of the requirements for the degree of Doctor (Ph.D.) in Pharmaceutical Sciences

Proefschrift voorgelegd tot het verkrijgen van de graad van Doctor in de Farmaceutische Wetenschappen

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Decaan: Prof. Dr. J-P. Remon **Promotor: Prof. Dr. H. J. Nelis**

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Gent, mei 2004

De auteur	De promotor
E. Vansnick	Prof. Dr. H. J. Nelis

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Abbreviations

AFB: acid fast bacterium

AFLP: amplified fragment length polymorphism

AGID: agar gel immunodiffusion

ATCC: American Type Culture Collection

BLAST: basic local alignment search tool

Bp: base pairs

CF: complement fixation test

CFU: colony forming unit

CMI: cell mediated immune response

CTAB: cetyl trimethyl ammonium bromide

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphates

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assay

GI: growth index

HEYM: Herrold's egg yolk medium

HPC: hexadecyl pyridinium chloride

IFN-γ: interferon gamma

IPTG: isopropyl-β-D-thiogalacto pyranoside

IS: insertion sequence

ITM: Institute of Tropical Medicine

JD: Johne's disease

LB: Luria-Bertani

Maa: *Mycobacterium avium* subspecies *avium*

MAC: *Mycobacterium avium* complex

Map: *Mycobacterium avium* subspecies *paratuberculosis*

MLSSR: multi locus short sequence repeat

MLST: multi locus sequence typing

Mtb: *Mycobacterium tuberculosis*

NCBI: National Center for Biotechnology Information

NCTC: National Collection of Type Cultures

NHI: National Health Institute

NTCC: NHI Type culture collection

ns: not specified

PAGE: polyacrylamide gel electrophoresis

PFGE: pulsed field gel electrophoresis

PBS: phosphate buffered saline

PCI: phenol : chloroform : isoamylalcohol

PCR: polymerase chain reaction

PPD: purified protein derivate

RFLP: restriction fragment length polymorphism

rRNA: ribosomal ribonucleic acid

RT: room temperature

RZSA: Royal Zoological Society of Antwerp

SDS: sodium dodecyl sulphate

SE-AFLP: single enzyme AFLP

SSCP: single strand conformation polymorphism

SSR: short sequence repeat

TAE: tris acetate EDTA

Tb: tuberculosis

TBE: tris borate EDTA

UV: ultraviolet

vol: volume

X-gal: 5-bromo-4-chloro-3-indolyl-ß-D-galactoside

1 Introduction

1.1 General features of *Mycobacterium avium* subsp. *paratuberculosis*

Mycobacterium avium subspecies *paratuberculosis* (Map) is an acid-fast micro-organism measuring 0.5 to 1.5 µm (Figure 1) (Cocito *et al.*, 1994). With its long generation time of roughly 2 days, it is a very slowly growing, fastidious bacterium.

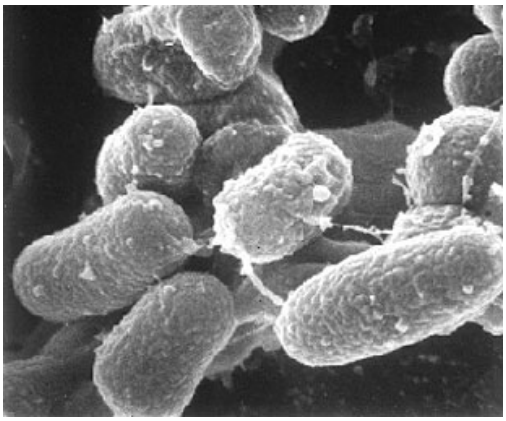


Figure 1: Scanning electron micrograph of *Mycobacterium avium* subspecies *paratuberculosis* magnified over 50,000 times

The bacterium is surrounded by a characteristic complex lipid-rich mycobacterial cell wall (Figure 2). Peptidoglycan is overlying the cell membrane and is responsible for the shape and rigidity of the cell. External to the peptidoglycan is a layer of arabinogalactan. The terminal arabinose units are covalently linked to the mycolic acids. The mycolic acids are a complex group of long-chain α-alkyl, β-hydroxy fatty acids and give the cell wall its thickness and pronounced hydrophobicity. The mycolic acids are largely responsible for the acid-fastness of the mycobacteria (Grange, 1996). The outer layer is composed of heterogeneous peptidoglycolipids or phenolic glycolipids termed mycosides. The thick cell wall enables the Map

organism to resist low pH, high temperatures and chemical agents (Manning *et al.*, 2001a).

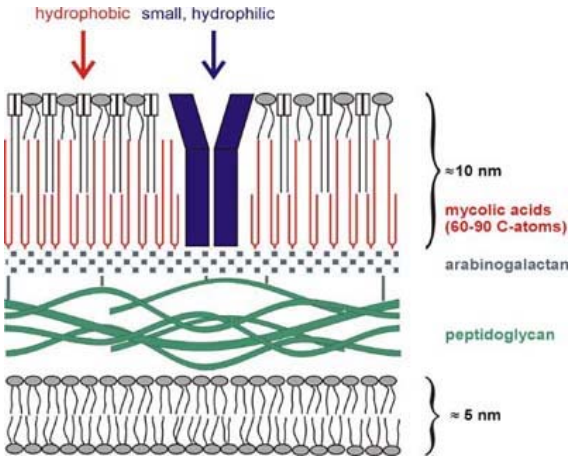


Figure 2: Cell wall of mycobacteria
© http://www.biologie.uni-erlangen.de/mycolab/bacicsd.html

Map belongs to the genus of Mycobacterium which is the single genus within the family of mycobacteriaceae. The taxonomic classification of Map is as follows (Rastogi *et al.,* 2001):

Superkingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteria
Subclass: Actinobacteridae
Order: Actinomycetales
Family: Mycobacteriaceae
Genus: *Mycobacterium*
Species: *Mycobacterium avium*
Subspecies: Map

The mycobacterial species avium is currently subdivided into four subspecies: *M. avium* subspecies *avium* (Maa), *M. avium* subspecies *hominissuis*, Map and *M. avium*

subspecies *silvaticum*. The subspecies designation of Map is based on DNA-DNA hybridisation studies.

The *M. avium* complex (MAC) contains the two species *M. avium* (with the four subspecies) and *M. intracellulare*.

Analysis of the 16s rRNA genes of mycobacteria has resulted in the division of this genus in two separate clusters. These correspond to the traditional fast-growing mycobacteria, represented by non-pathogenic environmental isolates and the slow-growing mycobacteria containing most of the overt pathogens.

Figure 3 shows a phylogenetic tree based on the 16S rRNA analysis of rapidly and slowly growing mycobacteria.

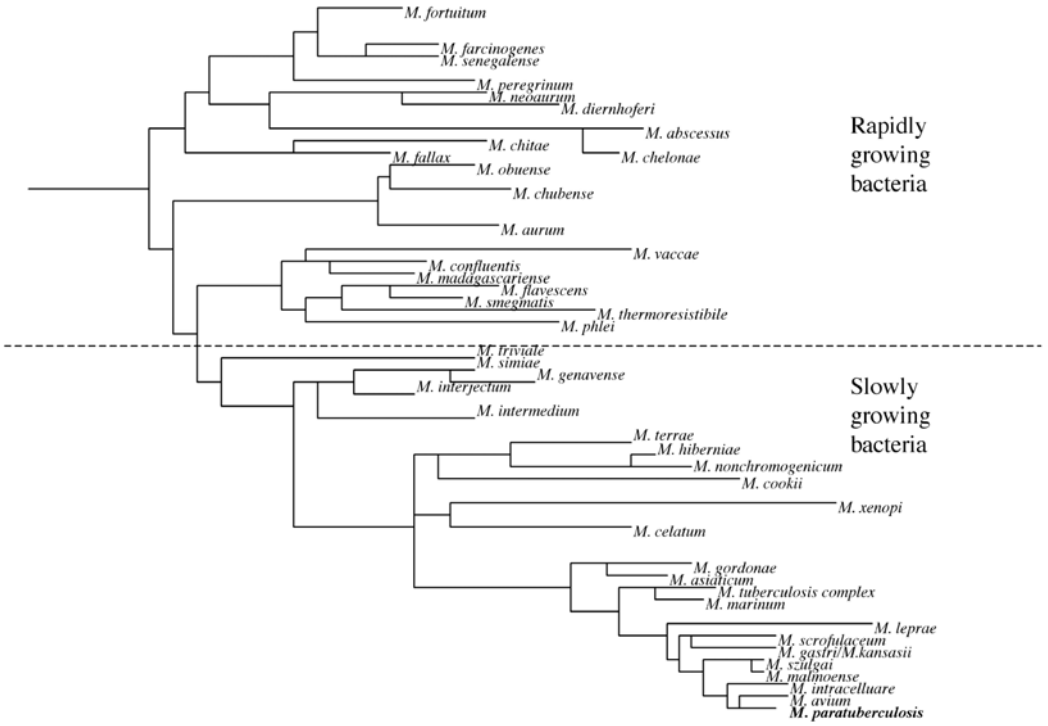


Figure 3: Phylogenetic tree based on the 16S rRNA analysis of 44 mycobacterial species (according to Rastogi *et al.*, 2001)

The Map genome is very similar to the genome of *M. avium* subsp. *avium* (Maa), the etiologic agent of tuberculosis in birds (Bannatine *et al.*, 2002). Genotypically, Map can be distinguished from Maa by the presence of multiple copies of an insertion element IS*900* (Vary *et al.*, 1990). At subspecies level, Map can be differentiated phenotypically from Maa by its dependence on mycobactin J (Barclay *et al.*, 1985).

Mycobactins are complex high molecular weight lipids confined to the cell membrane containing a core to which Fe^{3+} is co-ordinately linked. The iron requirement of pathogenic mycobacteria is peculiar in that an organic source of this metal ion is needed for its uptake and utilization (Lambrecht *et al.*, 1992). Map is essentially the only member of the acid-fast mycobacterial family that is unable to produce mycobactin J. Therefore, Map will multiply freely in parasitised hosts while it requires an exogenous supplement of mycobactin J for *in vitro* proliferation (Cocito *et al.*, 1994).

1.2 Host range, clinical signs and pathology

Map is the etiologic agent of Johne's disease (JD) or paratuberculosis, a chronic enteritis in animals. The natural hosts for Map are wild and domesticated ruminants (cattle, sheep, goat, deer, bison…) (Rajya *et al.*, 1961; Temple *et al.*, 1979; Thomas *et al.*, 1983; Buergelt *et al.*, 2000). However, non-ruminant wildlife (rabbit, horse, mouse, crow, fox, macaque…) has also been infected with Map (Larsen *et al.*, 1972; McClure *et al*., 1987; Beard *et al.*, 2001).

Young animals (i.e. less than six months) are more susceptible to infection with Map due to their immature cellular immunity and their limited ability to cope with intracellular pathogens (Manning, 2001a). Only a small dose of the organism may be required for infection in newborn calves, whereas for adult animals a sufficiently large and/or frequent dose of the organism is necessary. Not all exposed animals become infected and not all infected animals develop clinical disease.

The disease is most studied in cattle and shows three distinct clinical stages. In stage I, the infection process develops without appreciable shedding of the bacteria. Stage I is followed by a subclinical stage II in which the concentration of mycobacterial cells in the intestinal mucosa is increasing and the animal is intermittently shedding the bacteria in the faeces. In stage III, i.e. the terminal stage, clinical symptoms appear and the excretion of Map in the faeces increases, animals suffer from chronic diarrhoea, emaciation, decreased milk production and anaemia (Figure 4). Eventually, animals will die in a cachectic state (Cocito *et al.*, 1994). Clinical manifestations of JD are highly variable in different animal species. Clinical disease

in sheep and goats has been reported to be similar to that of cattle with the exception that diarrhoea is less frequent and the onset of the disease occurs in younger animals. Little has been published specifically on the clinical signs of JD in non-ruminant wildlife.

Figure 4: Cows with clinical signs (diarrhoea and emaciation) of Johne's disease
©Prof Haesebroeck and Johnes.org

Exposure to the organism can occur by a variety of routes. The oro-faecal route is the main route. As the infection progresses in the animal, the frequency and the number of Map cells excreted in the faeces increase, thereby contaminating the environment (pasture, soil, water and feed). Due to their thermal tolerance, UV resistance and resistance to low pH (Manning *et al*., 2001a), Map bacteria can survive for long periods in the environment and remain infective to other animals. The longer an infected animal remains in a herd, the higher the opportunity becomes for transmission of Map to other animals. Milk from infected animals is another important source of infection, as Map is present in the milk of clinically ill cows (Streeter *et al.*, 1995). Sweeney *et al.* (1992a) even isolated Map from milk and supramammary lymph nodes of asymptomatic cows. Milk is a very important route in the transmission of Map to newborn calves which are very sensitive to Map infection. Vertical transmission during pregnancy (*in utero* infection) has also been reported in the final stage of clinical disease (Sweeney *et al.*, 1992b). Another

possible route of transmission is the consumption of infected animals (e.g. rabbits) by carnivores (e.g. fox).

Once the Map bacteria are ingested, they enter the lymphatic system via the M-cells and are taken up by the macrophages. M-cells are located on the Peyers patches in the ileum and are specialized in the transcytosis of immunogens. Inside macrophages, the mycobacteria are able to resist enzymatic and toxic degradation and multiply until rupture of the infected macrophage occurs (Tessema *et al.*, 2001). Shortly after initial infection, most animals develop a T-cell response, characterised by release of the proinflammatory cytokines gamma interferon (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin 2 (IL-2). The animal now enters the subclinical phase during which, after a long incubation period of 2 to 5 years, the immune system shifts towards a Th2-like response with the production of cytokines coordinating non-protective B-cell immune responses (Coussens *et al.*, 2002). Due to influx of inflammatory cells in the intestine, the first clinical signs will appear in the infected animal, entering now the clinical phase. Given the long incubation time, clinical animals are usually older than two years.

1.3 Epidemiology

Johne's disease has been reported on every continent of the world and in an increasing range of animal species. However, the disease does not occur in all species in all parts of the world and some countries or regions have very little or no endemic infection. Prevalence studies were conducted in various countries but differences in sampling design and diagnostic strategies make a direct comparison of the results difficult. A study based on an absorbed ELISA test conducted in 1998 in the Belgian cattle population found a herd prevalence of 18.0 % and a within herd prevalence of 2.9 % (Boelaert *et al.*, 2000).

Paratuberculosis is a major problem in dairy farming. Indeed, it has a significant impact on the global economy due to premature disposal of animals and reduced milk production. In the United States, the annual losses in dairy industry due to

paratuberculosis are estimated at 250 million US$ (Ott *et al.,* 1999). Recent dairy herd prevalence data of some countries are summarized in Table 1 (BANR, 2003).
Large-scale surveys in the United States confirm that dairy herds are more likely to be infected than beef herds (BANR, 2003). A study in Georgia (USA) found a Map seroprevalence of 9.58 % in dairy cattle, whereas the seroprevalence in beef cattle was 3.95 % (Pence *et al*., 2003).

Country	Herd prevalence (%)
Australia	14-17
Austria	7
Belgium	22
England	17
Germany	10-30
Netherlands	55
New Zealand	60

Table 1: Seroprevalence of Map in dairy cattle in different countries (adapted from BANR, 2003)

Besides dairy and beef livestock, other domesticated animals are also susceptible to infection with Map. Ovine Johne's disease is important and well studied in Australia. A survey conducted in 2000 suggested that ovine JD had a highly clustered geographical distribution in Australia. Six to 10 % of sheep flocks in New South Wales were infected, whereas only 2.4 to 4.4 % of flocks Australia-wide were infected (Sergeant *et al*., 2002).
Although Map is predominantly known as a pathogen of domesticated ruminants, natural infection of wildlife has also been well documented. A study conducted in Italy on roe deer (n=94) detected 13 ELISA positive animals (13.8%) (Robino *et al.*, 2003). In Belgium, a serological survey revealed 13 positives out of 167 wild red deer (7.8%) (Linden *et al*., 2003).
The host range of Map is not limited to ruminants, as infection in horses, rabbits and non-human primates have been reported (Larsen *et al.*, 1997; Beard *et al.*, 2001; Zwick *et al.*, 2002). In a study about the potential role of wildlife in the spread of paratuberculosis in Scotland, Beard *et al.* (2001) found lesions typical for JD in foxes from which Map positive cultures of the intestine were obtained. These carnivores had probably been infected by eating infected rabbits. The rabbits were found to be

infected with the same Map strain as the infected cattle in that particular area. In the same study, the same Map was found in stoats, weasels, crows, rooks and jackdaws. This adds a new class of wildlife as reservoirs.

In addition, captive wildlife ruminants are also at risk for infection. Up to one third of zoos accredited by the American Zoo and Aquarium Association have reported at least one case of infection since 1995 (Manning *et al.*, 2001a). In a study conducted by Weber *et al*. (1992), 10.9% of zoo ruminants (n= 174) were found culture positive for Map. Infections were found in dwarf goats, mouflon sheep, alpine ibex and Cameroonian sheep. Due to the specific housing situation in zoo facilities, i.e. limited space for different animals, this is not surprising. The major risk factor is the introduction of new animals into the herd. Between zoos, animals are frequently exchanged and factors such as concentration of animals and stress during transport enhance the possibilities of Map transmission. In the past, the Royal Zoological Society of Antwerp (RZSA) had occasional problems with *Map*. After a Map positive faecal culture for an okapi (Tom) in 1999, the RSZA had some serious concerns about the prevalence of Map in their animal collection.

1.4 Map and Crohn's disease

Public health issues have been raised about the transmission of Map from animal products and the potential for subsequent infection and perhaps disease. Crohn's disease is thought by some to be linked to Map.

Crohn's disease in man is an inflammatory disorder of the intestine of unknown etiology. It is a chronic and incurable life-long disease progressing with remissions and relapses. The disease usually begins early in life with a peak incidence in young adults between 16 and 25 years. The major symptoms are abdominal pain, chronic weight loss and diarrhoea. The disease is most common in industrialised countries. A summary of the clinical features of Crohn's and Johne's disease is given in Table 2. It can be seen that the clinical symptoms of Crohn's disease are quite similar to those of Johne's disease (Figure 5) (BANR, 2003).

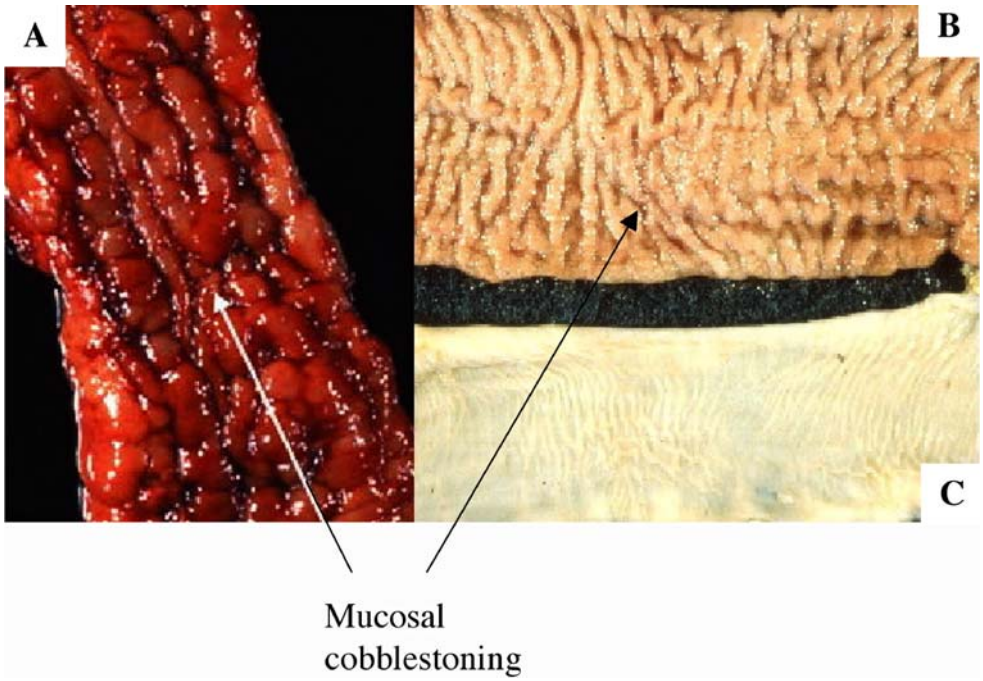


Figure 5: Clinical resemblances between Crohn's and Johne's disease
Affected ileum from Crohn's disease (A) and affected ileum from Johne's disease (B), unaffected ileum cattle (C) (©Greenstein, 2003 and Johnes.org)

Feature	Crohn's disease	Johne's disease
Preclinical stage		
Symptoms and signs	not known	decreased milk yield
Incubation period	not known	min. 6 months
Clinical stage		
Diarrhoea	chronic	chronic
Blood in stool	rare	rare
Abdominal pain	yes	unknown
Obstruction	yes	no
Amyloidosis	yes, but rare	yes
Skin lesions	yes	no
Renal involvement	yes, but rare	yes
Clinical course		
Remission and relapse	yes	yes

Table 2: Overview of Johne's and Crohn's disease symptoms
(adapted from BANR, 2003)

Although the cause of Crohn's disease in man remains unknown, it is likely to be due to a combination of genetic predisposition, an abnormal immune response and environmental factors. A long standing controversy about whether Map plays a role in Crohn's disease in humans is still not resolved and the demonstration of a link would have enormous implications on human-health. It is known that Map can survive pasteurisation of milk (Grant *et al.,* 2002) and it has been found in water supplies (Whan *et al.*, 2001; Greenstein, 2003), indicating that humans are at least exposed to the Map bacteria. The actual role of Map in the etiology of Crohn's disease is still subject to debate.

Two reports in Nature (Hugot *et al*., 2001; Ogura *et al*., 2001) have linked Crohn's disease to a mutation in the NOD_2 gene located on chromosome 16. This gene encodes for an intracellular receptor for microbial pathogens and plays a role in the launch of immune responses. A frameshift variant and two missense variants of the gene were found in families with a history of Crohn's disease. These mutations were responsible for a 20-fold or higher increase in susceptibility to Crohn's disease in NOD_2 homozygotes and could explain the involvement of micro-organisms like *Listeria*, measles virus or Map in the pathogenesis of Crohn's disease. In this context, Map is a good candidate micro-organism for a number of reasons. First of all there is the resemblance between JD and Crohn's disease symptoms. Furthermore, several studies (Sechi *et al.*, 2001) have found IS*900* positives in up to two third of intestinal samples of Crohn's disease patients. Bull *et al.* (2003a) even detected 92% of Crohn's patients (n=37) as IS*900* positive. Several papers suggest that Map survives in the intestine as a spheroplast or cell wall deficient form (Wall *et al.,* 1993; Hulten *et al.*, 2000; Hines *et al.*, 2003). These spheroplasts cannot be acid-fast stained (Markesich *et al.*, 1988), a fact that could explain why acid-fast bacteria are rarely found in intestinal tissue from Crohn's disease patients. Moreover, the spheroplast form is difficult to culture and thus far, only a few papers have reported a successful isolation (Wall *et al.*, 1993; Bull *et al.*, 2003a).

The above-mentioned arguments suggest a potential link between Map and Crohn's disease and warrant further research in this area. With current awareness about food safety and human health, it would unethical to neglect this possible link. Alltogether, these facts have led to an increased research interest in paratuberculosis.

1.5 Treatment and prevention

Treatment of paratuberculosis in animals is expensive and unrewarding (Harris *et al.*, 2001). Failure of antibiotic therapy could be due to the *in vivo* inaccessibility of mycobacteria to the drugs. Treatment requires daily medication for extended periods and can only be considered for animals of exceptional genetic value (Cocito *et al.*, 1994).

Vaccines (heat-killed and live attenuated strains) have been commercially available for many years but are not very effective in preventing the disease. Vaccination does not completely protect the animal from infection. Nonetheless, vaccines appear to provide partial protection since they reduce faecal shedding in cattle (Kormendy, 1994), minimize the number of clinically affected cows as well as the number of Map positive animals on histological and bacteriological tests (van Schaik *et al.*, 1996). In sheep, vaccination can modify the immunopathogenesis of paratuberculosis and can stop the progression from early subclinical infection to open disease. In the United States, vaccination is still a controversial control method due to the interference with several diagnostic tests for paratuberculosis and bovine tuberculosis (Collins *et al.*, 1994; Muskens *et al*., 2002). Vaccination cannot be used as a preventive tool but it can help to control paratuberculosis in already infected herds.

Adequate management is the best option to control the presence of paratuberculosis in domestic livestock herds (Kennedy *et al.*, 2001). Measures to stop the spread of the disease consist of the early removal of faecal shedders from the herd and prohibition of grazing on pastures occupied by infected animals. Nevertheless, the identification of infected animals is a problem using the currently available diagnostic methods. The overall cleanliness of the farm, proper manure handling and restriction of contact between calves and mature animals should be the general rule in preventing Map infections. Calves should be born in a clean and dry environment with minimal faecal contamination and colostrum for feeding calves should come from assumed paratuberculosis negative animals. Another major risk factor is the introduction of new animals into the herd. Animals from herds with an unknown paratuberculosis status should not be allowed to enter a herd free of paratuberculosis.

Several countries have introduced programmes for the control and eradication of paratuberculosis in sheep, goat and cattle. Almost all these programmes focus on the

early identification and the rapid elimination of clinically infected and excreting animals and the implementation of preventive measures. A good example is the Voluntary Paratuberculosis Programme for cattle in The Netherlands.

1.6 Existing diagnostic tests

Diagnostic tests for Map can be divided in two major categories: those that detect the organism itself and those that determine the host response to infection.
The first category includes the use of faecal smears and acid-fast staining, culture and PCR. The second category is based on observation of clinical signs in combination with gross and microscopic pathology, the use of serology, lymphocyte proliferation tests and tests to detect increased cytokine production.

1.6.1 Microscopic examination

The Ziehl-Neelsen staining technique is routinely used to detect mycobacteria. The technique is based on the fact that, due to the mycolic acids in their cell wall, mycobacteria retain the primary stain (carbol fuchsin) after exposure to a decolorising acidic alcohol. A counterstain (methylene blue) is usually employed to colour the background flora and to highlight the red stained mycobacteria (Volk, 1992). However, this method also stains other acid-fast bacteria such as *Nocardia* sp. and *Corynebacterium* sp. and will not differentiate between mycobacterial species. A positive diagnosis of paratuberculosis is based on the presence of clumps (three or more micro-organisms) of small, strongly acid-fast bacilli (Figure 6). The clumps are formed due to the hydrophobic features of the Map cell wall. The phenomenon of clumping is not well studied but it has been seen that clumping is important in the survival of Map during milk pasteurisation. Direct acid fast staining of faecal samples is difficult to interpret and has a poor detection limit (Manning *et al.*, 2001b). The success of microscopic diagnosis depends on the number of bacteria present in the sample. The presence of acid-fast organisms in tissue smears suggests but does not confirm infection. Although direct observation is insufficiently sensitive or specific, it is a quick, simple and cheap technique.

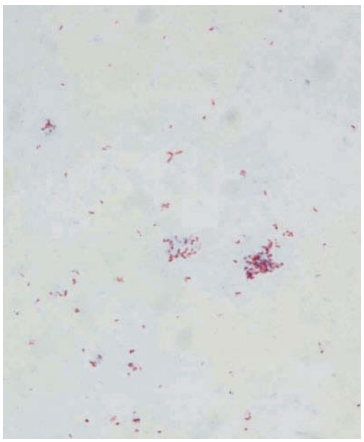


Figure 6: Ziehl-Neelsen stain of Map showing typical clumps of cells
(Richter *et al*., 2002)

1.6.2 Bacteriological culture

Culturing the bacteria is an extremely slow procedure due to the long generation time of Map. All culture methods require long incubation (4 to 16 weeks, in some cases even up to 32 weeks). Enriched media supplemented with mycobactin J are essential for the isolation of Map. Decontamination of the samples is necessary to prevent non-mycobacterial overgrowth and a variety of culture methods and decontamination protocols have been reported in literature. It is also recommended to supplement the media with an antibiotic mixture like PANTA plus (Becton Dickinson, Franklin Lakes, NJ, USA) to create additional selectivity against non-mycobacterial overgrowth.
The sensitivity of the culturing assays is strain dependent, as some strains (sheep and bison) are more difficult to isolate than others (Juste *et al.,* 1991).

1.6.2.1 Decontamination of Map containing clinical samples

The small intestine, intestinal lymph nodes and faeces are highly contaminated with bacteria and fungi and must be decontaminated before Map can be recovered on special media (Olsen *et al.*, 2002). Various chemicals and antibiotics which are more

toxic to the background flora than to Map have been included in the various decontamination protocols described.

Hexadecylpyridinium chloride (HPC) is widely accepted as the least harmful compound to Map and the most detrimental to other micro-organisms (Whittington *et al.*, 1999a; Grant *et al.*, 2001; Barrington *et al.*, 2003). A mixture of sodium hydroxide and oxalic acid is also commonly used in decontamination protocols (Halldorsdottir *et al.*, 2002; Kalis *et al.*, 2002).

Upon decontamination, a considerable reduction in the number of culturable Map cells may occur due to the sublethal effects of the chemicals or to loss during the centrifugation steps (Reddacliff *et al.*, 2003). This loss caused by decontamination is reported to be of the order of 1-2 $\log_{10}$ reductions in the number of Map cells (Merkal, 1973; Jorgensen, 1982; Whitlock and Rosenberger, 1990).

1.6.2.2 Conventional culture of Map

Conventional culture of Map on a more or less selective growth medium is time-consuming (12 to 32 weeks), requiring several months of incubation especially for samples with low Map load. Most laboratories use Herrold's egg yolk medium (HEYM) (Choy *et al.*, 1998; Donaghy *et al.*, 2003). Other commonly used media are modified Löwenstein-Jensen medium (Halldorsdottir *et al.*, 2002; Kalis *et al.*, 2002) and Middlebrook 7H10 agar (Donaghy *et al.,* 2003). All isolates should be confirmed as Map by mycobactin J dependency testing or by IS*900* based PCR identification.

1.6.2.3 Radiometric culture

The most commonly used radiometric system is the BACTEC® system of Becton Dickinson. This system, originally designed for diagnosis of human tuberculosis, has been modified to culture Map (Collins *et al.*, 1990). In this system a liquid culture medium (BACTEC 12B) containing an isotopically labelled nutrient source ($^{14}CO_2$ labelled palmitic acid) is used. The BACTEC® apparatus detects the ^{14}C labelled CO_2 that is produced by the metabolisation of the labelled palmitic acid. BACTEC detects positive samples sooner than convential culture (4-8 weeks) because metabolic products occur long before a colony is formed. For radiometric Map culture, egg yolk suspension and mycobactin J need to be added to the BACTEC 12B vials.

Confirmation of positive cultures should be done by Ziehl-Neelsen staining or by a Map specific PCR because many other non-Map organisms can also metabolise the palmitic acid.

This system is used in only a few laboratories because the equipment is expensive and requires the use and disposal of radioactive waste (Whittington *et al.*, 1998b; Manning *et al.*, 2001a; Grant *et al.*, 2003).

1.6.2.4 Other culture systems

Recently, several non-radiometric automated culture systems have become available. The ESP® culture system II (Trek diagnostic systems, Cleveland, OH, USA) monitors growth using a unique, non-radiometric, pressure-sensing detection system. BACTEC MGIT 960 (Grant *et al.*, 2003) and 9000MB (Becton Dickinson) are based on a fluorometric technology while the MB/BacT (Organon-Technika, Cambridge, UK) is based on a proprietary colorimetric technology. These culture systems are very expensive and are only useful in laboratories processing high numbers of samples.

1.6.2.5 Sensitivity of culture

The faecal culture method is the gold standard and detects animals shedding more than 100 colony-forming units (CFU) per gram of faeces with a diagnostic sensitivity of 50% and a specificity of near 100% (Merkal, 1973). Sockett *et al.* (1992) reported a slightly higher sensitivity for BACTEC than for conventional faecal culture (54.4 % versus 45.1%).

1.6.3 Molecular based diagnostic tests

A genetic element (IS*900*) unique to Map was discovered in 1989 (Green *et al.*, Collins *et al.*). This formed the basis for the development of PCR assays for the detection of Map.

The sensitivity of these assays is 100% and the specificity approaches 100% when applied in connection with the confirmation of Map cultures. However, PCR applied directly to biological samples has been hampered by difficulties in DNA isolation.

Therefore, these PCR assays lack sensitivity due to the extreme difficulty of removing PCR inhibitors from faecal and tissue samples (Fang *et al.*, 2002).

In general, PCR analysis has been unable to match the sensitivity of faecal culture in identifying small numbers of bacteria. However, it has proven to be very useful for the detection of Map in infected sheep, a significant benefit since recovery of Map from ovine samples is notoriously difficult (Choy *et al.*, 1998; Garrido *et al.*, 2000; Juste *et al*., 1991).

Various attempts have been made to improve the detection limit of the PCR. The use of magnetic beads for the extraction of Map DNA from faecal samples has partially solved the problem of inhibitors. A commercial kit based on IS*900* detection (HerdChek® Map DNA probe kit, IDEXX, Westbrook, ME, USA) has been evaluated in several host species (Collins *et al.*, 1993; Manning *et al.*, 2001a).

Other genetic targets like IS*1311* (Marsh *et al.*, 1999), f57 (Poupart *et al.*, 1993; Coetsier *et al.*, 2000) and hspX (Ellingson *et al.*, 1998) have been proposed as possible targets for the detection of Map.

Besides the low sensitivity (10^4 Map per g of sample), the high cost per sample (± 25 euro) and the requirement of special equipment and skilled technicians are other disadvantages of PCR. Moreover, this technique does not lead to isolation of the bacterium, thereby excluding subsequent testing.

1.6.4 Detection of immune responses

Map infections initiate both humoral and cell-mediated immune responses in the animal, which can be correlated with the stage of the disease and the observed lesions. In the subclinical stage of the disease, a strong cell-mediated immune response (CMI) and a weak humoral response are observed. However, in the clinically ill animal, the humoral response is strong and the cell-mediated one is weak (Manning *et al.*, 2001a).

Tests detecting serum antibodies, i.e the complement fixation test (CF), the agar gel immunodiffusion (AGID) and the enzyme linked immunosorbent assay (ELISA) are most useful in the clinical stage of the disease. Skin testing, determination of interferon gamma or lymphocyte transformation tests can be used to identify subclinically infected animals.

1.6.4.1 Cellular immunity tests

The initial and strongest response to mycobacterial infections in humans and animals is the cell-mediated immune response (CMI) mediated by T-lymphocytes. Various tests can be used to detect a CMI response to Map, but the most common assays are based on the detection of IFN-γ production. Currently, the intradermal skin test and lymphocyte transformation test are only rarely used.

1.6.4.1.1 Interdermal skin test

The intradermal skin test evaluates the delayed-type hypersensitivity (DTH) reaction of the animal to an injected Map extract. Skin testing has been used successfully in the fight against bovine tuberculosis in several countries, but the test is not specific for the diagnosis of paratuberculosis. Problems regarding antigenic cross-reactivity with non-pathogenic environmental mycobacteria have precluded its use as diagnostic tool for paratuberculosis (Monaghan *et al.*, 1994).

1.6.4.1.2 Lymphocyte transformation test

The lymphocyte blastogenesis transformation test is a relatively complex "*in vitro*" bio-assay that uses the Johnin purified protein derivate (PPD) antigen to stimulate lymphocytes present in fresh bovine whole blood co-incubated with H^3-thymidine. The incorporated thymidine is measured to determine the degree of lymphoblastogenesis. It must be done immediately after collection of the specimen and it suffers from specificity problems similar to those for skin testing (Alhaji *et al.*, 1974; Storset *et al.*, 2001).

1.6.4.1.3 Gamma interferon test (IFN-γ)

The IFN-γ test measures the production and release of IFN-γ by sensitised bovine lymphocytes in response to *in vitro* stimulation with mycobacterial antigens. A commercial IFN-γ test, using avian and bovine PPD in a comparative test is available for bovine tuberculosis. The IFN-γ test which can also be used for the diagnosis of paratuberculosis has a low specificity partly due to extensive cross-reaction with closely related environmental mycobacteria. Other limitations are the high cost and the practical complexity of using cellular assays (Manning *et al.*, 2001a).

1.6.4.2 Humoral immunity tests

Serologic assays are the best tools for surveillance of the paratuberculosis prevalence in herds. The main limitation of these antibody tests is the inability to identify affected animals early in the course of an infection.

1.6.4.2.1 Complement fixation test (CF)

The complement fixation test was one of the first serological tests developed for the diagnosis of Johne's disease. This assay detects complement-fixing antibodies to Map. First, complement in serum samples is inactivated by heating. The serum is subsequently mixed with a known amount of Map antigen and complement and the antibodies in the serum are allowed to fix the complement. In Map negative samples, the complement remains free in the serum and can be detected with a second antibody-antigen system (Volk, 1992).

The usefulness of the CF test is limited by the occurrence of false-positive results and its lack of sensitivity (Colgrove *et al.*, 1989). The CF test detects serum antibodies only 1-5 months later than does ELISA (Ridge *et al.*, 1991). Its sensitivity and specificity have been reported to be 38.4% and 99.0% respectively (Sockett *et al.*, 1992).

1.6.4.2.2 Agar gel immunodiffusion test (AGID)

The AGID test has been used successfully in control programmes of JD in cattle, sheep and goats. It is a simple test which requires no special equipment.

Antigen and antibody samples are placed in separate small wells punched in agar. The antigen and the antibody diffuse within the agar and the anitigen-antibody complex forms a line of precipitate in case of a positive result (Volk, 1992).

The AGID is reportly less sensitive than ELISA, particularly in subclinical infections (Nielsen *et al.*, 2001). Due to the low sensitivity, AGID is not a good test for screening subclinically infected herds, but it can be useful in confirming suspected clinically ill animals (Ferreira *et al*., 2002).

1.6.4.2.3 Enzyme linked immuno sorbent assay (ELISA)

ELISA is the most commonly used immunological test in routine diagnosis. It has a high specificity and the sensitivity is dependent on the clinical stage. It is a useful technique for large numbers of samples.

To reduce cross-reaction with non-specific mycobacterial antigens, the test serum is pre-absorbed with *M. phlei* (Bech-Nielsen *et al*., 1992). The pretreated serum samples are transferred to a microtitre plate coated with Map antigen. Antibodies against the Map antigen bind the antigen and can be detected with a conjugate. Research efforts are currently focusing on the application of the ELISA technology to the detection of antibodies in a wide range of non-domestic animals by using a non-specific binding conjugate (e.g. protein G) (Kramsky *et al.*, 2003). Several commercial ELISA kits are available, notably Herdchek® (IDEXX), SERELISA M ParaTB® (Synbiotics, San Diego, CA, USA), Parachek® (Biocor, Yardley, PA, USA) and ParaTB-Ab kit® (SVANOVA, Uppsala, Sweden).

1.6.5 Conclusion diagnostics

In general, diagnostic tests for the detection of paratuberculosis suffer from low accuracy in comparison to tests for many other veterinary diseases. This is due more to the biology of Map infections than to the quality of the tests. Because immunological reactions of the host vary and the seroconversion occurs only in the later stage of infection, diagnostic tests based on humoral immune responses suffer from considerable limitations. Detection of CMI responses could have a great potential in the early diagnosis of paratuberculosis if problems of non-specific antigens and uncertainty in interpretation were solved. Culture of faeces is a so-called one-sided gold standard, meaning it can be used to prove an animal is infected but cannot be used to prove the animal is not infected. If the inhibition problems could be overcome, PCR would be a reliable, fast and sensitive diagnostic tool.

As diagnosis in clinically ill animals is usually relatively simple, the actual challenge of diagnosis lies in the identification of subclinically infected animals. These animals shed small numbers of bacteria only intermittently and will usually be negative in standard immunological tests (Figure 7).

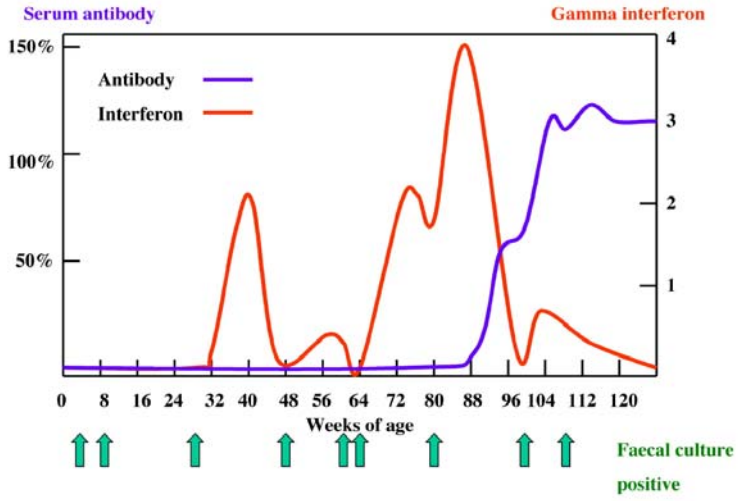


Figure 7: Antibody and interferon responses of an experimentally infected animal versus faecal culture (adapted from © Johnes.org)

Barrington *et al.* (2003) reported a daily variation in faecal culture and ELISA results for Map in cows. Therefore, testing animals more than once at different time intervals could increase test sensitivity. An ideal diagnostic test for control programs should identify most animals in stage I. Presently, no test meets this standard, as can be concluded from Table 3. In selecting the most appropriate test, it will be necessary to consider the purpose of testing. An immunological test like ELISA should be used for screening purposes and for obtaining herd level information. A technique based on the demonstration of Map itself, should be used for animal identification and for diagnosis confirmation.

	Stage I	**Stage II**	**Stage III**
Signs of disease	no	no	yes
Faecal culture	no	maybe	yes
PCR	no	maybe	yes
Acid-fast bacilli	no	maybe	yes
Interferon gamma	maybe	yes	maybe
Serology	no	maybe	yes

Table 3: Detectability of Map during the clinical stages by different tests (adapted from BANR, 2003)

1.7 Molecular biology of Map

Although the complete Map genome has been recently sequenced by Kapur and coworkers (2003), a fully edited and annotated public version of the genome has not yet become available. The size of the complete genome is 4.83 Mb with a GC content of 69.30% and containing over 4000 open reading frames. DNA-DNA hybridisation studies have shown a genetic similarity of more than 97% between *Mycobacterium avium* subspecies *avium* (Maa) and Map (Bannantine *et al.*, 2002). 16S rRNA sequences from Map and Maa are indistinguishable from each other (Van der Giessen *et al.*, 1992). An analysis of 48% of the Map genome revealed the presence of only 27 extra predicted coding sequences in comparison with Maa. Further analysis of this limited number of unique coding sequences will be critical for developing specific diagnostic reagents (Bannantine *et al.*, 2002).

1.7.1 Unique genetic markers

1.7.1.1 Insertion sequences

Insertion sequences are small, mobile genetic elements containing genes related to transposition functions. The first insertion sequence described in mycobacteria was IS*900* for Map, demonstrated independently by two research groups (Collins *et al.*, 1989, Green *et al.*, 1989). IS*900* is a 1.45 kb insertion sequence present in 15-20 copies in the Map genome and is considered to be specific. IS*900* lacks both inverted terminal repeats and direct repeats commonly associated with other transposable DNA elements (Mahillon *et al.,* 1998). The sequence of IS*900* codes for two proteins. A single open reading frame (ORF) on the positive strand encodes a protein of 339 amino acids (transposase). A second protein is encoded by an ORF2 (*hed* gene) on the complementary strand of IS*900* (Harris *et al.*, 2001).

IS*900* belongs to the same family of insertion sequences as IS*901*, IS*902* and IS*1110* which have been described in *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *avium,* respectively.

IS*900* is the most widely used target in PCR detection of Map organisms. Vary *et al*. (1990) described the first commercially available PCR (IDEXX) based on IS*900*. Using the same primers, Cousins *et al.* (1999) reported an IS*900*-like PCR amplicon

from environmental mycobacteria, thus demonstrating that false positive results could be obtained from strains other than Map. Naser *et al.* (1999) detected IS*900*-like sequences in Maa from HIV patients. Moreover, Englund *et al.* (2002) characterised a mycobacterial isolate harbouring one copy of a sequence showing 94% identity to IS*900* at the nucleic acid level. These data indicated that PCR results based on the IS*900* sequence alone should be interpreted with caution. The currently used IS*900* primers may not be specific for Map as IS*900*(-like) elements may be present in other closely related mycobacteria.

An explanation could lie in the fact that insertion elements may have spread through horizontal transfer from Map to environmental non-tuberculous mycobacteria. This phenomenon has already been reported in other mycobacteria (Chemlal *et al.*, 2003).

The only other insertion sequence described in the Map genome is IS*1311* (7-10 copies), but this insertion sequence is not unique to Map since it is also found in Maa and *M. intracellulare* (Marsh *et al.*, 1999). IS*1311* is somewhat smaller than IS*900* (1317 bp) and appears to contain a single ORF encoding a putative transposase. There is no homology between IS*900* and IS*1311* at the nucleotide level.

1.7.1.2 Other unique sequences

Two additional unique sequences have also been characterised, notably the f57 fragment and the hspX gene.

The f57 insert described by Poupart *et al.* (1993) is specific for Map and has not been found in other mycobacteria, including Maa. The insert is 620 bp long, has a GC content of 58.9% and does not resemble other known sequences. This f57 fragment has been used to develop a diagnostic duplex PCR capable of differentiating *M. bovis*, Maa and Map (Coetsier *et al.*, 2000).

The hspX gene (Ellingson *et al.*, 1998) encodes a putative heat shock-like protein. BLAST (basic local alignment search tool) analysis of hspX against Maa and other mycobacterial genomes shows that hspX has approximately 60 % homology to similar sequences in other mycobacteria indicating its uniqueness for Map.

1.7.2 Isolate typing

Typing of Map isolates originating from different host species and from different geographical locations is a prerequisite to study and understand the epidemiology of Map. It is important to identify the source of a new infection because that information will often determine the strategy of corrective actions. It will also be a key factor in the debate concerning the role of Map as a zoonotic micro-organism.

Molecular strain characterisation has a great influence on the studies of Map because very few phenotypical differences among Map isolates are found. Different fingerprinting techniques have been developed for Map strain characterisation with RFLP-IS*900* being the most commonly used.

1.7.2.1 Restriction fragment length polymorphism (RFLP-IS*900*)

This technique uses frequently cutting restriction enzymes to cut DNA in small, non-random fragments that are subsequently separated by gel electrophoresis. Specific probes will anneal to multiple copy regions providing different profiles. The Map RFLP fingerprinting technique uses IS*900* based probes. In 1999, Pavlik *et al.* proposed a standardization of the RFLP using two enzymes (*Pst*I and *BstE*II). The technique could differentiate 13 *Pst*I types and 20 *BstE*II types among 1008 Map isolates and a combination of both restriction types revealed a total of 28 different RFLP types.

This technique differentiates sheep and bovine Map field isolates, suggesting that Map strains underwent host adaptation. However, the occurrence of sheep isolates has been reported in cattle with Johne's disease, indicating that host specificity is not absolute (Bauerfeind *et al.*, 1996). Pavlik *et al.* (1995) isolated two different strains of Map, each with a unique RFLP profile, from the same animal, suggesting the existence of co-infections in some animals.

As high quantities of pure DNA are needed for this technique, its application is limited by the very slow growth of most strains and the lack of growth of others. Another drawback of the proposed RFLP is the need to use radioisotopes for probe labelling.

1.7.2.2 Pulsed field gel electrophoresis (PFGE)

The PFGE technique is used to fractionate and separate large DNA fragments. The DNA needs to be isolated carefully to minimize artificial shearing. Therefore, samples of cells are mixed with molten agarose and the agarose blocks are incubated with hydrolytic enzymes to digest cellular components. The blocks containing chromosomal DNA are incubated with rare cutter restriction enzymes. During the PFGE run, the relative orientation of the gel and the electric field is periodically altered. Due to the discontinuity of the electric field, the DNA molecules are intermittently forced to change their conformation and direction of migration. The time taken to alter conformation is size-dependent (Olive *et al.,* 1999) and generates a size-dependent banding pattern. Thus far, PFGE has not yet been extensively studied as a mean for isolate typing, although it has been useful in differentiating *M. avium* isolates (Stevenson *et al.*, 1997).

Stevenson *et al.* (2002) used a multiplex PFGE (*Spe*I, *SnaB*I) in a study of pigmented and non-pigmented Map strains. They were able to detect 16 multiplex PFGE profiles in a panel of reference strains, whereas only 6 profiles were obtained when using the IS*900*-RFLP approach on the same panel. This multiplex PFGE could subdivide IS*900* RFLP types. Multiplex PFGE explores the whole genome and may be more appropriate and successful for discriminating organisms with limited genetic heterogeneity but further research is needed to confirm this. A disadvantage of the PFGE is the need for large quantities of DNA.

1.7.2.3 Random amplified polymorphic DNA (RAPD)

RAPD reactions are PCR reactions that randomly amplify DNA segments of unknown origin. A single, short oligonucleotide primer annealing to many different loci is used to amplify random sequences in extracted DNA. No sequence info is required to design specific primers. The technique is fast, simple and efficient, requiring only small amounts of DNA. Moreover the procedure can be automated (Kristensen *et al*., 2001). Pillai *et al.* (2001) observed 6 major fingerprint patterns in 208 Map isolates using RAPD.

However, poor reproducibility between laboratories is the major criticism. Small errors occurring during dilution and pipetting of PCR reagents may contribute to this

lack of reproducibility. The need to use low annealing temperatures often generates non-specific bands due to misannealing of the primers, which makes the interpretation of profiles difficult (Olive *et al*., 1999).

1.7.2.4 Multiplex PCR of IS*900* integration loci (MPIL)

Bull *et al.* (2000) have sequenced flanking regions for 14 different IS*900* loci present in Map and have used the sequence information to develop a multiplex PCR typing method which reveals the presence or absence of IS*900* at each locus. The IS*900* sequence becomes integrated into conserved loci in the Map genome and these integration sites are unique to the Map genome.

Bull *et al.* differentiated 10 different MPIL types, whereas *Pst*I/*Bst*EII RFLP could detect 17 types. Nine of the MPIL types corresponded to 9 different RFLP types, whereas the 10th MPIL type corresponded to the 8 remaining RFLP-types. An advantage of this technique is the fact that MPIL typing could be done straight on field samples without prior culturing. This technique is not widely used yet and more research is necessary on the integration sites of IS*900*.

1.7.2.5 Mycobacterial interspersed repetitive units (MIRU)

MIRU's are mini-satellite sequences that are distributed throughout the genome as single copies or as multiple tandem repeats. MIRU's were first identified in *M. tuberculosis* and were shown to be present in up to 40 loci (Frothingham *et al*., 1998). Bull *et al.* (2003b) identified MIRU in Map and developed a simple PCR method to distinguish Map from Maa and *M. intracellulare*.

MIRU typing of Map readily distinguishes ovine-pigmented isolates from other Map isolates and has provided the first genetic evidence of a difference between pathogenic Map isolates and the 316F vaccine strain. Further research is necessary to evaluate the applicability of MIRU for fingerprinting of Map isolates (Bull *et al.*, 2003b).

1.7.2.6 IS*1311* restriction endonuclease analysis (IS*1311*-REA)

Whittington *et al.* (1998a) used the IS*1311* sequence to distinguish between ovine and bovine isolates. A polymorphism (C to T) in basepair 167 in the ORF (Genbank

accesion number AJ223974, ORF from 35 to 1219) of the insertion sequence IS*1311* is unique to bovine Map, whereas ovine Map is identical to Maa at this locus. This results in a gain of an HinfI restriction site in Map of bovine origin. After PCR amplification of IS*1311*, a restriction with *Hinf*I will show 4 bands in the bovine strain and 3 bands in the ovine strain. This fingerprinting method can only be used to differentiate sheep from cattle strains.

1.7.2.7 Multilocus sequence typing (MLST)

The most important limitation of current typing methods is the difficulty of comparing results achieved by different laboratories.

MLST involves the sequencing of several internal fragments of housekeeping genes and has been developed for several pathogenic bacterial species including *Neisseria meningitidis* and *Streptococcus pyogenes*. Each unique sequence is given a unique and arbitrary allele number and the combination of allele numbers at the different loci is known as the allelic profile. Each allelic profile is similarly assigned a number which is known as the sequence type. Validation studies have shown that these sequence types correspond to the lineages determined from other methods (Chan *et al*., 2001). Because sequence data are used, the results are directly comparable with little or no investigator interpretation required making the method very robust. Variants of each gene used can be stored and used for comparison over the internet allowing labs worldwide to access the data (Maiden *et al.*, 1998).

MLST has also its limitations, e.g. being unsuitable for highly uniform species which lack sequence variation in housekeeping genes. Similarly, allele assignments are not suitable for species with so much recombination that each sequence is unique. The technique is also time-and money-consuming.

Multilocus sequence typing is not applicable to Mycobacteria (including Map) because the gene sequence polymorphism is very restricted among strains of this species (Mazars *et al*., 2001, Kremer *et al*., 1999).

1.7.2.8 Multilocus short sequence repeat (MLSSR)

Because MPIL, AFLP and RFLP techniques are generally unable to resolve Map isolates into meaningful epidemiologic groups due to the apparently restricted genetic diversity within the subspecies, the possibility of using short sequence repeats for the typing of Map was investigated by Amonsin *et al.* (2004).

Short sequence repeats in bacterial DNA have been used as markers for differentiating and subtyping strains of several bacterial species including *Mycobacterium tuberculosis* (Gascoyne-Binzi *et al*., 2001). SSRs consist of simple homopolymeric tracts of a single nucleotide or multimeric tracts such as di- or trinucleotide repeats.

Preliminary bioinformatic analyses on the genome of Map led to the identification of numerous SSRs. The result of the study suggests that MLSSR is a useful approach for strain differentiation and enables the rapid and facile discrimination of epidemiologically and geographically distinct strains of Map.

In a parallel investigation, Motiwala *et al.* (2004) used the MLSSR approach for the study of Map in wild animal species. The authors concluded that the MLSSR can differentiate Map isolates from different host species and can provide evidence for the host specificity of some Map strains as well as sharing of strains between wild and domesticated animal species.

1.7.2.9 Amplified fragment length polymorphism (AFLP)

Motiwala *et al.* (2003) used a commercial AFLP kit for the fingerprinting of Map strains (in comparison with MPIL) and could differentiate isolates clustering together in the MPIL analysis. On the other hand, MPIL fingerprinting discriminated between isolates in one cluster of AFLP. This indicates the need to use more than 1 fingerprinting technique to distinguish between Map strains. The AFLP fingerprints of human Map strains were unique and did not cluster with either bovine or ovine strains, whereas RFLP could not discriminate between human strains (Pavlik *et al.*, 1995; Whittington *et al.*, 2000).

The principles of AFLP will be discussed in further detail in Chapter 6.

2 Objectives

This research originated from a concrete request by the RZSA. After the positive faecal culture from an okapi (1999), the RZSA had some serious concerns about the prevalence of paratuberculosis in their animal collection. The RZSA is the studbook keeper for these valuable and almost extinct animals and coordinates the worldwide breeding programme.

In the United States, one third of the zoo and animal parks have reported at least one paratuberculosis case since 1995. Due to the typical housing situation of zoo animals, this is not surprising. In European zoo and animal parks, no data on Map prevalence are available.

A survey on the detection of animals carrying Map became necessary after this unfortunate finding because the animals of the RZSA are frequently exchanged with other zoos and animal parks. To prevent the spread of Map, it is advisable to test animals before transport to other zoo's or animal parks. Therefore a simple, fast and specific and sensitive test was essential. If positive animals would be found, it will be of paramount importance to investigate the spread of Map among different zoo-species. To this end, the detailed typing of isolates would be essential. Although the commonly used RFLP-IS*900* for Map characterisation can be used for this purpose, this technique has some serious disadvantages. Map isolates should be grown before extraction of the genomic DNA. To increase discriminatory power, two restrictions need to be performed. Taken together all these facts makes the whole RFLP time-consuming. There is a clear need for a good characterisation technique that can be used straight on field samples.

Given the deficiencies of the existing diagnostic methods, no such test was available at the start of our work. The above starting points have led to the definition of the following objectives for this thesis:

1. To develop a rapid, specific and sensitive PCR test for the detection of Map in zoo animals. To this end, new specific primers for IS*900* amplification are required as the commonly used primers described by Vary *et al*. (1990) are known to result in non-specific amplification products.

2. To combine the new PCR method with a suitable procedure to extract enough Map DNA, in the absence of inhibitory substances, from animal faeces.

3. To assess the occurence of Map in the RZSA, using the new PCR method as well as a conventional culture and radiometric culture for comparison.

4. To collect data on seropositive animals using a commercial ELISA test (Herdchek® ELISA kit, IDEXX) on sera from zoo animals.

5. To type Map isolates in order to investigate their potential spread among zoo animals. Specifically, to compare AFLP patterns and to clone and sequence informative polymorphic bands. Such information on sequences could lead to the development of new PCR assays for typing Map isolates directly in field samples.

In general, our work hopes to contribute to organizing future epidemiological studies in zoo environments.

3 Design of new diagnostic primers for the detection of Map

3.1 Background and aim

Molecular methods for the diagnosis of paratuberculosis are under constant development and evaluation. A number of genes and sequences unique to Map have been identified over the years. IS*900* is the most commonly used and studied Map specific sequence. However, the currently used IS*900* primers (Vary *et al.,* 1990; Millar *et al.,* 1995) may not be specific for Map as IS*900*(-like) elements could be present in closely related mycobacteria (Cousins *et al.*, 1999; Englund *et al.*, 2002). Using these primers, Cousins *et al.* (1999) reported an IS*900*-like PCR amplicon from environmental mycobacteria, hereby demonstrating that false positive results can be obtained from strains other than Map. Englund *et al.* (2002) characterised a *Mycobacterium* isolate harbouring one copy of a sequence showing 94% identity to IS*900* at the nucleic acid level. The existence of sequences related to IS*900* was also reported in wood pigeon mycobacteria (IS*902*) and Maa (IS*901*). The closest related insertion sequence to IS*900* is the insertion sequence IS*1626* (found in Maa and *M. intracellulare*) (Puyang *et al.*, 1999).

To overcome these non-specific PCR amplification of IS*900*-like sequences, new specific primers for IS*900* amplification will be designed. The specificity of these primers will be evaluated with a panel of Map and non-Map isolates. A confirmatory test for the IS*900* amplicon based on restriction analysis will be designed. Furthermore, computer simulations will be performed to evaluate the PCR results for the IS*900*-related sequences.

As an alternative molecular test, a second primer set will be designed for the amplification of another Map specific gene i.e. f57. The f57 sequence has no homology with any known sequence in other bacteria (Poupart *et al.*, 1993; Coetsier *et al.*, 2000). The specificity of these new primers will also be tested with a panel of Map and non-Map isolates. A combination of both PCR assays in a multiplex PCR would be ideal.

Over the last few years, multiplex PCR-based assays have been designed to co-identify distinct mycobacterial strains in the same PCR tube (Yeboah-manu *et al.*, 2001). The multiplex PCR procedure offers an easy, rapid and inexpensive way of detecting two different genes at the same time. The possibility of using a multiplex PCR for the simultaneous detection of the two Map specific fragments IS*900* and f57 will be evaluated.

3.2 Demonstration of the IS*900* sequence

3.2.1 Materials and Methods

3.2.1.1 Design of primers

IS*900* sequences (NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) accession numbers AF305073, AF416985, AJ251437, X16293) were aligned using the ClustalW program (EMBL-EBI, Cambridge, UK). This multiple sequence alignment was used as the template for designing primers in the Primer Premier DNA program (Premier Biosoft International, Palo Alto, CA, USA). The proposed primers were tested and rated with the PCR simulating program Amplify (Engels, 1993) and checked for possible homologous sequences with a standard nucleotide BLAST (NCBI). The primers selected for the first round were IS*900*S1 (5'GGG TTG ATC TGG ACA ATG ACG GTT A 3') and IS*900*R3(2) (5'AGC GCG GCA CGG CTC TTG TT 3') and for the nested PCR IS*900*S2 (5'GGA GGT GGT TGT GGC ACA ACC TGT 3') and IS*900*R1 (5'CGA TCA GCC ACC AGA TCG GAA 3'). The predicted amplicon lengths were 572 bp for the first round and 452 bp for the nested PCR.

3.2.1.2 Bacterial isolates

Mycobacterial isolates from the collection of the Belgian Mycobacterium Reference Laboratory (Institute of Tropical Medicine, Antwerp, Belgium) were used. The non-Map isolates are listed in Table 4, whereas the Map isolates are given in Table 5. Map isolates were grown on slopes of modified Löwenstein-Jensen medium

supplemented with mycobactin J (1 mg/l) (Synbiotics), PANTA plus (40 ml/l) and sodium pyruvate (4 g/ l) and cultured at 37°C. The non-Map isolates were cultured on Löwenstein-Jensen slants or on Stonebrink medium (*M. bovis*), Ogawa medium supplemented with Fe ammonium citrate (1.5%) (*M. haemophilum*) and Middlebrook 7H11 (*M. genavense*, *Corynebacterium* sp.). All mycobacterial and bacterial species were identified with biochemical and / or molecular tests.

Species	Isolate (ITM nr)	Source	Origin	Provided by	Year of isolation
M. abscessus	00-0828	Human	USA	CDC	ns
M. abscessus	95-0553	Human	Belgium	ITM	1995
M. africanum	01-0012	Human	Belgium	ITM	2001
M. avium	00-1342	Human	Belgium	Hospital Aalst	2000
M. avium	00-0568	Human	Belgium	Hospital Aalst	2000
M. avium	00-0570	Human	Belgium	Hospital Aalst	2000
M. avium	00-0571	Human	Belgium	Hospital Aalst	2000
M. bovis	00-1060	ns	ns	Dr Haddad	2000
M. bovis	96-0770	Human	Belgium	Hospital Passendaele	1996
M. canetti	9818	Human	Belgium	ITM	1993
M. celatum	94-0123	Human	Belgium	ITM	1994
M. celatum	95-0142	Human	USA	Butler, Atlanta, JCM0899	ns
M. chelonae	7286	Human	Belgium	Sint-Elisabeth Hospital, Turnhout	1991
M. chelonae	7701	Human	Belgium	ITM	1992
M. chelonae	97-0245	Human	Brazil	CDC	ns
M. chelonae	00-0476	Human	Belgium	ITM	2000
M. chelonae	02-0265	Human	Belgium	ITM	2002
M. chitae	7735			ATCC 19627	
M. duvalii	4974			ATCC 43910	
M. flavescens	98-1294			ATCC14474	
M. fortuitum	97-0998	Human	Belgium	Vesalius Hospital Brasschaat	1997
M. fortuitum	97-0461	Human	The Netherlands	ITM	1997
M. haemophilum	95-1070	Human	Spain	Pedro Idigoras	1995
M. haemophilum	95-1071	Human	Spain	Pedro Idigoras	1996
M. haemophilum	95-1072	Human	Spain	Pedro Idigoras	1997
M. haemophilum	95-1141	Human	Belgium	ITM	1995
M. haemophilum	95-1142	Human	Belgium	ITM	1995
M. heidelbergense	98-1295	Human	Germany	Rusch-Gerdes Borstel 5636/96	1996

Non-Map isolates …/…

Species	Isolate (ITM nr)	Source	Origin	Provided by	Year of isolation
M. gadium	4983			NCTC 10942	
M. gastri	97-0937	Environment	Italy	ITM	1997
M. genavense	95-0608	Human	Switserland	Hirschel 8859	1995
M. genavense	95-0975	Human	Switserland	Hirshel	1995
M. genavense	96-0823	Human	USA	ns	1996
M. genavense	95-0614	Bird	Belgium	Zoo antwerpen 163/94	1995
M. gordonae	7838	Human	Belgium	ITM	1992
M. gordonae	4996			ATCC 14470	
M. gordonae	97-0246	Human	Brazil	Dr Suffys CD9	1997
M. gordonae	1430	Monkey	USA	ITM	1990
M. gordonae	8671	Human	Belgium/zaire	ITM	1992
M. interiectum	96-0116	Human	Germany	letter Borstel	1996
M. intermedium	961170	Human	Germany	Aken, BN886	1996
M. intracellulare 1	8718	Human	Australia	ITM	1992
M. intracellulare 1	8721	Human	Australia	ITM	1992
M. intracellulare 1	4199	Human	Australia	ITM	1990
M. intracellulare 1	4208	Human	Australia	ITM	1990
M. intracellulare 1	8732	Human	Australia	ITM	1996
M. intracellulare 2	6776	Environment	Italy	ITM	1991
M. intracellulare 2	6777	Environment	Italy	ITM	1991
M. intracellulare 2	94-1123	Environment	India	Paramasivan	1994
M. intracellulare 2	94-0788	Bovine	Burundi	ITM	1994
M. intracellulare 2	5922	Human	The Netherlands	ITM	1990
M. kansasii	96-1361	Human	Czech republic	Czech hospital	1996
M. kansasii	00-0836	Human	Belgium	Hospital Aalst 0285545	2000
M. mageritense	4990	ns	USA	Dr. Brown, Texas M0-1025	1998

Non-Map isolates …/…

Species	Isolate (ITM nr)	Source	Origin	Provided by	Year of isolation
M. malmoense	96-1633	Human	England	J Norton 6622/96	1996
M. malmoense	00-0827	Human	USA	CDC	ns
M. malmoense	6033	Environment	Zaire	ITM	1991
M. malmoense	6035	Environment	Zaire	ITM	1991
M. malmoense	96-1630	Human	England	J Norton 6527/96	1996
M. malmoense	96-1635	Human	England	J Norton 7330/96	1996
M. marinum	98-0852	Human	Belgium	ITM	1998
M. marinum	99-0822	Human	Belgium	ITM	1999
M. marinum	99-2570	Human	Belgium	ITM	1999
M. marinum	99-3021	Human	Belgium	ITM	1999
M. marinum	00-0533	Human	Belgium	ITM	2000
M. microti	99-2426	Animal	The Netherlands	ITM	1999
M. nonchromogenicum	4980			ATCC 19530	
M. parafortuitum	96-0484			ATCC 25808	
M. peregrinum	97-0462			ATCC 14467	
M. peregrinum	01-0086	Human	USA	CDC	ns
M. peregrinum	96-0098	Human	USA	CDC	ns
M. peregrinum	96-1258	Human	USA	Santiago Estrador, Colombia	1996
M. peregrinum	96-1259	Human	USA	Santiago Estrador, Colombia	1996
M. peregrinum	4150	Seagull	Belgium	ITM	1989
M. phlei	4973			NCTC 8151	
M. scrofulaceum	00-1478	Human	Belgium	Dr. H Vanachtere 67637	2000
M. scrofulaceum	01-0529	Human	Belgium	Dr A-M Vints 01032452	2001
M. simiae	6610	Human	Belgium/Zaire	ITM	1991
M. simiae	94-1219	Human	Australia	Danson Red W55 S93/172	1994
M. simiae	7052	Human	Belgium	ITM	1991
M. simiae	96-1873	Human	Belgium	ITM	1996
M. simiae-like	95-0447	Human	Benin	ITM	1995

Non-Map isolates …/…

Species	Isolate (ITM nr)	Source	Origin	Provided by	Year of isolation
M. smegmatis	01-0003	Human	Belgium	Dr De Beenhouwer 0471001	1999
M. smegmatis	98-1314	Human	Belgium	ITM	1998
M. smegmatis	98-1315	Human	Belgium	ITM	1998
M. smegmatis	98-1316	Human	Belgium	ITM	1998
M. smegmatis	98-1317	Human	Belgium	ITM	1998
M. smegmatis	4995			ATCC 607	
M. szulgai	94-0267	Human	Belgium	ITM	1994
M. terrae	7369			ATCC 15755	
M. triplex	97-9660			ATCC 700071	
M. triviale	99-0796	Human	Irak	L-sh-24	1999
M. tuberculosis	02-0251	Human	Belgium	ITM	2002
M. ulcerans	9537	Human	PapuaNG	ITM	1994
M. ulcerans	94-0662	Human	Ivory coast	ITM	1994
M. ulcerans	94-0886	Human	Benin	ITM	1994
M. ulcerans	94-1324	Human	Australia	ITM	1994
M. ulcerans	97-9680	Human	Angola	ITM	1997
M. vaccae	4978			ATCC 15483	
M. xenopi	02-0260	Human	Belgium	ITM	2002
M. xenopi	9741	Human	Czech republic	Dr Mulan Kubin	1993
Nocardia asteroides	99-0737			ATCC 19297	
N. brasiliensis	99-0738			ATCC 19296	
N. nova	99-0739			ATCC 33726	
N. farcinica	99-0740			ATCC 3318	
Rhodococcus equi	99-0741			ATCC 6939	

Non-Map isolates …/…

Species	Isolate (ITM nr)	Source	Origin	Provided by	Year of isolation
Corynebacterium equi	4512	Horse	Zoo Belgium	Zoo Antwerp	ns
Corynebacterium equi	4513	Baikal seal	Zoo Belgium	Zoo Antwerp	ns
Corynebacterium equi	4514	Rhinoceros	Zoo Belgium	Zoo Antwerp	ns
Corynebacterium equi	4515	Sea lion	Zoo Belgium	Zoo Antwerp	ns
Corynebacterium equi	4516	Dybowski deer	Zoo Belgium	Zoo Antwerp	ns
C. huayni	1038			NTCC 10673	
C. flaccumfaciens	1017			NTCC 559	
C. pyogenes	1024			NTCC 5224	
C. xerosis	1046			NTCC 9755	
C. diphteriae	1018			NTCC 3984	

Table 4: Non-Map isolates used for the evaluation of the new primers

(ATTC American Type Culture Collection, NCTC: National Collection of Type Cultures, NTCC: NHI Type Culture Collection (= New Zealand Reference Culture Collection), ns: not specified)

Isolate (ITM nr)	Year of isolation	Isolated by	Origin	Source	Sample
02-0904	2002	ITM	Belgium	*Cervus elaphus*	large intestine
02-0909	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1466	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1444	2002	ITM	Belgium	*Cervus elaphus*	small intestine
02-1461	2002	ITM	Belgium	*Cervus elaphus*	spleen
02-1463	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1445	2002	ITM	Belgium	*Cervus elaphus*	spleen
02-0910	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1462	2002	ITM	Belgium	*Cervus elaphus*	spleen
02-1465	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1447	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1451	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1458	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1663	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1448	2002	ITM	Belgium	*Cervus elaphus*	mes. lymph node
02-1441	2002	ITM	Belgium	*Cervus elaphus*	spleen
02-0706	2002	ITM	Belgium	cattle	faeces
02-0806	2002	ITM	Belgium	cattle	faeces
02-0702	2002	ITM	Belgium	cattle	faeces
02-0710	2002	ITM	Belgium	cattle	faeces
02-1696	2002	ITM	Belgium	cattle	faeces
02-1717	2002	ITM	Belgium	cattle	faeces
02-0700	2002	ITM	Belgium	cattle	faeces
02-1715	2002	ITM	Belgium	cattle	faeces

Map isolates …/…

Isolate	Year of isolation	Isolated by	Origin	Source	Sample
02-0699	2002	ITM	Belgium	cattle	faeces
02-1680	2002	ITM	Belgium	cattle	faeces
02-0705	2002	ITM	Belgium	cattle	faeces
02-1675	2002	ITM	Belgium	cattle	faeces
02-1679	2002	ITM	Belgium	cattle	faeces
02-1673	2002	ITM	Belgium	cattle	faeces
02-0900	2002	ITM	Belgium	cattle	faeces
02-0708	2002	ITM	Belgium	cattle	faeces
02-0711	2002	ITM	Belgium	cattle	faeces
02-0697	2002	ITM	Belgium	cattle	faeces
02-1041	2002	ITM	Belgium	cattle	faeces
02-0808	2002	ITM	Belgium	cattle	faeces
02-1042	2002	ITM	Belgium	cattle	faeces
02-0698	2002	ITM	Belgium	cattle	faeces
02-1678	2002	ITM	Belgium	cattle	faeces
02-0701	2002	ITM	Belgium	cattle	faeces
02-0807	2002	ITM	Belgium	cattle	faeces
02-1040	2002	ITM	Belgium	cattle	faeces
02-0811	2002	ITM	Belgium	cattle	faeces
02-0810	2002	ITM	Belgium	cattle	faeces
02-0370	2002	ITM	Belgium	cattle	faeces
02-0369	2002	ITM	Belgium	cattle	faeces
2666			ATCC 19698	cattle	faeces
9403	1992	ITM	Burundi	cattle	lymph node
99-0901	1997	Cerva-Coda, 97010	Belgium	cattle	faeces

Map isolates …/…

Isolate	Year of isolation	Isolated by	Origin	Source	Sample
99-0912	1997	Cerva-Coda, 97152	Belgium	cattle	faeces
99-0932	1997	Cerva-Coda, 97274	Belgium	cattle	faeces
99-0933	1997	Cerva-Coda, 97287	Belgium	cattle	faeces
99-0937	1996	Cerva-Coda, 96017	Belgium	cattle	milk
99-0938	1996	Cerva-Coda, 96022	Belgium	cattle	faeces
99-0941	1996	Cerva-Coda, 96025	Belgium	cattle	faeces
99-0942	1996	Cerva-Coda, 96026	Belgium	cattle	faeces
99-0943	ns	Cerva-Coda, 43015	Belgium	cattle	faeces
99-0946	1998	Cerva-Coda, 196/98	Belgium	cattle	faeces
99-0947	1998	Cerva-Coda, 357/98	Belgium	cattle	faeces
99-0948	1998	Cerva-Coda, 358/98	Belgium	cattle	faeces
99-2678	1999	ns	Australia	cattle	faeces
99-2679	1999	ns	Australia	sheep	faeces
99-2681	1999	ns	Australia	cattle	faeces
00-0010	1999	Cerva-Coda, 99/80	Belgium	cattle	faeces
00-0012	1999	Cerva-Coda, 99/62	Belgium	cattle	faeces
00-1671	2000	ITM	Belgium	cattle	faeces
00-0002	1999	Cerva-Coda, 99/31	Belgium	cattle	faeces
00-0003	1999	Cerva-Coda, 99/30	Belgium	cattle	faeces
00-0324	1999	Cerva-Coda, 99/531	Belgium	cattle	faeces
00-0004	1999	Cerva-Coda, 99/23	Belgium	cattle	faeces
00-0451	2000	ITM	Belgium	cattle	faeces
00-0452	2000	ITM	Belgium	cattle	faeces
00-0453	2000	ITM	Belgium	cattle	faeces
00-0005	1999	Cerva-Coda, 99/22	Belgium	cattle	faeces

Table 5: Map isolates used for the primer specificity tests

(ATCC: American Type Culture Collection, ns: not specified)

3.2.1.3 Extraction of genomic DNA

One loop of bacterial cells was suspended in 600 µl of disruption buffer (4 M guanidine thiocyanate (ICN biochemicals, Costa Mesa, CA, USA), 0.025 M sodium citrate pH 7, 0.5% sarkosyl (Sigma, St. Louis, MO, USA), 0.1 M 2-mercapto-ethanol (Sigma), 20 mM EDTA (Sigma) pH 8). DNA was isolated from the disruption buffer by a phenol:chloroform:isoamylalcohol (PCI) extraction. Six hundred µl of a PCI mixture (25:24:1, Acros organics, Geel, Belgium) was added to the disruption buffer and vortex mixed for 5 s. After centrifugation at 12,000 x *g* for 5 min, the upper aqueous phase was transferred to a new tube and mixed with an equal volume of PCI mixture. The sample was vortex mixed and centrifuged at 12,000 x *g* for 5 min. The upper aqueous phase was then transferred to a new tube and mixed with 1 vol of a chloroform:isoamylalcohol mixture (24:1). After centrifugation, the aqueous phase was transferred to a new tube followed by a DNA precipitation in 0.1 vol of 3 M sodium acetate and 1 vol of ice-cold isopropanol. After centrifugation during 10 min at 12,000 x *g*, the supernatant was discarded. The pellet was initially dried in an exsiccator and then dissolved in 50 µl of 1x TE (10mM Tris-HCl pH 8, 1 mM EDTA). The concentration of the extracted DNA was measured with a GeneQuant spectrophotometer (Amersham Bioscience, Freiburg, Germany).

3.2.1.4 PCR

A nested PCR approach was chosen to increase the sensitivity of the PCR assay. Nested PCR means that two pairs of PCR primers are used for a single locus. The first pair amplifies the locus as seen in any PCR protocol. The second pair of primers binds within the first PCR product and produces a second PCR product that will be shorter than the first one. In a semi-nested PCR, one primer of the first PCR is re-used in the second PCR.

Primary PCR was performed in a final volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 200 µM of each dNTP, 20 pM of each primer (IS900S1 and IS900R3(2)), 0.5 U Taq polymerase (Silverstar, Eurogentec, Seraing, Belgium) and 1 µl bacterial DNA extract. The tubes were placed in a thermocycler (PTC 100, MJ Research, MA, USA) and amplification was as follows: one cycle of denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 45 s,

annealing at 68°C for 45 s and extension at 72°C for 45 s and a final extension at 72°C for 10 min.

The secondary PCR was performed in a final volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 200 µM of each dNTP, 20 pM of each primer (IS*900*S2 and IS*900*R1), 0.5 U Taq polymerase (Silverstar) and 1 µl of the first PCR solution. The tubes were placed in a thermocycler (PTC 100) and amplification was as follows: one cycle of denaturation at 94°C for 4 min followed by 25 cycles of denaturation at 94° C for 45 s, annealing at 68°C for 45 s and extension at 72°C for 45 s and a final extension at 72°C for 10 min. A PCR mixture with MilliQ water as template was used as negative control. Bacterial DNA of the Map reference strain ATCC 19698 (American Type Culture Collection, Manassas, VA, USA) was used as positive control. PCR results were analysed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised by ultraviolet trans-illumination.

3.2.1.5 Detection limit of the PCR

A serial dilution of Map isolate M02-0904 ranging from 10^6 to 10^{-1} CFU per PCR volume was used to estimate the detection limit of the PCR. The counts in the serial dilution were determined with solid phase cytometry. It is difficult to obtain single cell suspensions of Map due to their very hydrophobic cell wall. For this reason, the sensitivity will be expressed in colony forming units which can however consist of more than 1 Map cell. Several negative controls (MilliQ water) were always included in the PCR reaction. A strict procedure was followed to avoid cross-contamination between samples or carry-over of PCR products.

3.2.1.6 Confirmatory test for IS*900* amplicon

After IS*900* amplification, an extra test can be performed to be sure the amplicon is originating from the IS*900*. A restriction endonuclease reaction was chosen for this confirmatory test. Ideally, the enzyme cuts the amplicon only once approximately in the middle of the amplicon. The Gene Jockey DNA program (Biosoft, Cambridge, UK) recommended to use *Hph*I (5´ ...G G T G A (N)8^... 3´). This restriction

enzyme cuts at basepair 160 of the IS*900* amplicon resulting in two fragments of 160 bp and 290 bp, respectively.

Four µl of PCR product was mixed with 6 units *Hph*I and the supplied buffer according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). The tubes were incubated overnight in a water bath at 37°C. Four µl of the mixture was loaded on a 10 % polyacrylamide gel (PAGE) and run at 100 V for 2 h 40 min. Three µl of a DNA ladder (GeneRuler 100 bp DNA Ladder, MBI Fermentas, St. Leon-Rot, Germany) was included on every gel. Finally, the PAGE gel was silver stained.

3.2.1.7 Computer simulation of the PCR assays

Computer analysis of the PCR assays using the Vary (Vary *et al*., 1990), the Millar (Millar *et al.,* 1995) and the newly developed primers on IS*900* related sequences were done using the PCR software Amplify (Engels, 1993). Various DNA sequences from the literature showing considerable homology to the IS*900* sequences were selected to be tested in the analysis. The following sequences were used: IS*900* (Genbank AF 416985 and AF 305073), IS*900*-like (Genbank AF 455252, Englund *et al.*, 2002), IS*900*-like WA-1 and IS*900*-like WA-2 (Cousins *et al.*, 1999), IS*1613* (Genbank AJ011837), IS*901* (Genbank X59272) and IS*902* (Genbank X58030). These sequences have given false positive IS*900* PCR results in the past (Cousins *et al*., 1999; Englund *et al*., 2002).

The PCR software Amplify allows simulating and rating conditions and results of polymerase chain reactions. Amplify uses two parameters to measure the quality of a primer match. "Primability" is the parameter rating the ease of extension at the 3' end of the primer by the DNA polymerase. The "stability" is the parameter measuring the strength of the primer and target bond. The primability and stability rates need to exceed a given cut-off point in order to conclude about a good primer match.

3.2.2 Results

3.2.2.1 Specificity and detection limit of the PCR assay

The specificity of the PCR assay was confirmed by the use of DNA templates from a panel of 190 mycobacterial isolates (74 Map isolates and 116 non-Map isolates). Results show that Map specific primer pairs amplify only DNA from Map and not from the non-Map isolates. The amplicons were of the expected length, according to the published sequence information and no non-specific bands were detected. It is unlikely that negative results with the new IS*900* primers on DNA other than Map would be due to an inadequate PCR amplification, as positive controls were always amplified correctly.

Tests to determine the detection limit for the new IS*900* primers were repeatedly performed. PCR with 1 or more CFUs per PCR clearly showed positive PCR results on agarose gel.

3.2.2.2 Confirmatory test for IS*900* amplicons

All IS*900* amplicons were subjected to the restriction analysis with *Hph*I. All amplicons revealed two bands of 160 and 290 bp on the PAGE gel, confirming that the amplicons were all IS*900* derived.

3.2.2.3 Computer simulations

A summary of the results of the computer simulation analysis with Amplify is given in Table 6. The newly developed primers gave very good results with the IS*900* sequences (only bands of the expected size). The Vary and Millar primers gave rise to many non-specific bands. For the IS*900*-like sequence (AF 455253), amplification could be obtained under sub-optimal PCR conditions with the new primers. For the WA-1 and WA-2 IS*900*-like sequences (Cousins *et al.*, 1999), there was no annealing of the new primers, whereas the Vary and Millar primers did give an amplification product. The Millar primers and the new primers yielded no amplification for IS*901* and IS*1613*. With the Vary-primers, the IS*1613* was amplified. All primers gave expected results for IS*902* (no amplification).

	Amplify score		
Sequences	**New primers**	**Vary primers**	**Millar primers**
IS*900* (AF416985)	5	3	4
IS*900* (AF305073)	5	2	4
IS*900*-like (AF455252)	3	2	2
IS*900*-like (WA-1 Cousins *et al.,*1999)	4	1	1
IS*900*-like (WA-2 Cousins *et al.,* 1999)	5	1	1
IS*901* (X59272)	5	4	5
IS*1613* (AJO11837)	5	1	4
IS*902* (X58030)	5	4	5
IS*1626* (AF071067)	5	5	4

Table 6: Results for the PCR computer simulations on IS*900* and IS*900*-like sequences (Amplify score : 5: expected result, no mispriming; 1: non-specific bands mispriming)

3.3 Demonstration of the f57 sequence

3.3.1 Materials and Methods

3.3.1.1 Primers design

The primers developed by Coetsier *et al*. (2000) gave non-specific amplification products in our hands. The f57 sequence (NCBI accession number X70277) was used as a template in the Right Primer DNA program (Biodisk, San Francisco, CA, USA) to design new primers. A semi-nested PCR approach was chosen to obtain a higher sensitivity. In the first round, primers F57 (5' CCT GTC TAA TTC GAT CAC GGA CTA GA 3') and R57 (5' TCA GCT ATT GGT GTA CCG AAT GT 3') amplified a fragment with the predicted length of 432 bp. In the semi-nested PCR F57 was re-used in combination with F57Rn (5' TGG TGT ACC GAA TGT TGT TGT CAC 3') to amplify a fragment of 424 bp.

3.3.1.2 Bacterial isolates and genomic DNA extraction

The same bacterial isolates and genomic DNA extracts as for IS*900* primers were used for the evaluation of the primers for f57.

3.3.1.3 PCR

The primary and nested PCRs were performed following the standard PCR protocols described in 3.2.1.4 with following variables:

Primary PCR			
Primer: F57			
Primer: R57			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	64	45	
Extension	72	45	

Secondary PCR			
Primer: F57			
Primer: F57Rn			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	25
Annealing	64	45	
Extension	72	45	

3.3.2 Results

All 74 Map isolates gave an amplicon of the expected length and no non-specific bands were detected. The 116 non-Map isolates gave no amplification with the f57 PCR assay. These results confirm the specificity of the primers for f57.

The detection limit was experimentally determined to be 1 CFU per PCR for pure cultures.

3.4 Multiplex IS*900*-f57 PCR

3.4.1 Materials and Methods

3.4.1.1 Primer design

A simultaneous assay for the f57 sequence and IS*900* would be very useful for a specific diagnostic test for Map. In a multiplex PCR, two or more fragments are amplified during the same PCR reaction. In the development of a multiplex PCR it is important to choose primer pairs that are compatible. The primers should have approximately the same annealing temperature and the amplicons should be distinguishable in size. Due to the similar fragment sizes (424 bp and 452 bp), it was not possible to use the f57 PCR primers with the new IS*900* PCR. New f57 primers had to be designed giving an amplicon of a different size. In the first round, primer F57 (5' CCT GTC TAA TTC GAT CAC GGA CTA GA 3') was used in combination with F57M2 (5' GGT GTA CCG AAT GTT GTT GTC AC 3'). This gave an amplicon of 395 bp. A semi-nested PCR was performed in the second round using the new primer F57M1 (5' CGC CGC TGA CGC ACC GAA CGA C 3') and F57M2 resulting in an amplicon of 269 bp. The first multiplex PCRs were performed under the same primer conditions as in a normal PCR, whereas the primer concentrations for the IS*900* primers were later adapted.

3.4.1.2 Bacterial isolates and genomic DNA extraction

The same bacterial isolates and genomic DNA extracts as for the IS*900* and f57 primers were used for the evaluation of the multiplex PCR.

3.4.1.3 PCR

The primary and nested PCR were performed following the standard PCR protocols described in 3.2.1.4 with following variables:

Primary PCR			
Primers: IS*900*S1 and IS*900*R3(2) (10 pM)			
Primers: F57 and F57M2 (20 pM)			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	68	45	
Extension	72	45	

Secondary PCR			
Primers: IS*900*S2 and IS*900*R1 (10 pM)			
Primers: F57M1 and F57M2 (20 pM)			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	25
Annealing	68	45	
Extension	72	45	

3.4.2 Results

For all Map isolates, amplicons of the expected size were generated after amplification of the target sequences. The 452 bp and the 269 bp fragments were easily distinguishable on agarose gels (Figure 8). The multiplex PCR assay gave no amplicons for the non-Map isolates. The sensitivity of the assay was the same as in the one fragment PCR for IS*900* and f57.

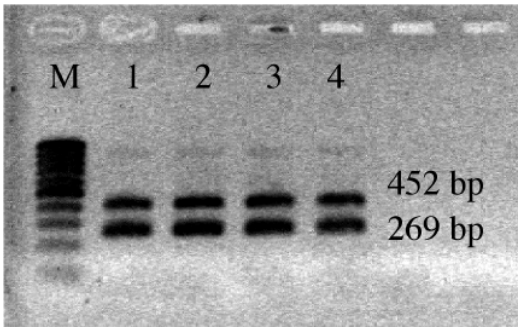


Figure 8: Agarose gel with amplification products of the multiplex PCR
(M: 100 bp Marker, 1-4: amplification products of Map isolates, 452 and 269 bp respectively)

3.5 General discussion

Map diagnosis using PCR assays has been reported using various protocols and samples: detection of IS*900* in formalin-fixed paraffin-embedded tissues (Whittington *et al.*, 1999b), after BACTEC 12B radiometric culture (Whittington *et al.*, 1998b), in commercial samples of bulk milk (Grant *et al.*, 2000) and in human intestinal biopsies (Collins *et al.*, 2000). It has been shown that some *Mycobacterium* spp. contain DNA sequences with considerable homology to the IS*900* sequence (Cousins *et al.*, 1999; Englund *et al.*, 2002). Our *in vitro* analysis of IS*900*-like sequence showed that, indeed, the Millar and Vary primers gave rise to many non-specific bands and that the primers could amplify the IS*900*-like sequences. These results clearly demonstrated that these IS*900* assays can not be used as the only confirmatory diagnostic tool.

The primers developed in our study resulted in a PCR assay with a very low detection limit (1 CFU per PCR volume) and gave only amplification of the target sequence in Map isolates. The IS*900* amplicons were confirmed with an extra test, i.e. restriction endonucleasis (3.2.1.6). The computer simulations confirmed the good specificity of the new developed primers for IS*900*.

Englund *et al.* (2002) recommended that a positive IS*900* PCR should be confirmed by subsequent sequencing or by a PCR assay targeting another gene in Map. In our study, the PCR assay on the f57 sequence was chosen as a complementary test to confirm or exclude the presence of Map in a sample after a positive IS*900* PCR-test. All Map isolates generated a f57 PCR product of the expected size and the non-Map isolates, including Maa, did not yield positive amplification.

The results of this study indicate that the newly developed assays are a useful tool for the identification of Map. Although the IS*900* primers were specific in our hands, we also recommend to include the f57 PCR assay to confirm the presence of Map after a positive IS*900* PCR. *In vitro* analysis with the computer programme Amplify showed that it is possible to amplify the IS*900*-like sequence described by Englund *et al.* (2002) with our newly designed primers if PCR conditions are suboptimal.

To avoid a second PCR test after a positive IS*900* sample, we investigated the possibility to amplify the two Map specific sequences in one PCR. The results obtained with our multiplex PCR targeting IS*900* and f57 were very promising but a validation on field samples is needed.

3.6 Conclusion

The combination of the both presumed specific PCR assays (IS*900* PCR assay and f57 assay) with our newly developed primers has proven to be superior to the Vary an Millar primers in the identification of *Mycobacterium avium* subspecies *paratuberculosis*. Furthermore, our multiplex PCR for the simultaneous detection of Map is very promising. A specific PCR assay was the first requirement for a good molecular diagnostic test. This requirement has been now fulfilled and the development of an extraction method to isolate from faeces enough Map DNA devoid of inhibitory substances could now be tackled.

4 Development of a DNA extraction method for the detection of Map in faecal samples

4.1 Background and aim

Tissue samples from the site of infection (ileum and mesenteric lymph node) are probably the most appropriate samples for diagnosing Map infections in animals. However, collecting tissue (biopsy) samples is not practical in living animals, especially when it concerns valuable zoo animals. Using these tissue samples for culture and PCR amplification, however, can be very useful in confirming diagnosis at necropsy (*post mortem* diagnosis). Serological testing in zoo animals and free-living animals is also impractical. It is ethically not acceptable to anaesthetize apparently valuable healthy animals for bleeding if the test is known to be insufficiently sensitive in the early stages of the disease. For these reasons, faeces are the most appropriate specimens for the detection of Map infection in living animals. Bacterial concentrations in stool of infected animals may exceed 10^8 organisms per g of faeces (Cocito *et al.*, 1994).

PCR on faeces could be a good alternative for the very slow culture technique. PCR results can be obtained much faster than culture results (one week instead of 12 weeks) but faeces are one of the most difficult specimens for recovering DNA. First of all, the faeces matrix is complex and its composition is dependent on the nourishment of the animal. A second important problem is the presence of PCR inhibitors like haemoglobin, bilirubin, bile salts, chelating agents and humic materials (Widjojoatmodjo *et al.*, 1992; Shames *et al.*, 1995). These inhibitors interfere with the Taq DNA polymerase and hence, affect the amplification of the DNA. Faeces also contain a lot of non-specific DNA originating from other bacteria, food rests and the host. A large amount of these non-specific nucleic acids can easily cause inhibition in PCR reactions (Wilson, 1997).

In view of some properties of Map bacteria, the use of a faecal DNA extraction as the basis of a diagnostic test is even more difficult than for other bacteria. The rigid cell wall renders the bacteria extremely resistant to disruption and hampers the release of DNA during the extraction procedure. The hydrophobic Map cell wall consists of a complex mixture of lipids and polysaccharides and is responsible for a considerable "clumping" of Map cells. Due to these clumps, Map bacteria are not equally distributed in the faeces. It should also be remembered that in the early stage of the disease, only a small amount of bacteria is shed in the faeces. This means that frequent sampling over time will be necessary to increase the sensitivity of any PCR test for faeces.

Ideally, a rapid, specific, sensitive and high yield DNA extraction method should include homogenisation of the sample, disruption of the Map bacteria and removal of all PCR inhibitors. At present, no such test is available.

A new extraction method will be developed using spiked samples and the detection limit will be determined. The new faecal extraction method will subsequently be evaluated on a wide range of clinical samples.

4.2 Preparation of Map spiked samples

The different extraction methods were compared using Map spiked samples. Map culture negative faeces were mixed with a defined suspension containing Map organisms. The suspensions were counted microscopically after Ziehl-Neelsen staining and by means of solid phase cytometry.

4.2.1 Materials and methods

Map cells were cultured on Löwenstein-Jensen supplemented with mycobactin J (1 mg/l). Culture tubes were harvested in 2 ml of a 10% Tween-20 solution containing glass spheres. The bacteria were subsequently mixed on a vortex mixer to destroy the hydrophobic clumps, followed by the preparation of tenfold dilutions in physiological saline up to a dilution of 10^{-8}.

4.2.1.1 Microscopical counting

An in-house protocol of the Mycobacterium laboratory of the ITM (Antwerp) was used to stain the suspensions before microscopical counting. Two drops (0.1 ml) of a dilution were mixed with two drops of formaldehyde milk (10% milk, 0.4% formaldehyde). Three circles of a multispot microscope slide (Hendley, Essex, United Kingdom) were spotted with 5 µl of each mixture. The slides were allowed to dry at room temperture (RT). The slides were subsequently exposed to 10% formaldehyde vapours for 3 min, followed by the exposure to steam for 2 min. One drop of gelatin phenol was added to each circle on the slides and the slides were incubated for 4 min at RT. The slides were again exposed to steam for 2 min followed by the exposure to formaldehyde vapours for 3 min in a box and to steam 2 min. The slides were allowed to cool down and were stained using the Ziehl-Neelsen method with carbol fuchsine, acidic alcohol and methylene blue.

In total, 60 microscope fields were counted (20 fields per circle of the multispot microscope slide). The number of bacteria in one ml can be calculated with a formula that takes into account the diameter of the circle (5.45 mm), the applied volume and the microscope optics.

For the microscope used in this study (Leitz laborlux D with field objective 0.17 mm Ø), the calculation factor was 6.4×10^3. The detection limit of the test is 1 counted AFB in 60 fields or 6.4×10^3 Map cells per ml.

4.2.1.2 Solid phase cytometry counting

Visual counting of the slides is time-consuming and has an unfavourable detection limit. Solid-phase cytometry allows rapid detection of bacteria at the single cell level without the need for a growth phase. The bacteria are isolated from a filterable matrix by membrane filtration, fluorescently labelled and automatically counted by a laser-scanning device (Chemscan®, Chemunex, Ivry-sur-Seine, France) (Figure 9). The entire membrane filter is scanned, yielding a theoretical detection limit of 1 cell per membrane filter and hence, per filtered sample volume (D'Haese *et al*., 2002). The viability of the filtered cells can be demonstrated by the use of ChemChrome V6. This substrate is an initially non-fluorescent carboxy fluorescein derivative, which is taken up by metabolically active cells and cleaved by intracellular esterases to yield an

intensely green fluorescent product. Hence, viability labeling is based on enzymatic activity as well as membrane integrity. After membrane filtration of 1 ml Map suspensions, the filters were incubated for 45 min at 30°C on a cellulose pad containing 600 µl B16 buffer and 6 µl ChemChrome V6 (Chemunex), followed by analysis with the ChemScan®. Each filter was visually inspected with an epifluorescence microscope (Olympus BX40, Tokyo, Japan) connected to the ChemScan® by a computer driven moving stage.

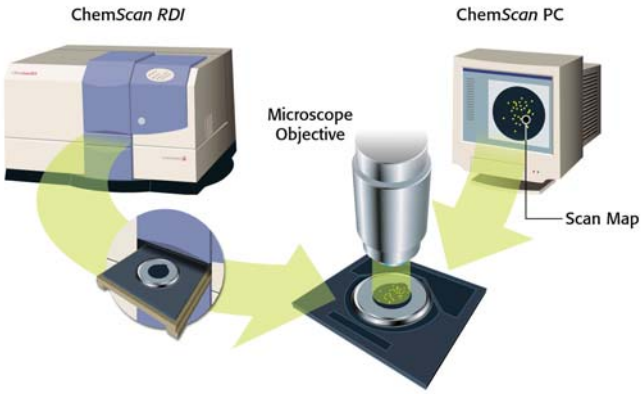


Figure 9: Chemscan (Chemunex): Solid phase cytometry for the detection of filtered micro-organisms

4.2.2 Results and conclusion

The number of cells of the different suspensions was determined using microscopy and solid phase cytometry. The tenfold dilution range could be confirmed and the microscopic results agreed with the solid phase cytometry results. Solid phase cytometry is a good, reliable and rapid alternative for the time consuming visual counting of bacteria.

The Map dilutions could be used for spiking the Map culture negative faeces. Aliqouts of the dilution were kept at –20°C and were thawed just before use.

4.3 Development of a faecal DNA extraction procedure

The ideal extraction method should allow good homogenisation of the sample, release of the total DNA content of the bacterium and the removal of all inhibitory substances. The extraction can be based on chemical, mechanical and enzymatic techniques or a combination of them.

The first step in the extraction includes homogenisation of samples and concentrating the bacteria. The DNA is subsequently removed, leaving the inhibitory substances in the suspension. Several homogenisation procedures were tested in combination with two DNA isolation methods. Two combined methods (homogenisation and isolation in only one step) were also tested.

4.3.1 Homogenisation methods

4.3.1.1 Use of NaOH

Sodium hydroxide is used in a Map extraction protocol developed by Bleumink-Pluym *et al*. (1994). NaOH is a strong base and treatment of the sample with NaOH will destroy a fraction of the faecal bacteria except mycobacteria. Due to their thick cell wall, mycobacteria are resistant to acidic and alkaline solutions. NaOH is commonly used as a decontamination solution prior to culturing mycobacteria.

Materials and methods

One gram of faeces was suspended in 20 ml of a 0.2 M NaOH solution, filtered through a sterile gauze and centrifuged at 3,000 x *g* for 1 h. The supernatant was discarded and the pellet was suspended in 1 ml MilliQ water. The suspension was transferred to a 1.5 ml tube and centrifuged for 10 min at 12,000 x *g*. The supernatant was discarded and the pellet was used in the DNA isolation.

4.3.1.2 Use of NaOH and CTAB

Cetyl trimethyl ammonium bromide (CTAB) is a cationic-active desurfactant and detergent. CTAB complexes cell wall debris, denatured protein and polysaccharides and has been described to effectively remove PCR-inhibiting factors from stools (van Zwet *et al*., 1994; Jiang *et al*., 1992). CTAB is also commonly used in plant cell extractions (Ranamukhaarachchi *et al*., 2000). To the best of our knowledge, CTAB has not yet been used for the extraction of Map from faeces.

Materials and methods

One gram of faeces was suspended in 20 ml of a 0.2 M NaOH solution. The samples were filtered through a sterile gauze and centrifuged at 3,000 x *g* for 1 hour. The supernatant was discarded and the pellet was suspended in 1 ml MilliQ water. The suspension was transferred to a 1.5 ml eppendorf tube and centrifuged for 10 min at 12,000 x *g*. The supernatant was discarded and the pellet was suspended in 147 µl TEN buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM EDTA), 20 µl of a 5 M NaCl solution and 33 µl of a 10% CTAB solution. The samples were incubated for 10 min at 60°C, followed by centrifugation for 10 min at 12,000 x *g*. The supernatant was discarded and the pellet was used for the DNA isolation.

4.3.1.3 Use of NaOH and Stomacher

Stomacher lab blenders offer fast, reliable blending and homogenisation of laboratory samples. Through their unique action, in which paddles apply pressure to a sample within a sterile, disposable sample bag, contents are blended and homogenised, and deep-seated organisms are effectively released. Stomacher lab blenders are commonly used in food microbiology for the homogenisation of food samples. Garrido *et al.* (2000) have used the stomacher to homogenise faecal samples for Map detection. A combination of the NaOH protocol (Bleumink-Pluym *et al*., 1994) and the protocol of Garrido *et al*. (2000) was evaluated.

Materials and methods

One g of faeces was homogenised in a Stomacher lab blender (Seward, Norfolk, UK) for 30 s in 20 ml of 0.2 M NaOH. After sedimentation for 15 min, 18 ml supernatant was transferred from the sample bag to a 50 ml tube and centrifuged for 30 min at 3,000 x *g*. The supernatant was discarded and the pellet was resuspended in 1 ml MilliQ water, transferred to a 1.5 ml eppendorf tube and washed twice with 1 ml MilliQ water. The tubes were then centrifuged for 10 min at 12,000 x *g*. The supernatant was discarded and the pellet was used for further DNA isolation.

4.3.1.4 Use of Stomacher and SDS

Garrido *et al*. (2000) used sodium dodecyl sulphate (SDS) as the chemical reagent in the homogenisation of faeces with the stomacher lab blender. SDS is a detergent and destroys the integrity of the lipid bilayer. In the presence of sodium acetate in high molarity, SDS forms insoluble complexes with proteins and polysaccharides.

Materials and methods

One g of faeces was homogenised in a Stomacher lab blender for 30 s in 20 ml of a solution containing 5% SDS (Sigma). After sedimentation for 15 min, 18 ml of supernatant was transferred to a 50 ml tube and centrifuged for 15 min at 3,000 x *g*. The supernatant was discarded and the pellet was resuspended in 1 ml of phosphate buffered saline (PBS). The suspension was transferred to a 1.5 ml eppendorf tube and washed twice with PBS. The tubes were centrifuged for 10 min at 9,400 x *g*, the supernatant was discarded and the pellet was used for further DNA isolation.

4.3.1.5 Use of trypsin

Trypsin is a serine protease cutting protein chains in fragments of only just a few amino acids long. Cell wall proteins rich in serine will disintegrate and the cell wall will loose its integrity. Trypsin is commonly used to disaggregate tissues before cell culture (Paul, 1965). The usefulness of this enzyme for Map DNA extraction will be tested with the following protocol.

Materials and methods

Two g of faeces, 10 ml of physiological saline and glass beads were vortex mixed in a 50 ml tube for 1 min. Two ml of a 4% trypsin solution (Sigma) was added and the samples were mixed again for 1 min. The 50 ml tubes were incubated in a shaking water bath (300 rpm) at 37°C for 1 hour. After sedimentation for 15 min, 1 ml of the supernatant was transferred to a new 1.5 ml tube, centrifuged at 12,000 x *g* for 10 min and the pellet was used for further DNA isolation.

4.3.1.6 Use of trypsin and Stomacher

This protocol is a combination of the mechanical (Stomacher) with an enzymatic (trypsin) homogenisation method.

Materials and methods

Five ml physiological water was added to 1 g of faeces, followed by mixing in a stomacher for 1 min. One ml of 4% trypsin solution (Sigma) was added and the sample was mixed again in the stomacher for 30 s. After an incubation of 1 h at 37°C, the samples were again mixed with a stomacher for 30 s. Sedimentation was done at RT for 15 min. One ml of the supernatant was transferred to an 1.5 ml tube and centrifuged at 12,000 x *g* for 10 min. The pellet was used for further DNA isolation.

4.3.1.7 Use of Tween-20

Polyoxyethylene sorbate compounds (Tweens) belong to a class of non-ionic surfactants used to lyse cells by solubilising the membranous lipids and proteins. Tween-20 is the most commonly used Tween in extraction procedures (Purohit *et al.*, 2003).

Two different approaches were tested. In the first approach, the whole supernatant after sedimentation was used requiring a long centrifugation step. The second procedure was more rapid and easier, because only 1 ml of supernatant obtained after sedimentation was used.

Materials and methods

Protocol A

One g of faeces was vortex mixed with glass beads (0.4 mm Ø) and 10 ml of a 2% Tween-20 solution during 1 min. The samples were left for 30 min at RT to allow sedimentation. The supernatant was transferred to a new 50 ml tube and centrifuged for 30 min at 1,500 x *g*. The supernatant was discarded and the pellet was suspended in 1 ml MilliQ water. The suspension was transferred to a 1.5 ml eppendorf tube and centrifuged for 5 min at 12,000 x *g*. The supernatant was discarded and the pellet was used for further DNA isolation.

Protocol B

One g of faeces was suspended in 10 ml of a 2% Tween-20 solution and glass beads (0,4 mm Ø) were added. The samples were mixed vigorously on a vortex mixer for 1 min. The samples were left for 30 min at RT. One ml supernatant was transferred to a new microcentrifuge tube and centrifuged at 6,000 x *g* for 1 min. The supernatant was transferred to a new microcentrifuge tube. The tube was centrifuged at 14,000 x *g* for 10 min. The supernatant was discarded and the pellet was used for DNA isolation.

4.3.1.8 Use of Tween–80

Van Boxtel *et al*. (1990) studied the influence of Tweens on Map colony morphology on Middlebrook 7H9 agar. The cell walls appeared less rough at higher concentrations of Tween-80 and the polysaccharide-rich outer layers were damaged. The colonies of Map grown in the presence of Tween-20 were rougher than those of Map grown with Tween-80. This implies that Tween-80 damages the Map cell wall more than Tween-20.

Materials and methods

One g of faeces and 10 ml of a 2% Tween-80 solution were vortex mixed during 1 minute. The samples were left standing for 30 min at RT to allow sedimentation. The supernatant was transferred to a new 50 ml tube and centrifuged for 30 min at 1,500 x *g*. The supernatant was discarded and the pellet was suspended in 1 ml MilliQ water. The suspension was transferred to a 1.5 ml eppendorf tube and centrifuged for 5 min

at 12,000 x *g*. The supernatant was discarded and the pellet was used for further extraction.

4.3.1.9 Use of Sample buffer

The sample buffer, used by Singh (1998) and Fang *et al.* (2002) for DNA extraction of Map, consists of NaOH, Tween-80 and antifoam. NaOH will destroy bacteria except mycobacteria, while Tween-80 will break up the clumps. In combination with a vortex step and glass beads, the faecal samples will be homogenised.

Materials and methods

One g of faeces was suspended in a 50 ml tube containing 10 glass beads (4mm ∅) and 25 ml sample buffer (0.2 M NaOH, 0.1% Tween-80, 1 µg/l antifoam 289 (Sigma)) by heavy vortexing for 2 min. The samples were allowed to settle for 30 min at RT. The supernatant was filtered through a sterile gauze and the filtrate was collected in a fresh 50 ml tube. The tubes were centrifuged at 3,000 x *g* for 30 min. The supernatant was discarded and the pellet was resuspended in 20 ml MilliQ water. After centrifugation at 3,000 x *g* for 30 min, the supernatant was discarded and the pellet was resuspended in 1 ml MilliQ water. The suspension was transferred to a 1.5 ml tube and centrifuged for 10 min at 14,000 x *g*. The supernatant was removed and the pellet was used for further extraction.

4.3.2 DNA isolation

After homogenisation and concentration of the faecal samples, the DNA needs to be recovered from the sample and should be free from PCR inhibitors. First, the DNA should be released from the bacteria. The rigid bacterial cell wall can be broken up by agitation at high speed in the presence of very small beads with an optimal size of 0.1 mm Ø (Biospec: technical information, 2001). Disruption occurs by shearing and the crushing action of the beads as they collide with the cells.

A total DNA isolation can thereafter be performed with a mixture of phenol : chloroform : isoamylalcohol (PCI). However, we preferred not to use hazardous reagents like PCI and chose to use a solid phase isolation for Map (zirconium beads)

described by Bleumink-Pluym *et al.* (1994). A second new DNA isolation technique for Map tested was the Mangiapan-extraction based on a sequence-capture (Mangiapan *et al.,* 1996).

4.3.2.1 Zirconium beads

Zirconium beads are used to disrupt the cell wall of mycobacteria. The beads can also bind DNA allowing its simple purification by washing the beads. Afterwards the DNA can be eluted of the beads. This DNA isolation method has been used by Bleumink-Pluym *et al.* (1994) for the detection of Map in faeces and tissues.

Materials and methods

The pellet was suspended in 750 µl of 7 M NaI (Sigma), 500 µl of 0.1 mm ∅ zirconium beads (Biospec, Merlin Diagnostics, Breda, The Netherlands) were added and the samples were left to stand on ice for 30 minutes. The sample was then beated for 3 min in a Mickle apparatus (HH Mickle, Gomshall, Surrey, UK). The tubes were left at RT for 5 min to allow the zirconium beads to settle down. The supernatant was discarded and the beads were washed three times with 900 µl of a solution containing 150 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 50 % ethanol. The beads were subsequently washed twice with 900 µl of 70% ethanol and once with 900 µl of acetone. The acetone was discarded and the beads were allowed to dry for 20 min at 50°C. The DNA was then eluted from the beads using 120 µl MilliQ water. The supernatant was transferred to a new tube and 5 µl was used in the PCR.

4.3.2.2 Sequence capture DNA isolation (Mangiapan)

Magnetic particles covalently bound to streptavidin are used for the directed immobilisation of both double-stranded and single-stranded biotinylated DNA. The remarkable stability and strength of the non-covalent biotin-streptavidin interaction allows DNA manipulation reactions without interfering with the binding of the DNA to the beads (Uhlen, 1989). Beads containing biotinylated oligonucleotides can capture single-stranded DNA out of a complex mixture containing large numbers of host cells and other organisms. This will result in the elimination of irrelevant DNA and potential inhibitors present in the original sample prior to amplification (Figure

10). The technique is based on hybridisation of the oligonucleotide to the specific DNA under specific conditions of time and temperature. Mangiapan *et al.* (1996) used this method for the detection of *Mycobacterium tuberculosis* in paucibacillary clinical samples from patients. These investigators also found that the addition of magnetic beads had no deleterious effect on amplification during PCR.

The sequence capture approach has not yet been used in the isolation of DNA from faecal samples.

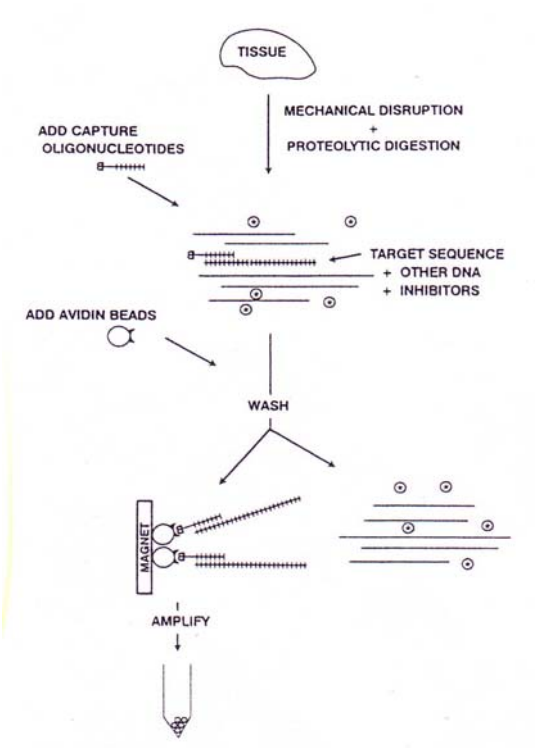


Figure 10: The sequence capture technique
(Mangiapan *et al*., 1996)

Materials and methods

The pellet resulting from the homogenisation step was mixed with 500 µl of TEN-buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM EDTA), 500 µl of glass beads (0.1mm Ø) and 50 µl of proteinase K (Sigma, 20 mg in 1 ml MilliQ water). The tubes were subjected to vortex mixing for 50 s. After an overnight incubation at 50°C (1000 rpm, Thermomixer, Eppendorf, Hamburg, Germany), the tubes were

again agitated on a vortex for 50 s. The samples were boiled for 10 min and immediately cooled on ice.

Two hundred µl of a 3.75 M NaCl solution containing 2.5 pmol of biotinylated capture oligonucleotides IS*900*SB and IS*900*RB (Table 7) was added to the samples. Tubes were incubated at 60°C for 3 h under agitation to allow hybridisation. Ten µl of streptavidin Dynabeads (Dynal, Oslo, Norway), washed according to the manufacturer's instructions was added and the incubation was continued for 2 h at RT. The beads were captured with a magnet, washed twice with 900 µl of a 10 mM Tris-HCl solution containing 0.1 mM EDTA, pH 8 and were resuspended in 20 µl MilliQ water. Ten µl was used for amplification.

4.3.3 Combined methods

In the combined methods, homogenisation and DNA isolation occur in only one step instead of two separate ones.

4.3.3.1 Roman-protocol

Several lysis techniques and DNA extraction procedures for environmental samples (soil) were developed in the Mycobacteriology laboratory of the ITM (Roman Kotlowski, unpublished results). Of each chemical, the optimal concentration was determined allowing cell lysis without inhibiting the PCR reaction. The lysis buffer (Triton X-100, sarkosyl, SDS, guanidine thiocyanate) is used in conjunction with phenol : chloroform : isoamylalcohol (PCI). The lysis buffer releases the DNA from the cells (total lysis) and the PCI separates the DNA from the rest of the sample.

Materials and methods

One gram of faeces was mixed with 7 ml lysis buffer (5% sarkosyl (Sigma), 0.5% Triton X-100 (Sigma), 0.5 % sodium citrate, 0.25% SDS, 4 M guanidine thiocyanate, 1 mM Tris-HCl, 0.5 mM EDTA), 7 ml phenol : chloroform : isoamylalcohol (25:24:1) and 10 glass beads (0.4 mm Ø). After incubation at –20°C for 1 h, the mixture was centrifuged at 1,600 x *g* for 4 min. The aqueous phase was transferred to a new tube and mixed with 5 ml chloroform : isoamylalcohol (24:1). The mixture was

subsequently centrifuged at 1,600 x *g* for 4 min. The resulting aqueous phase was transferred to a new tube and mixed with 0.1 vol of 3 M sodium acetate and 2 vol of ice cold ethanol. After an overnight incubation at –20°C, the precipitated DNA strands were transferred to a small eppendorf tube. The tube was centrifuged for 5 min at 1600 x *g*. The supernatant was discarded and the pellet was washed twice with 900 µl of 70% ethanol (4°C). The pellet was subsequently dried and suspended in 100 µl TE buffer (10 mM Tris-HCl,0.1 mM EDTA pH 8).

4.3.3.2 Roman protocol with additional PCI step.

The protocol as described in 3.4.3.1 can be combined with an additional PCI step to remove more inhibiting substances.

Materials and methods

One gram of faeces was mixed with 7 ml lysis buffer (5% sarkosyl, 0.5% Triton X-100, 0.5 % sodium citrate, 0.25% SDS, 4 M guanidine thiocyanate, 1 mM Tris-HCl, 0.5 mM EDTA), 7 ml phenol:chloroform:isoamylalcohol (25:24:1) and 10 glass beads (0,4 mm Ø). After incubation at –20°C for 1 h, the mixture was centrifuged at 1,600 x *g* for 4 min. The upper aqueous phase was transferred to a new 50 ml tube and mixed with 5 ml phenol:chloroform:isoamylalcohol (25:24:1). The mixture was then centrifuged at 1,600 x *g* for 4 min. The aqueous phase was transferred to a new tube and mixed with 5 ml chloroform : isoamylalcohol (24:1). The mixture was subsequently centrifuged at 1,600 x *g* for 4 min. The resulting aqueous phase was transferred to a new tube and mixed with 0.1 vol of 3 M sodium acetate and 2 vol of ice cold ethanol. After an overnight incubation at –20°C, DNA strands were transferred to a small eppendorf tube. The tube was centrifuged for 5 min at 1,600 x *g*. The supernatant was discarded and the pellet was washed twice with 900 µl of 70% ethanol (4°C). The pellet was subsequently dried and suspended in 100 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8).

4.3.3.3 Lysis solution

Another combined DNA extraction protocol was composed based on literature research. Lysis (Tween-20, Triton X-100) was combined with a Chelex-100 step.

Triton-X-100 is a non-ionic detergent that affects the cytoplasmatic membrane. Chelex-100 is a chelating resin specifically designed for extraction of PCR-ready template DNA. Chelex-100 binds contaminating metal ions that can catalyse the digestion of DNA during boiling (Walsh *et al*., 1991). The boiling step is necessary to lyse the cells and to release the genomic DNA. After boiling, the resin needs to be removed from the sample because Chelex can inhibit the PCR reaction by binding the Mg^{2+} ions.

Materials and methods

One g of faeces was suspended in 2 vol lysis solution (100 mM Tris-HCl, 0.5% Tween-20, 1.0% Triton X-100) and centrifuged for 90 s at 1,000 x *g*. The supernatant was subsequently transferred to a new tube and centrifuged for 15 min at 12,000 x *g*. The pellet was resuspended in 1 ml of lysis solution with addition of 100 µl of 2% SDS and 100 µl of Proteinase K (Sigma, 20 mg /ml in MilliQ water). The tubes were then incubated for 4 h at 58°C. After centrifugation for 10 min at 12,000 x *g*, the supernatant was discarded. In a next step, 250 µl of a 20% Chelex-100 solution in MilliQ water (Biorad, Hercules, CA, USA) was mixed with the pellet on a vortex mixer and incubated during 15 min at 100°C. The samples were then centrifuged for 90 s at 1,400 x *g* and the supernatant was transferred to a new tube. Five µl of the supernatant was used in the PCR reaction.

4.3.4 PCR

A nested PCR approach was used for the amplification of Map DNA from the extracts, but protocols differed depending on the extraction method used. Primary PCR on extracts of faeces with zirconium beads and the combined methods were performed in a final volume of 25 µl with 5 µl of sample. The primary PCR after faecal extraction with the Mangiapan procedure was performed in a final volume of 50 µl with 10 µl of sample. PCR conditions for the primary and secondary PCR were as described in 3.2.1.4 with following variables:

Primary PCR			
Primer: IS*900*S1			
Primer: IS*900*R3(2)			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	68	45	
Extension	72	45	

Secondary PCR			
Primer: IS*900*S2			
Primer: IS*900*R1			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	25
Annealing	68	45	
Extension	72	45	

Primer	**DNA sequence**	**Amplicon size**
IS*900*S1	5' GGG TTG ATC TGG ACA ATG ACG GTT A 3'	575 bp
IS*900*R3(2)	5' AGC GCG GCA CGG CTC TTG TT 3'	
IS*900*S2	5' GGA GGT GGT TGT GGC ACA ACC TGT 3'	453 bp
IS*900*R1	5' CGA TCA GCC ACC AGA TCG GAA 3'	
Probe	**DNA sequence**	
IS*900*SB	5' biotin-GTT CGG GGC CGT CGC TTA GGC T 3'	
IS*900*RB	5' biotin-GAG GAT CGA TCG CCC ACG TGA 3'	

Table 7: DNA sequences of primers and biotinylated probes used in the PCR procedure for faeces

4.3.5 Results

The detection limits obtained with the different homogenisation and DNA isolation methods are summarized in Table 8.

The NaOH homogenisation protocol in combination with the zirconium DNA isolation and with a centrifugation step for 15 min at 1,000 x *g* gave no PCR results. A faecal sample spiked with 10^6 Map per g faeces was used to modify the extraction. The number of Map cells in each fraction was evaluated by Ziehl-Neelsen staining and microscopic counting. Using the centrifugation step described in the protocol of

Bleumink-Pluym *et al.* (1994), too many Map bacteria remained in the supernatant. Several time periods and centrifugal forces were tried but centrifugation for one h at 3,000 x *g* gave the best results. After amplification, a detection limit of 10^4 Map cells per g faeces was obtained. The same homogenisation step in conjunction with the Mangiapan DNA isolation yielded a detection limit of 10^3 Map cells per g faeces, i.e. a tenfold improvement.

The addition of CTAB to the NaOH homogenisation did not improve the detection limit. Moreover, no amplification was observed when using zirconium beads. With the Mangiapan procedure, the detection limit became 10^4 Map cells per g faeces.

NaOH in combination with the stomacher and the zirconium DNA isolation gave a variable detection limit of 10^4 – 10^5 Map cells per g. After extension of the centrifugation step to 1 h at 3,000 x *g* the detection limit was 10^4 Map cells per g faeces. In combination with the Mangiapan procedure, the detection limit was 10^4 Map cells per g faeces. Sporadically 10^3 Map cells per g faeces could be detected.

The same observation was made for SDS homogenisation in combination with the stomacher. The detection limit for the zirconium procedure was 10^4 Map cells per g faeces. Similar detection limits were observed for Mangiapan DNA isolation but sporadically 10^3 Map cells per g faeces could be detected.

The use of the stomacher on faeces was not practical. The plant material sticked to the sample bag and it was impractical to transfer the supernatant from the sample bag to the falcon tube with a pipet.

The trypsin protocol with stomacher and the zirconium DNA isolation gave a negative result. With the Mangiapan procedure, only sporadic amplification occurred. The trypsin protocol in conjunction with zirconium DNA isolation using a falcon tube gave no amplification. With the Mangiapan DNA isolation the detection limit was determined at 10^4 Map cells per g faeces. Sporadic 10^3 Map cells per g faeces could be detected.

As the zirconium DNA isolation was less performant, the Tween homogenisations were only tested in combination with Mangiapan DNA isolation. Of all homogenisations, the Tween homogenisation gave the best results. A detection limit of 100 Map cells per g faeces was obtained. We could not observe differences between Tween-20 protocol A, Tween-20 protocol B and a procedure with Tween-80. The homogenisation with Tween-20 (protocol B) was shorter and was chosen for further evaluation of the test.

The Roman extraction with one PCI step gave no amplification. With an additional PCI step, a detection limit of 10^4 Map cells per g faeces could be observed. In an attempt to obtain a higher detection limit, the DNA extracted with the roman protocol was subjected to Mangiapan DNA isolation. With this combination a detection limit of 10^3 Map cells g faeces could be obtained. However, the Roman extraction method uses hazardous agents and is time-consuming (especially in combination with the Mangiapan extraction). When using the lysis buffer, no amplification could be detected.

Homogenisation method	**Detection limits (number of cells) for 2 DNA isolation protocols**	
	Zirconium procedure	**Mangiapan procedure**
NaOH	10^4	10^3
NaOH + CTAB	/	10^4
NaOH + stomacher	10^4	10^3– 10^4
SDS + stomacher	10^4	10^3– 10^4
Trypsin	/	/
Trypsin + stomacher	/	10^4
Tween-20 protocol A	ND	10^2
Tween-20 protocol B	ND	10^2
Sample buffer	ND	10^2

Table 8: Detection limit (Map cells per g faeces) obtained after different homogenisation and DNA isolation protocols.
(ND: not done, /: no amplification)

4.3.6 Conclusion

The Mangiapan DNA isolation was found to give the best results. Most likely, this is due to the selective binding of the Map DNA to a solid phase allowing an optimal removal of inhibitory substances and non-specific DNA. With the other extraction methods, much of the non-specific DNA remains in the extract and can inhibit the PCR reaction. It was also noticed that many steps in the homogenisation of the samples were not always the best option. The centrifugal forces and duration of centrifugation steps should be carefully optimized to avoid loss of bacteria.

The best extraction method, i.e. homogenisation with Tween-20 in combination with the Mangiapan DNA isolation, gave a detection limit of 100 Map cells per g faeces. The new extraction method needed to be evaluated on a range of clinical samples.

4.4 Evaluation of the extraction method

The faecal DNA extraction method was developed using spiked samples, but these artificially made samples differ from clinical field samples. Therefore, a large number of faecal field samples were tested with the newly developed method and compared with conventional culture results.
Because of the promising results of the Mangiapan DNA isolation method for faeces, the same approach was used and evaluated to extract Map DNA from tissues.

4.4.1 Materials and methods

4.4.1.1 Collection of samples

Spiked faecal samples

Faecal samples negative for Map culture from Belgian cattle were collected. These faecal samples were spiked with a known amount of Map cells ATCC 19698 (range 10 – 10^6 Map cells per g faeces). The faecal spiked samples were tested ten times with PCR and were used in each DNA extraction as a positive extraction control.

Faeces of experimentally infected cattle

At CERVA (Veterinary and Agrochemical Research Centre, Ukkel, Belgium), 5 young calves were experimentally infected by the oral route by Dr. J. Godfroid and colleagues. In the framework of a collaborative study, we were provided with periodically taken faecal samples of these calves (n=230).

Faecal samples of a naturally infected dairy herd

Four sample collections (n=154) were organized in two Belgian dairy herds from two different provinces with a known paratuberculosis history. Sample collections 1 to 3 were performed in Hamont-Achel. Sample collection 4 was performed by Dr. J. Wullepit and colleagues (Dierengezondheidszorg Vlaanderen, Torhout).

Faecal and tissue samples from wild red deer (Cervus elaphus)

During necropsy of hunter-killed free-living cervids (from southern Belgium), faecal and tissue samples were collected by Dr. A. Linden and colleagues (University of Liège, Belgium). A total of 81 mesenteric lymph nodes, 69 spleens, 2 small intestines, 2 large intestine and 25 faeces were sampled from Map suspected animals, based on macroscopic inspection and positive serology (HerdChek, IDEXX).

4.4.1.2 Decontamination and culture

All faecal samples (except the spiked samples) were cultured on Löwenstein-Jensen media supplemented with mycobactin J (1 mg/l), PANTA plus (40 ml/l) and sodium pyruvate (4g/l). Two commonly used decontamination methods were used. The double incubation method of Whitlock and Rosenberger (1990) was used for decontamination of the faecal samples from the Belgian diary herds. Briefly, 2-5 g faeces were mixed with 10 ml of sterile saline. The tubes were allowed to stand for 30 min at RT. Five ml of the supernatant was transferred to a new tube containing 25 ml of a 0.9% hexadecylpyridinium chloride solution (Sigma) in half-strength Brain Heart Infusion Broth (LabM, Lancaster, UK). Samples were left for 24 h at 37°C. After centrifugation at 900 x *g* for 30 min, the pellet was resuspended in 1 ml of an antibiotic mixture containing 100 µg vancomycin, 100 µg nalidixic acid and 50 µg amphotericin B (all Sigma reagents) in 1 ml distilled water. The sediment was used to seed the culture tubes.

Faecal samples from deer and the experimentally infected cattle were decontaminated with the oxalic acid method (Beerwerth *et al.,* 1969). Briefly, 0.5 g of faeces was suspended in 5 ml distilled water. Five ml of the surface liquid was transferred to a fresh tube and mixed with 5 ml of 1 M sodium hydroxide, 5 ml of a 0.2% malachite green solution (Merck, Overijse, Belgium) and 1 ml of a 0.8% acitidone solution (Sigma). The samples were incubated for 30 min at RT. After centrifugation at 3,000

x *g* for 15 min, the supernatant was transferred to a fresh tube and mixed with 10 ml of a 5% oxalic acid solution. After centrifugation at 3,000 x *g* for 15 min, the supernatant was discarded and the pellet was resuspended in 1 ml of distilled water. This suspension was used to inoculate culture tubes.

The tissue samples were decontaminated with the reverse Petroff method (adapted from Petroff, 1915). About 1 g of minced tissue was suspended in 10 ml of PBS. Five ml of the supernatant was transferred to a fresh tube and mixed with 5 ml of a 1M HCl solution. After 20 min incubation, the suspension was neutralized with a 1M NaOH solution.

After centrifugation at 3,000 x *g* for 20 min, the supernatant was discarded and the pellet was resuspended in 1 ml of sterile distilled water. This suspension was used to inoculate the Löwenstein-Jensen media supplemented with mycobactin J (1 mg/l), PANTA plus (40 ml/l) and sodium pyruvate (4 g/l).

The tubes were incubated at 37°C for up to 8 months and examined weekly for signs of bacterial growth.

4.4.1.3 Sample preparation before sequence capture

In case of tissue samples, 250 µl of decontaminated tissue suspension was transferred to a 1.5 ml tube and mixed with 250 µl of buffer A (200 mM Tris-HCl pH 7.4, 300 mM NaCl, 100 mM EDTA). In case of faecal samples, 1 g faeces was suspended in 10 ml of a 2% Tween-solution and glass beads were added. The samples were mixed vigorously on a vortex mixer for 1 min and left for 30 min. One ml of supernatant was transferred to a new microcentrifuge tube and centrifuged at 6,000 x *g* for 1 min. The supernatant was transferred to a new microcentrifuge tube and centrifuged at 14,000 x *g* for 10 min. The pellet was suspended in 500 µl of a 100 mM Tris-HCl solution containing 150 mM NaCl and 50 mM EDTA.

4.4.1.4 Sequence capture DNA isolation

Sequence capture DNA isolation was performed as described in 4.3.2.2.

4.4.1.5 PCR

A nested PCR was used. Primary PCR on extracts of tissue was performed in a final volume of 25 µl with 5 µl sample. The primary PCR after faecal extraction was performed in a final volume of 50 µl with 10 µl of sample. PCR conditions for the primary and secondary PCR were as described in 3.2.1.4 with following variables:

Primary PCR			
Primer: IS*900*S1			
Primer: IS*900*R3(2)			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	68	45	
Extension	72	45	

Secondary PCR			
Primer: IS*900*S2			
Primer: IS*900*R1			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	25
Annealing	68	45	
Extension	72	45	

4.4.2 Results

The PCR procedure yielded a positive PCR for 98 out of 230 faecal samples from experimentally infected animals, of which only 47 samples were found positive on culture. Only three culture positive samples remained negative on PCR. Evaluation of the method on faecal specimens from Belgian dairy herds with a known history of paratuberculosis was not possible in a reliable way due to fungal overgrowth of the cultures from sample collections 2 and 3. In sample collection 1 (n=20), where no overgrowth was observed, 4 PCR positive samples were detected of which 3 were culture positive. In sample collections 2 and 3 (n=34), suffering from massive fungal contamination, 11 PCR positive faecal samples were obtained but culture results were not available. In sample collection 4 (n=100), 4 samples were both PCR and culture positive. When extractions were redone on the same faecal sample, results were the same.

PCR and culture results on tissue and faecal samples of the wild red deer are summarized in Table 9.

	Number of samples	**Number of PCR positive samples**	**Number of culture positive samples**
Experimentally infected animals	*230*	*98*	*47*
Belgian Dairy herds	*154*	*19*	*7*
sample collection 1	20	4	3
sample collections 2 and 3	34	11	contamination
sample collection 4	100	4	4
Cervids	*179*	*83*	*65*
lymph node	81	41	28
spleen	69	20	17
small intestine	2	2	2
large intestine	2	2	2
faeces	25	18	16
Total	*563*	*200*	*119*

Table 9: Overview of the PCR and culture results for different field samples (contamination: fungal overgrowth)

Eighteen out of 25 faecal specimens were PCR positive, whereas only 16 were culture positive. All tested small and large intestine samples were both PCR and culture positive. Forty-eight lymph node samples were PCR positive, only 28 culture positive. For spleen samples, 20 samples were PCR positive, whereas 17 were culture positive. Seven culture positive tissue samples were PCR negative. Table 10 summarises the results for all faecal and tissue samples.

Sample	Number of samples	Number of PCR positive samples	Number of culture positive samples
Tissue	154	65 (42.2%)	49 (31.8%)
Faeces	409	135 (33.0%)	70 (17.1%)
Total	563	200 (35.5%)	119 (21.1%)

Table 10: Overview of PCR and culture results on tissue and faecal samples using DNA extractions according to Mangiapan *et al.*, 1996

4.4.3 Discussion

We report a DNA-extraction method for the detection of Map in faecal and tissue samples. In general, PCR assays may give false negative results when applied to field samples. This may be due to a variety of inhibitory substances such as haemoglobin, bilirubin, plant components and chelating agents (Widjojoatmodjo *et al.*, 1992; Christopher-Hennings *et al.*, 2003). Difficulties are experienced in recovering DNA from small numbers of micro-organisms in field specimens, especially in complex samples as faeces with their extremely varying composition (van der Hoek *et al.*, 1995; Englund *et al.*, 2001). Excessive non-specific DNA derived from the host or other microbes can also prevent amplification (Marsh *et al.*, 2000).

Using a nucleic acid sequence capture technique, highly purified templates were recovered by preliminary binding the target DNA to a biotinylated DNA probe followed by capture of the probe on streptavidin-coated magnetic beads, hereby separating Map DNA from non-specific DNA and PCR inhibitory substances (Marsh *et al.*, 2000). Although magnetic-particle technology has been reported as a tool for isolating specific DNA targets from complex mixtures, it results rarely in a complete absence of PCR inhibition. In our study, 10 culture positive (7 tissue and 3 faeces) samples were negative with PCR. This could be explained by the inhibition of the PCR assay.

Our faecal extraction method has a detection limit of 100 Map per g faeces for spiked samples. This is comparable with the detection limit reported for faecal culture (Merkal *et al.*, 1973). Millar *et al.* (1995) also described a sequence capture technique

for detecting Map in faeces samples, based on the use of a IS*900* probe of 513 bp, giving a detection limit of 2500 Map per g of faeces.

In 2002, Halldorsdottir *et al.* described a detection method for Map in faeces using buoyant density centrifugation, sequence capture PCR based on the one described by Mangiapan *et al.* (1996) and dot blot hybridization. They reported a sensitivity of 1000 CFU per g of faeces. The differences with our sequence capture procedure are the probe sequence and the dot blot hybridisation step. We used two 20 bp probes, while Halldorsdottir used a probe of 60 bp. We have chosen to perform a nested PCR instead of dot blot hybridisation. By using the nested PCR approach, i.e. a two step amplification procedure, the influences of inhibitory substances can to some extent be overcome. But due to the high sensitivity of this method, false positive results resulting from carry-over or cross contamination could occur more frequently. Precautions must be taken to circumvent this problem (Belak *et al.*, 1993) and real-time PCR could be an alternative to reduce the chance of DNA contamination. We could detect more positive faecal samples with sequence-capture PCR (33.0%) than with culture (17.1%). There is a slight possibility that the higher percentage of positives in PCR is due to amplicon contamination during PCR. Negative controls were included in every PCR run and the PCR facilities in our laboratory have a "three room system". A possible explanation for the higher number of negative samples in culture could be the heterogenous distribution of Map organisms in faecal samples due to clumping. This phenomenon has been described previously (Sockett *et al.,* 1992) and seems more pronounced in light shedders. Another explanation may relate to the decontamination step before culture. Decontamination reduces the number of viable cells meaning that they can not be cultured but can still be detected by PCR. We were unable to obtain culture results of 34 faecal samples on a total of 409 faecal samples due to fungal overgrowth. Contamination of media is one of the major problems in the culture of Map. Overgrowth by non-acid fast organisms can easily mask the presence of Map colonies and may inhibit Map growth. The use of a PCR overcomes this contamination problem associated with culture.

The sequence capture technique can be used on a variety of other clinical samples when combined with an appropriate sample preparation. We tested the performance of sequence capture PCR on tissues by comparing culture and PCR on 154 samples from cervids. More tissue samples were found positive with PCR (42.2%) than with culture (31.8 %). The use of the new extraction method in combination with the new

specific primers could reveal more information about Map in tissues from patients with Crohn's disease. A large-scale survey in Crohn's disease and non-Crohn's disease patients and their environment could clarify the hypothesis of a possible link between the disease and Map. The spheroplast forms of Map are difficult to isolate from Crohn's diseased tissue (Hines *et al.*, 2003). A RNA extraction could be used as an alternative method if it is important to demonstrate viability of the Map spheroplasts in the gut.

The sequence-capture DNA isolation is based on capture with IS*900* probes. When f57 is used as a back up system, it is recommended to adapt the biotinylated probes for f57 sequence capture. The detection limit for f57 sequence capture might be higher because f57 is not a multicopy sequence like IS*900*. Also, a sequence capture with f57 and IS*900* probes should be tested in combination with the multiplex PCR.

The usefulness of the sequence capture technique was also shown in experiments in the Veterinary laboratory of the ITM for the detection of *Taenia saginata* and *Taenia solium* cells in human faeces.

4.5 Conclusion

A sensitive two-day PCR procedure was developed to detect Map in faecal and tissue samples without the use of hazardous reagents. The detection limit was 100 Map cells per g of faeces, comparable with the sensitivity obtained by faecal culture and better than that of the existing PCR methods. Moreover, the PCR method allowed the detection of more positive samples than the culture method. It can be concluded that the reported faecal DNA extraction method is a valid alternative to the time-consuming Map culture method. Furthermore, the Mangiapan approach was successfully applied to tissue samples.

At this stage, the newly developed faecal DNA extraction in combination with the specific primers could now be used in an epidemiological survey on the presence of Johne's disease in the Antwerp Zoo.

4.6 Alternative approach: concentration on a hydrophobic column

The special composition of the cell wall makes Map bacteria very hydrophobic. This feature could be explored as an alternative to separate and concentrate Map bacteria. It is assumed that Map present in a matrix would bind more strongly to hydrophobic materials than do other bacteria, which could supposedly be eluted more easily. The Map cells will remain on the hydrophobic column and could be subsequently eluted with a strong (i.e. a non-polar) eluent. A concentration step before DNA extraction would be advantageous when dealing with faecal samples of subclinically infected animals. These animals only shed a small amount of Map cells in their faeces.

The hydrophobic C8 polymeric beads (Biorad, Hercules, CA, USA) were previously used to concentrate and purify Map bacteria from milk samples (D'Haese *et al*., 2003) before enumeration of viable bacteria with the ChemScan®.

4.6.1 Materials and methods

A plastic column for solid phase extraction was filled with 0.5 g of C8 polymeric beads (Biorad) and sterilised. The column was washed with 2 ml of a 0.5M NaCl solution and then loaded with 2 ml sample. After a washing step with 3 ml of 0.5 M NaCl, the Map bacteria were eluted with 2 ml n-propanol.

The Map positive faeces samples were first homogenised with a 2 % Tween-20 solution before being loaded on the column. As results were disappointing, some additional experiments were performed using pure bacterial suspensions.

The bacterial suspensions containing different numbers of bacteria (Table 11) were loaded on the columns. For each Map dilution, one loaded column (before elution) was checked with PCR to detect absorbed bacteria. A second column was washed and eluted with n-propanol. The column after elution was also checked with PCR for residual bacteria. All fractions (first-pass fraction upon loading, elution fraction) were checked with PCR. DNA extractions of all fractions were done according to the Mangiapan method (see 4.3.2.2).

The C-8 beads were extracted by boiling. One half of a column was mixed with 250 µl MilliQ and boiled for 15 min while shaking at 1,400 rpm (Thermomixer, Eppendorf). After centrifugation for 5 min at 12,000 x *g*, the supernatant was transferred to a new tube and 5 µl was used for PCR amplification.

4.6.2 Results

The first tests were performed on homogenised faeces samples. The eluate still contained background flora and faecal material and no PCR amplification of Map could be detected. In order to analyse the interaction between Map and the column with the C8-beads, it was decided to use defined bacterial suspensions for loading the column. Inconsistent results were observed (Table 11). The column still contained Map bacteria even after elution with a non-polar solvent like n-propanol. Moreover, the PCR amplification was already positive with an initial bacterial load of 10^3 Map cells whereas minimally 10^5 Map cells were required for PCR detection on a loaded column. Map bacteria were detected in the first-pass fractions collected upon loading of the column and loss of bacteria during the wash steps was also observed. All tests were repeated and gave comparable results.

	Presence of Map DNA in the fraction and on the columns				
Number of Map loaded	**Column before elution**	**Column after elution**	**First-pass fraction**	**Wash fraction**	**Eluate**
10^6	+	+	+	+	+
10^5	+	+	+	-	+
10^4	-	+	-	-	-
10^3	-	+	-	-	-
10^2	-	-	-	-	-
10	-	-	-	-	-

Table 11: Results of PCR detection on the fraction and the hydrophobic column
(+: positive PCR result, -: negative PCR result)

4.6.3 Discussion

A saturation effect of the column was probably the reason that Map bacteria were found in the first-pass fraction during loading of the columns. N-propanol will disrupt the hydrophobic forces between Map and the C8 beads but also among the Map bacteria (clumps). This could explain the results obtained for the column after elution. A possible explanation may be that n-propanol damages the bacterial cell wall so that the bacteria will easily release their DNA during the boiling step. On the other hand, the Map bacteria on the column before elution are not treated with n-propanol and the release of DNA will be much less. It is obvious that the C8 beads can bind the hydrophobic bacteria but that n-propanol is not a good eluent for the recovery of the Map bacteria. Homogenised faecal samples were difficult to pass through the column due to clogging.

5 Occurence of Map in the zoos of the Royal Zoological Society of Antwerp

5.1 Background and aim

The Royal Zoological Society Antwerp (RZSA) was founded in 1843 and coordinates the Antwerp Zoo and the animal park Planckendael (Muizen). The Zoo is situated in the centre of Antwerp and is a typical "city" zoo with small cages and artificial soils. Planckendael is a park where animals have more space and live on natural soils. Five thousand animals of 950 species are present in the zoos of the RZSA and the animals are frequently exchanged between the Zoo and Planckendael.

The specific housing situation in zoo facilities, i.e. limited space for different animals, is a cause of concern. The major risk factor is the introduction of new animals into the herd. Animals are frequently exchanged between zoos and factors such as concentration of animals and stress during transport enhance the possibility of Map transmission.

In the past, the zoos of the RZSA have had occasional problems with Map. In 1979 during post mortem examination, Johne's disease was diagnosed in a skinny gayal (*Bibos frontalis*). A few years later (1982), Map was isolated from a pudu herd (*Pudu pudu*) with diarrhoea (De Meurichy *et al.*, 1985). In 1999, an okapi (*Okapia johnstoni*), exchanged with Blijdorp (Rotterdam zoo, The Netherlands) as part of a breeding program, developed diarrhoea. A faecal sample of this okapi Tom was sent to ID-DLO (Lelystad, the Netherlands) and tested positive on culture for Map.

This chapter describes a survey on the presence of Map in the both zoos of the RZSA using the previously developed PCR method in combination with other diagnostic tools. Culture and PCR will be applied to the detection of Map in *post mortem* (tissue and faeces) samples of ruminants. A non-species dependent ELISA test (HerdChek Map ELISA kit, IDEXX) will be used and evaluated for its use in a zoo environment.

5.2 Culture and PCR

For the tissue samples, a classical mycobacterial DNA extraction was used. The faecal samples for PCR were kept frozen until the new faecal DNA extraction was developed and evaluated. Because culture is still the gold standard, all tissue and faecal samples were cultured with conventional and radiometric techniques after decontamination.

5.2.1 Materials and Methods

5.2.1.1 Sample collection

Faeces, mesenteric lymph node and intestinal tissue (ileum and rectum) were collected from 48 ruminants during routine post mortem examination (Table 12). All samples for PCR were kept frozen until processed.

Additionally, 22 faecal samples from okapi Tom taken monthly in the period November 2000 - August 2002 were collected.

Nr	Species (Latin)	Species (English)	Date of birth	Date of death	Remark
1	*Bison bison*	Bison	1/03/1999	23/08/2000	culling
2	*Bison bison*	Bison	12/05/1984	12/09/2000	culling
3	*Bison Bonansus*	European bison	11/07/1998	19/05/2000	culling
4	*Gazella leptoceros*	Slender-horned gazelle	28/03/1993	2/09/2000	pneumonia
5	*Cervus elaphus*	Wapiti/ Elk	15/06/1999	12/10/2000	culling
6	*Cervus elaphus*	Wapiti/ Elk	3/06/1998	12/10/2000	culling
7	*Bison bison*	American bison	2/04/1999	12/10/2000	culling
8	*Synercus caffer*	African buffalo	4/05/1979	2/11/2000	culling
9	*Oryx leucoryx*	Arabian Oryx	9/01/2000	15/11/2000	emaciated, worms
10	*Gazella leptoceros*	Slender-horned gazelle	15/03/1999	16/11/2000	skinny
11	*Tragelaphus spekii*	Sitatunga	16/02/1989	26/11/2000	skinny
12	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	1/06/1992	22/12/2000	heart-insuffiency
13	*Bos mutus*	Yak	20/04/1999	22/12/2000	culling
14	*Oryx leucoryx*	Arabian oryx	22/10/1999	10/01/2001	culling
15	*Cervus nippon dybowskii*	Sika deer	1/09/1997	10/09/2001	culling
16	*Dama dama*	Fallow deer	1/05/1996	11/01/2001	culling
17	*Syncerus caffer nanus*	Dwarf forest buffalo	3/05/1999	11/01/2001	culling
18	*Rangifer tarandus*	Reindeer	1/06/1989	17/01/2001	died of old age
19	*Dama Dama*	Fallow deer	1/05/1997	11/01/2001	culling
20	*Boselaphus tragocamelus*	Nilgai	1/05/1994	26/01/2001	culling
21	*Tragelaphus spekii*	Sitatunga	1996	21/02/2001	culling
22	*Bos taurus*	Domestic cow	1997	29/01/2001	culling
23	*Bison bison*	Bison	1998	21/02/2001	culling
24	*Bison bison*	Bison	1998	21/02/2001	culling

Zoo animals …/…

Nr	Species (Latin)	Species (English)	Date of birth	Date of death	Remark
25	*Bison bison*	Bison	1998	21/02/2001	culling
26	*Bos taurus*	Domestic ox	3/03/2000	6/04/2001	culling
27	*Bison bison*	Bizon	1/09/1998	21/02/2001	culling
28	*Okapia Johnstoni*	Okapi	1/09/1983	3/05/2001	anemia
29	*Tragelaphus spekii*	Sitatunga	1996	10/05/2001	culling
30	*Connochaetes gnou*	White tailed gnu	3/03/1998	10/05/2001	culling
31	*Bison bison*	American bison	1998	30/05/2001	culling
32	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	1999	15/06/2001	culling
33	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	1999	15/06/2001	culling
34	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	1999	15/06/2001	culling
35	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	1999	22/06/2001	culling
36	*Rangifer tarandus*	Reindeer	4/05/1989	26/08/2001	trauma
37	*Capra ibex*	Rock goat	31/05/2000	18/08/2001	trauma
38	*Tragelphus spekii*	Sitatunga	21/05/1997	29/08/2001	trauma
39	*Oryx leucoryx*	Arabian Oryx	23/05/1996	21/11/2001	skinny, dehydrated
40	*Alces Alces Alces*	European Elk	1/05/1985	24/11/2001	enteritis
41	*Okapia Johnstoni*	Okapi	5/07/1988	15/02/2002	colitis
42	*Oryx Dammah (Cretzschmar)*	Scimitar Oryx	26/04/1984	30/03/2002	Clostridium enterotoxemia
43	*Oryx Dammah (Cretzschmar)*	Scimitar Oryx	30/05/1988	17/07/2002	kidney insufficiency
44	*Rangifer tarandus*	Reindeer	24/04/1990	12/08/2002	gastro-enteritis
45	*Bison Bonansus*	European bison	5/09/1982	14/08/2002	culling
46	*Bibos javanicus domesticus*	Banteng	27/07/1983	29/09/2002	skinny
47	*Ovis canadensis canadensis Shaw*	Bighorn	4/05/1995	12/12/2002	skinny, died during anaesthesia
48	*Taurotragus oryx*	Eland	25/09/1993	7/03/2003	skinny, died during anaesthesia

Table 12: Zoo animals tested for Map using culture and PCR

5.2.1.2 Decontamination

The double incubation method of Whitlock and Rosenberger (1990) was used for decontamination. Briefly, 2-5 g faeces were mixed with 10 ml of sterile saline and kept for 30 min at RT. Five ml of the surface fluid was transferred to a fresh tube containing 25 ml of a 0.9% HPC solution (Sigma) in Half-Strength Brain Heart Infusion Broth (LabM). The sample was incubated for 24 h at 37°C. After centrifugation at 900 x *g* for 30 min, the pellet was resuspended in 1 ml of an antimicrobial mixture (100 µg vancomycin + 100 µg nalidixic acid and 50 µg amphotericin B in 1 ml distilled water). The samples were incubated for 48-72 h at RT. In case of tissue samples, the decontamination procedure was slightly different. Five g of tissue was homogenised in a blender for 30 s using sterile physiological water. Twenty-five ml of a 0.75% HPC solution was added and the sample was left at RT for 48-72 h, filtered through sterile gauze cloth, centrifuged at 900 x *g* for 30 min and the pellet was suspended in 1 ml antimicrobial mixture. The sample was incubated for 48 h at RT and the sediment was used to seed culture tubes.

5.2.1.3 Culture

For radiometric culture, 0.1 ml of the sediment was inoculated in BACTEC12 B-vials (Becton Dickinson). The BACTEC12 B-vials were supplemented with 200 µl of PANTA-plus (Becton Dickinson), 1 ml Egg Yolk (Becton Dickinson), 5 µg of mycobactin J (Synbiotics) and 0.7 ml of water and incubated at 37°C. The BACTEC instrument detects the amount of radioactivity and records it as a growth index (GI) on a scale from 0 to 999. Every week, the Growth Index (GI) was determined with an automatic ion chamber (BACTEC 460). Smears were made from all vials with a GI > 20 and stained with the Ziehl-Neelsen method. If acid-fast bacilli were detected, the samples were processed for PCR-testing.

For culturing on solid medium, 0.1 ml of the sediment was inoculated onto 4 tubes with Löwenstein-Jensen medium supplemented with 1 mg mycobactin J, 40 ml PANTA-plus and 4 g sodium pyruvate per litre.

The tubes were incubated at 37°C. Growth was determined weekly and Ziehl-Neelsen staining was performed on positive tubes. If acid-fast bacilli were detected,

the samples were processed for PCR-analysis. The two culture methods were evaluated using spiked samples.

5.2.1.4 Tissue DNA extraction

An in-house protocol of the Mycobacterium laboratory at the ITM (Antwerp) was used for the extraction of tissue samples. Tissue samples were cut in 1-3 mm^2 pieces and transferred to a 1.5 ml microcentrifuge tube. Two hundred fifty µl of lysis buffer (60 mM Tris pH 7.4, 60 mM EDTA, 10% Tween 20, 1% Triton-X 100, 1.6 M guanidine-HCl), 50 µl of proteinase K (20 mg in 1 ml MilliQ water, Sigma) and 500 µl of 0.1 mm Ø glass microspheres were added. The tubes were incubated for 1 h at 60°C. The samples were sonicated (Branson 1200, 47 kHz, Branson Ultrasonics, Danbury, CT, USA) for 5 min at RT and 40 µl of diatomaceous earth suspension (Sigma) (0.2 g/ml) was added. The tubes were incubated at 37°C for 60 min in a thermomixer (Eppendorf).

Each tube was subsequently centrifuged for 20 s and the supernatant was discarded. The pellet was washed twice with 900 µl of ethanol (70%) and once with 900 µl acetone. After drying in a thermoblock at 50°C for 20 min, 90 µl of TE-buffer (10 mM Tris and 1 mM EDTA, pH 8) was added and incubated for 20 min at 60°C under constant shaking at 1,000 rpm (Thermomixer, Eppendorf). The tube was centrifuged for 40 s and 60 µl of the supernatant was transferred to a new tube for subsequent PCR detection.

5.2.1.5 BACTEC DNA extraction

The positive BACTEC cultures with a GI > 20 were confirmed with an IS*900* PCR. The ethanol extraction method described by Whittington *et al*. (1998b) was used on the BACTEC medium. Briefly, the rubber stopper lid of the vial was wetted with 100% isopropanol. Two hundred µl of medium was removed with a sterile syringe and needle and transferred to a 1.5 ml microcentrifuge tube. Five hundred µl of absolute ethanol was added and the tube was left for 2 min before mixing vigorously on a vortex mixer for 5 s. After centrifugation at 8 x *g* for 10 min at RT, the supernatant was transferred to a new microcentrifuge tube and centrifuged at 18,000 x *g* for 5 min. The pellet was washed twice with 200 µl sterile PBS and resuspended in

50 µl of sterile MilliQ water. The tube was heated at 100°C for 20 min to lyse the mycobacteria and 5 µl of the lysate was used for PCR.

5.2.1.6 Faecal DNA extraction

The new developed faecal DNA extraction method was used for the analysis of the faecal samples (see 4.3.1.7A and 4.3.2.2).

5.2.1.7 PCR

A nested PCR was performed. Primary PCR on extracts of tissue and BACTEC was performed in a final volume of 25 µl with addition of 5 µl sample. The primary PCR after faecal extraction was performed in a final volume of 50 µl with 10 µl of sample. PCR conditions for the primary and secondary PCR were as described in 3.2.1.4 with following variables:

Primary PCR			
Primer: IS*900*S1			
Primer: IS*900*R3(2)			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	68	45	
Extension	72	45	

Secondary PCR			
Primer: IS*900*S2			
Primer: IS*900*R1			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	25
Annealing	68	45	
Extension	72	45	

5.2.2 Results

5.2.2.1 Culture

After 4 months a GI of > 20 was recorded in 10 of the 192 BACTEC 12B vials. Ziehl-Neelsen staining revealed the presence of acid-fast bacilli in only one vial

(mesenteric lymph node of a wapiti). A BACTEC DNA extraction was performed but the PCR assay with IS*900* primers was negative. To identify the mycobacterium, a panel of mycobacterium specific primers was used. A positive PCR was obtained using primers amplifying the IS*901* locus, i.e. an insertion sequence of *Mycobacterium avium* subspecies *avium*.

One hundred and six out of 608 Löwenstein-Jensen solid media became contaminated by fungal or bacterial overgrowth. Only the 4 tubes of the mesenteric lymph node of the wapiti were Ziehl-Neelsen positive. The PCR using the IS*900* assay was negative but IS*901* assay gave positive results. The acid-fast bacilli in the cultures were also diagnosed as *Mycobacterium avium* subspecies *avium*. No Map was detected in the faecal cultures of okapi Tom.

The two culture methods were also tested with spiked samples (positive controls) and positive results were obtained.

5.2.2.2 DNA extraction and PCR

All tissue samples were extracted and tested in triplicate. Only three samples were IS*900* positive (nr 41: lymph node okapi, nr 45: rectum European bison and nr 46: ileum banteng). A second PCR on the same sample was performed and confirmed the positive results. The faecal DNA extracts of the 48 animals were all negative.

Only one faecal sample (June 2002) out of the 22 monthly samples from okapi Tom was IS*900* positive.

5.3 Serology

ELISA is a rapid and relatively inexpensive test but has a low sensitivity when used on sera of subclinically ill animals. The ELISA test has only been validated on cattle and small ruminant samples (Payeur *et al.,* 1998). Research efforts are currently focusing on the application of ELISA to detect antibodies in non-domestic species, by using a non-specific binding conjugate or by developing a monoclonal/polyclonal conjugate that can bind antibodies produced by all artiodactylids (Manning *et al*., 1999, Schroen *et al*., 2002, Kramsky *et al*., 2003). The commercial kit Herdchek ELISA test kit is based on protein G conjugate, which reacts with antibodies of

multiple animal species. Protein G is a non-species specific protein binding the Fc region of multi-species IgG in a non-immunological reaction without interfering with the antigen binding sites. The HerdChek ELISA has only been validated for small ruminants and bovine serum samples. For these animals, the specificity of the kit is 99% but the sensitivity is affected by the stage of infection. The sensitivity on low level faecal shedders is only 15%, whereas for clinically affected animals it is 87%, with an overall sensitivity of 45% (Collins *et al.*, 1996). Thus far the HerdChek kit has not been validated for zoo animals.

5.3.1 Materials and methods

5.3.1.1 Sample collection

448 sera from 51 different ruminant species were randomly chosen from the sera collection bank of the Royal Zoological Society of Antwerp. The sera were collected between 1976 and 2002 and kept at –20°C (Table 13). The collection included the sera from the gayal (post mortem diagnosis of Johne's disease in 1979) and the pudu herd (Map culture positive in 1982).

5.3.1.2 ELISA

The serum samples were tested for the presence of antibodies against Map using the Herdchek ELISA test kit (IDEXX). This commercially available kit is an indirect ELISA performed in Map antigen coated microtitre wells. The detection of bound antibodies is based on a horseradish peroxydase (HRPO) protein G conjugate, which reacts with antibodies of multiple animal species. All samples were tested according to the manufacturer's instructions supplied with the kit. Fifteen µl of sample was mixed with 135 µl of adsorbent solution and incubated for 60 min at RT. Hundred µl of each pre-incubated sample was transferred into a well of an antigen coated microtitre plate and incubated for 60 min at RT. The wells were then rinsed 5 times with diluted wash solution. Hundred µl HRPO conjugate was added to each well and incubated during 30 min at RT. The wells were washed 5 times with diluted wash solution. Hundred µl of substrate solution was added and the plates were incubated for 10 min at RT in the dark. The reaction was stopped with 100 µl of stop solution. The absorbance was measured at 450 nm.

The test kit manual provides cut-off values of S/P ratios for two groups of mammals. An S/P ratio is the ratio of the absorbance of the samples to the absorbance of the provided positive controls. For bovine samples, an S/P ratio less than 0.150 is classified as negative, an S/P ratio between 0.150 and 0.300 as suspect and an S/P ratio higher than 0.300 as positive. For small ruminants, an S/P ratio between 0.300 and 0.400 is suspect and an S/P ratio greater than 0.400 is positive.

Species (Latin)	Species (English)	number of animals
Okapia johnstoni	Okapi	25
Rangifer tarandus tarandus	Reindeer	22
Bison bonasus	European bison	19
Bison bison	Bison	43
Alces alces alces	Elk/Moose	7
Elapurus davidianus Milne-Edwards	Père David's deer	29
Syncerus caffer caffer	African cape buffalo	20
Poëphagus mutus grunniens	Yak	23
Syncerus caffer nanus	Forest buffalo	7
Bos taurus Linnaeus	Domestic ox	8
Connochaetes taurinus	Blue gnu	8
Ovibos moschatus Wardi	Musk ox	11
Saiga tatarica tatarica	Saiga	3
Ammotragus lervia	Barbary sheep	3
Sika hortulorum	Dybowski's Sika deer	4
Bibos frontalis	Gayal	1
Pudu pudu	Pudu	7
Boselaphus tragocamelus	Nilgai	11
Ovis canadensis canadensis Shaw	Bighorn	7
Taurotragus oryx Derbianus	Derby eland	2
Ovis ammon musimon	European mouflon sheep	6
Cervus elaphus nelsoni Bailey	North American elk	15
Damaliscus dorcas phillipsi Harper	Blesbok	3
Boocercus euryceros	East African bongo	1
Bibos javanicus domesticus	Banteng	5
Tragelaphus spekei gratus Sclater	Sitatunga	12
Capra ibex ibex Linnaeus	Alpine ibex	4
Cervus elaphus elaphus Linnaeus	Red deer	1
Bubalus depressicornis (H. Smith)	Anoa	5
Taurotragus oryx	Eland	33
Cephalophus monticola	Blue duiker	3
Capra hircus Fl	Goat	4
Connochaetes gnou	White-Tailed Gnu	3
Ovis aries L.	Domestic sheep	27
Oryx dammah (Cretzschmar)	Scimitar oryx	2
Capra hircus linnaeus	Goat	5
Bos primigenius taurus linnaeus	Wild ox	4
Capra hircus Fl	Flemish goat	2
Hemitragus jemlahicus jemlahicus	Himalayan tahr	14
Oreamnos americanus	Mountain goat	3
Anoa depressicornis	Lowland anoa	1
Giraffa camelopardalis	Giraffe	2
Syncerus caffer brachyceros	Savannah buffalo	1
Bos taurus	Domestic ox	8
Gazella leptoceros	Slender-horned gazelle	6
Hippotragus niger	Sable antilope	4
Oryx leucoryx	Arabian oryx	10
Tapirus indicus	Tapir	1
Ovis dalli	Dall's sheep	1
Dama dama	Fallow deer	1
Tragulus javanicus	Lesser Malayan cevrotain	1

Table 13: Zoo animals tested for Map with serology

5.3.2 Results

An S/P ratio of > 0.400 (cut-off ratio for small ruminants according to the manufacturer) was considered as positive. An S/P ratio between 0.150 and 0.400 was considered as suspect.

The results of the ELISA on the 448 sera gave 21 positives and 15 suspects (Table 14).

Species (Latin)	Species (English)	Number tested	Number of positives	Number of suspected ones
Okapia johnstoni	Okapi	25	0	1
Rangifer tarandus tarandus	Reindeer	22	0	1
Bison bonasus	European bison	19	3	1
Bison bison	Bison	43	1	2
Elapurus davidianus Milne-Edwards	Père David's deer	29	0	1
Poëphagus mutus grunniens	Yak	23	3	2
Ovibos moschatus Wardi	Musk ox	11	0	1
Ammotragus lervia	Barbary sheep	3	3	0
Bibos frontalis	Gayal	1	1	0
Pudu pudu	Pudu	7	2	0
Boselaphus tragocamelus	Nilgai	11	1	1
Ovis canadensis canadensis Shaw	Bighorn	7	0	1
Damaliscus dorcas phillipsi Harper	Blesbok	3	3	0
Capra ibex ibex L.	Alpine ibex	4	0	1
Taurotragus oryx	Eland	33	1	2
Ovis aries L.	Domestic sheep	27	2	0
Bos taurus	Domestic ox	8	0	1
Tapirus indicus	Tapir	1	1	0
Total			21	15

Table 14: Serology results of positive zoo animals per species
(positive: cut-off ratio > 0.400, suspect: cut-off ratio between 0.150 and 0.400)

5.4 Discussion

This study was undertaken to investigate the presence of Map in the animal collection of the RZSA. To this end different diagnostic tests were used.

No growth of Map was detected on solid medium or in BACTEC 12B vials. The sensitivity of the Map culture method is estimated as only 50% (Collins *et al.*, 1996) and is obviously related to the likelihood of sufficient numbers of Map being shed from the intestinal lesions. The negative isolation of Map from the zoo samples may

have been due to the limited distribution of focal lesions, sporadic excretion of organisms or the high detection limit of the faecal culture methods employed (McDonald *et al.*, 1999). The decontamination and the culture protocols used in this study are well-established routine methods (Cousins *et al.*, 1995; Whittington *et al.*, 1999a; Pavlik *et al.*, 2000) and appropriate controls were incorporated using spiked samples. A higher percentage of fungal and bacterial contamination was found for solid medium cultures (17.4%) than for BACTEC 12B vials (5.2%), which has also been reported in other studies (McDonald *et al.*, 1999). On the other hand, the use of a stronger decontamination method could have negatively affected the growth of Map (Reddacliff *et al.*, 2003).

Our study shows that PCR is a practical, rapid, and specific test for detecting Map in field samples. Organisms can be detected without the need for culture and therefore this method speeds up the process of diagnosis and overcomes problems with fungal or bacterial contamination. Faeces, however, contain many PCR-inhibitors and difficulties are experienced in recovering DNA from small numbers of target organisms in such a complex matrix. Generally, PCR based tests on faeces have been found to be less sensitive than faecal culture (Harris *et al.*, 2001). However, when applied to tissue samples, PCR is more sensitive and reliable (Stevenson *et al.,* 1997). Although the use of biopsy samples is not practical in the case of field studies, the technique is useful for confirming diagnosis at necropsy.

Three autopsy samples from zoo animals of the RSZA were positive on PCR for IS*900*. One of the positive autopsy samples was a lymph node from an okapi (Kasindi). The okapi (14 years old) was very skinny and died after anaesthesia. At *post mortem* examination, no typical paratuberculosis lesions could be found, although there was inflammation in the colon. The okapi was born in 1987 in the Bristol zoo and traded to the Antwerp zoo in 1988. In the past, the Bristol zoo had serious problems with paratuberculosis (Van Puyenbroeck, personal communication). Newborn animals are at highest risk for infection and the okapi was probably infected with Map in the Bristol Zoo. In 1999, okapi Tom was found positive on culture. One faecal sample of okapi Tom was found positive on PCR in our study. Okapi Tom was born in the Antwerp zoo in 1992. It could well be that okapi Kasindi got infected in the Bristol zoo and became a shedder while she was at the Antwerp zoo, transmitting the infection to okapi Tom. Although we have monitored okapi Tom during 22 months using PCR and culture on faeces, we could only detect one PCR positive

sample. Moreover, no typical clinical signs of paratuberculosis could be detected in the okapi herd. This indicates that at the time of sampling there were only low-grade infections with minimal bacterial shedding. However, okapis are very valuable animals which are almost extinct in nature. Under farming conditions, it is known that Map infection can be dormant. At a certain moment, the infection becomes visible and the animal's health declines. The recommendation following our research on the okapis was to test the okapi herd at regular intervals with PCR and culture on faecal samples.

Samples from the RZSA serabank were screened with ELISA in order to obtain data on the prevalence of the disease in the past. The results contribute to the overall picture of the Map prevalence in the Antwerp zoo, as ELISA is a good method to be used on serum samples from time periods during which no PCR samples could be obtained. With the kit, we observed 21 animals with an S/P ratio > 0.400. We assume that these animals are positive when considering the results reported by Stöbel *et al.* (2002). These investigators described a non-species dependent ELISA for the detection of antibodies to *Borrelia burgdorferi* in zoo animals and clearly demonstrated that protein G is a useful alternative to species-specific secondary antibodies. Kramsky *et al.* (2003) concluded in their study that protein G conjugates might serve as useful reagents for serodiagnosis of Map, although they observed that protein G varied in its degree of binding to the Fc region of antibodies. A cut-off value has to be established for each species in order to correctly interpret ELISA results, but it is nearly impossible to do that for all zoo ruminants.

We have used the most conservative cut-off value to analyse the ELISA results (small ruminants > 0.400) and have classified all samples with a cut-off between 0.150 and 0.400 as suspected. Serum from the gayal (clinical Johne's disease in 1979) and sera from the pudu herd (Map positive culture in 1984) were positive in the ELISA. The ELISA was a very useful method to have an idea of animals that could have been infected in the past, but as mentioned before, ELISA results need to be confirmed with culture. ELISA is cheaper and can be done on a large number of samples, but the sensitivity is very low and dependent of the clinical stage of the infection.

Some zoo veterinarians question the importance of paratuberculosis in zoo animals. When considering the link with Crohn's disease, it is also important for zoos and animal parks to monitor and control paratuberculosis. Especially in children's farms,

where children are in contact with animals that could be infected. Moreover, skinny, unhealthy animals are not attractive for the general public. One of the tasks of a zoo is the conservation of almost extinct animals and healthy animals are indeed indispensable.

Following the results of our study, the RZSA has decided to test the ruminants on a regular basis using our newly developed faecal DNA extraction method. The plan also exists to initiate a survey to monitor Map infections in other European zoos. For the diagnosis of paratuberculosis in subclinically ill animals, repeated sampling will be necessary to declare a zoo paratuberculosis free.

5.5 Conclusion

To study the occurence of paratuberculosis in the zoos of the RZSA, a survey was conducted using two culture methods, PCR and ELISA. No Map positive cultures could be obtained. On the other hand, we observed positive PCR results in three tissue samples of different animals and in 1 faecal sample of okapi Tom. The ELISA also showed positive results in several animal species. In combination with the history of Map in the RSZA, we can conclude that there is evidence for the presence of Map infections in their animal collection. However, no high level faecal shedders were detected. A follow-up on the animals with PCR will be necessary, especially in animal herds with PCR and ELISA positive results. Repeated testing is necessary to complete the general picture of the Map infections at the RZSA. Moreover, further investigations are required in other European zoos and animal parks in order to have a general picture of Map infections in European collections of non-domestic hoofstock and to initiate control measures if necessary.

The next step is the analysis of the epidemiology of Map in the zoos. This will help to understand the spread of Map between the different species and zoos. When the spread of Map is clarified, control measures can be taken to overcome more Map infections. To this end, a rapid and easy molecular typing method for Map is necessary.

6 Molecular typing of Map isolates

6.1 Background and aim

Map infections do not only occur in ruminants. More and more reports describe the presence of infections in non-ruminant species (Beard *et al.*, 2001, Zwick *et al.*, 2002). To analyse the spread of Map in animal populations, strain typing is a prerequisite. Several molecular methods can be used for the typing of Map. The RFLP approach based on an IS*900* probe is widely used and could help to understand the spread of Map (Pavlik *et al.*, 1999). For example, RFLP-IS*900* was used in a wildlife survey in Scotland. Identical RFLP types were found in cattle, rabbits and foxes of the same geographical region, which led the authors to conclude that rabbits were infected by grazing on pastures contaminated by Map shedding cattle. The foxes were probably infected by eating infected rabbits (Beard *et al.*, 2001).

However, RFLP is limited by the requirement of sufficient amounts of pure genomic DNA. Therefore, all samples must be cultured before RFLP can be performed. This makes RFLP a time-consuming typing method.

As a consequence, PCR based methods that can be applied directly on clinical samples are therefore highly desirable for characterizing and discriminating among Map strains. As there is no need for prior culturing, PCR is considerably faster.

A modified AFLP (Amplified Fragment Length Polymorphism) method will be used to identify putative polymorphic loci. To this end, AFLP patterns will be analysed and informative fingerprint fragments will be identified. These fragments will be subsequently cloned and sequenced. The sequence information will be used to develop PCRs detecting the fingerprint fragments. With a panel of different PCR assays, it would be possible to unequivocally type the Map strains.

6.2 AFLP

AFLP was first described by Vos *et al.* in 1995. The AFLP technique is based on the selective amplification of restriction fragments from a total digest of genomic DNA. There are three major steps in the AFLP procedure: 1) restriction endonuclease digestion of genomic DNA and ligation to specific adapters, 2) amplification of the restriction fragments by PCR using primer pairs containing common sequences of the adapter followed by 1-3 arbitrary nucleotides and 3) analysis of the amplified fragments using gel electrophoresis. The possibility to combine different restriction enzymes and selective nucleotides in the primers for PCR makes AFLP a useful tool for molecular typing.

The major advantage of AFLP is that fingerprints can be produced without prior sequence knowledge using a limited set of generic primers. The AFLP technique is robust and reliable because stringent reaction conditions are used for primer annealing: thus, the reliability of the RFLP technique is combined with the power of the PCR technique (Olive, 1999).

In a conventional AFLP, two restriction enzymes (a frequent and a rare cutter) are used to cut the DNA, followed by a pre-selective amplification with primers without selective nucleotides. A second amplification occurs with primers containing one or more selective nucleotides. The electrophoresis is performed on a sequence apparatus and the detection of the fragments is done with fluorescently or radioactively labelled primers (Figure 11).

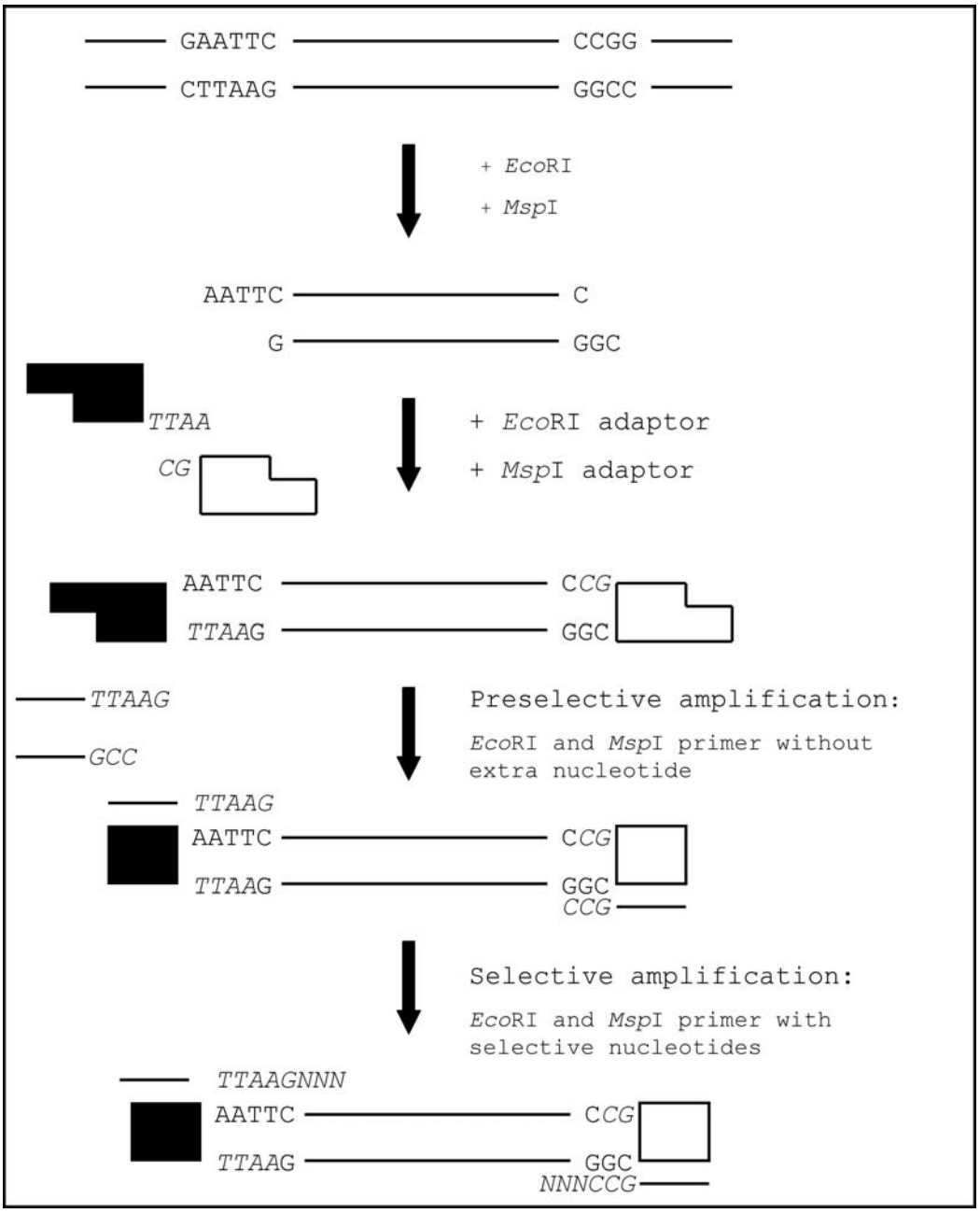


Figure 11: Procedure of a conventional AFLP

Besides the conventional AFLP (Huys *et al.*, 2000), several variations have been described for typing of bacteria. Van der Zee *et al.* (2003) used 4 restriction enzymes and 4 primers for the typing of *Klebsiella pneumoniae*. TE-AFLP (three enzymes AFLP) is described by van der Wurff *et al.* (2000). A SE-AFLP (single enzyme AFLP) was described by Ranamukhaarachchi *et al.* (2000) for genetic characterisation in plants. The SE-AFLP has been successfully used for typing different bacteria, including mycobacteria (Peters *et al.*, 2001; Gaafar *et al.*, 2003;

Moreno *et al.*, 2003). The obtained fingerprint patterns are easier to interpret because lesser fragments will be amplified relative to the conventional AFLP method. This will facilitate the identification of informative bands. The SE-AFLP (Figure 12) was chosen as a starting point to find polymorphic markers in the Map isolates. This technique was not previously used for Map.

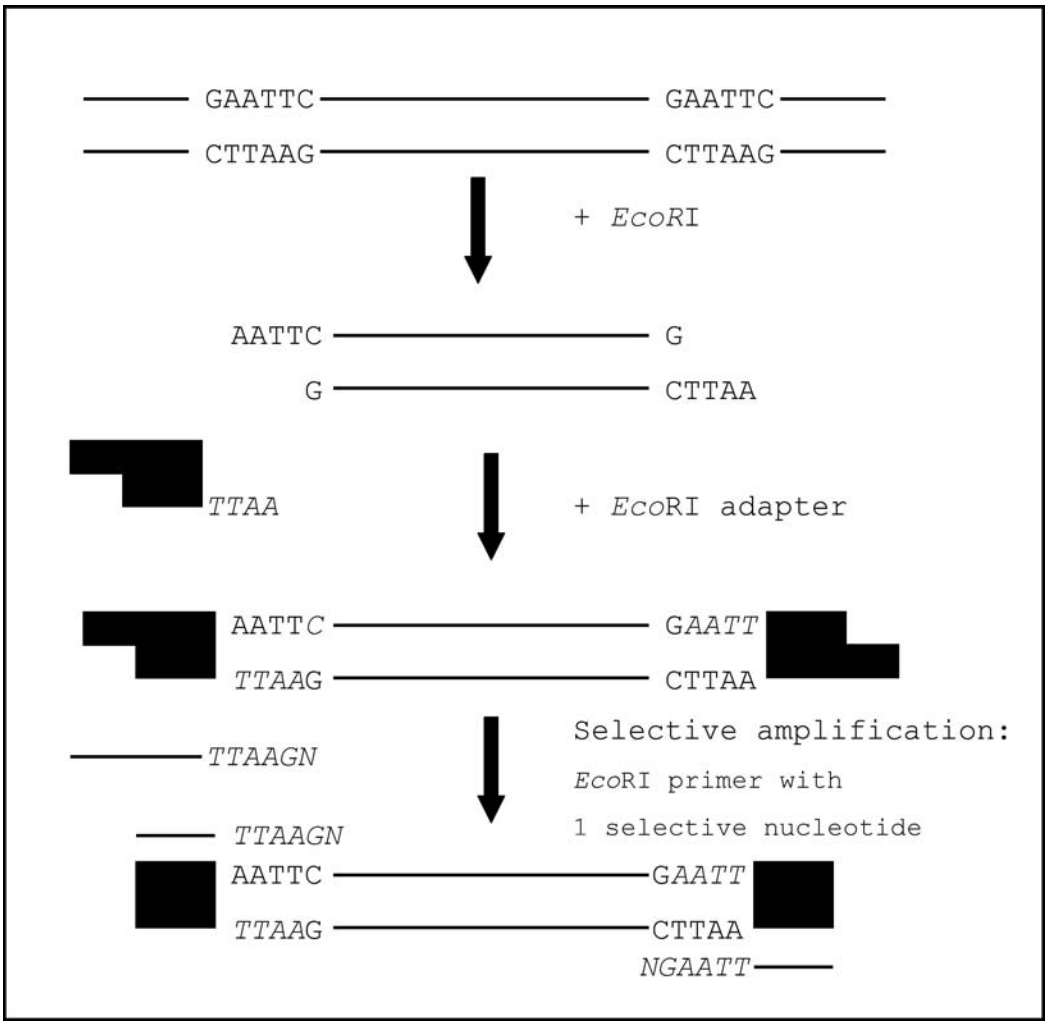


Figure 12: Procedure of single enzyme AFLP

6.2.1 Materials and methods

6.2.1.1 Isolate collection

A collection of 74 Map and 2 Maa isolates was used for the development of the AFLP typing method (Table 15). The isolates were grown on Löwenstein-Jensen medium supplemented with Mycobactin J. The collection included the reference strain ATCC 19698, two bovine isolates from Australia, one bovine isolate from Burundi, several cervid and cattle isolates from Belgium.
All isolates were tested for their mycobactin dependency and were confirmed as Map using an IS*900* PCR.

6.2.1.2 Genomic DNA isolation

Fingerprinting techniques require good quality DNA and so any shearing of the DNA during isolation should be avoided. It was necessary to slightly modify the method used for the isolation of DNA from pure culture described by Mve-Obiang *et al.* (2001). The clumps of Map did not disintegrate in methanol, which excluded further processing to a suspension. This problem was resolved by transferring the bacteria immediately to the disruption buffer containing 4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl, 0.1 M 2-mercapto-ethanol, 20 mM EDTA. DNA was isolated from the disruption buffer by a phenol:chloroform:isoamylalcohol (25:24:1) extraction (PCI). The PCI extraction was repeated twice followed by a precipitation in 0.1 vol of 3 M sodium acetate and 1 vol of ice-cold isopropanol.
After the genomic DNA isolation, the quality of the DNA was checked on 1% agarose gel and the 260/280 nm ratios were spectrophotometrically determined (GeneQuant). Calculations were made to quantify the amount of DNA in the sample.

6.2.1.3 Set up of a Single Enzyme AFLP

The SE-AFLP used in this study is based on the one described by Ranamukhaarachchi *et al.* (2000). To this end, genomic DNA was restricted by one restriction enzyme, one adapter was ligated followed by a PCR using a primer with one selective nucleotide. The PCR products were electrophoresed on an agarose-gel.

6.2.1.3.1 Restriction digestion of genomic DNA and ligation of the adapters

0.5 µg of genomic DNA was digested by 10 units of restriction enzyme (New England Biolabs, Hitchin, United Kingdom). Four different restriction enzymes, i.e. two frequent cutters (*Taq*I and *Msp*I) and two rare cutters (*EcoR*I and *Apa*I)) were used in the study. The digestion mixture was incubated for 6 h at the temperature specified by the manufacturer. After digestion, the mixture was heated to inactivate the restriction enzymes. Adapters were prepared by mixing equal volumes of two single stranded adapter-oligonucleotide solutions (25 pmol/µl). First the adapter-oligonucleotides were heated to 95°C and allowed to cool to RT to form double stranded adapters (see Table 16 for enzyme and adapter information). The ligation of the adaptors was done by mixing 2 µl of the adapter solution with 20 units of DNA ligase (Invitrogen, Merelbeke), followed by incubation for 16 h at RT. Afterwards, the template DNA was purified from excess adapters, enzymes and other contaminants using a 95% ethanol precipitation.

Isolate (ITM nr)	Year of isolation	Isolated by	Origin	Source	Sample
2666			ATCC19698	bovine	faeces
99-932	1997	Cerva-Coda, 97274	Belgium	bovine	faeces
00-452	2000	ITM	Belgium	bovine	faeces
00-451	2000	ITM	Belgium	bovine	faeces
00-03	1999	Cerva-Coda, 99/30	Belgium	bovine	faeces
00-04	1999	Cerva-Coda, 99/23	Belgium	bovine	faeces
99-941	1996	Cerva-Coda, 96022	Belgium	bovine	faeces
99-933	1997	Cerva-Coda, 97287	Belgium	bovine	faeces
9403	1992	ITM	Burundi	bovine	lymphnode
00-1675	***2000***	***ITM***	***Belgium***	***ns***	***geen paratuberculosis***
00-1671	2000	ITM	Belgium	bovine	faeces
00-1414	***2000***	***Cerva-Coda, 75T2000***	***Belgium***	***ns***	***geen paratuberculosis***
99-2681	1999	ns	Australia	bovine	faeces
00-324	1999	Cerva-Coda, 99/531	Belgium	bovine	faeces
99-2678	1999	ns	Australia	bovine	faeces
00-10	1999	Cerva-Coda, 99/80	Belgium	bovine	faeces
00-05	1999	Cerva-Coda, 99/22	Belgium	bovine	faeces
99-947	1998	Cerva-Coda, 357/98	Belgium	bovine	faeces
99-946	1998	Cerva-Coda, 196/98	Belgium	bovine	faeces
99-938	1996	Cerva-Coda, 96022	Belgium	bovine	faeces
99-912	1997	Cerva-Coda, 97152	Belgium	bovine	faeces
00-02	1999	Cerva-Coda, 99/31	Belgium	bovine	faeces
00-453	2000	ITM	Belgium	bovine	faeces
99-932	1997	Cerva-Coda, 97274	Belgium	bovine	faeces
00-12	1999	Cerva-Coda, 99/62	Belgium	bovine	faeces

Map and Maa isolates …/…

Isolate (ITM nr)	Year of isolation	Isolated by	Origin	Source	Sample
99-943	ns	Cerva-Coda, 43015	Belgium	bovine	faeces
99-942	1996	Cerva-Coda, 96026	Belgium	bovine	faeces
99-937	1996	Cerva-Coda, 96017	Belgium	bovine	milk
00-466	2000	ITM	Belgium	bovine	faeces
02-0904	2002	ITM	Belgium	Cervid	large intestine
02-0909	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1466	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1444	2002	ITM	Belgium	Cervid	small intestine
02-1461	2002	ITM	Belgium	Cervid	spleen
02-1463	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1445	2002	ITM	Belgium	Cervid	spleen
02-0910	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1462	2002	ITM	Belgium	Cervid	spleen
02-1465	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1447	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1451	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1458	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1663	2002	ITM	Belgium	Cervid	mes. lymphnode
02-1448	2002	ITM	Belgium	Cervid	mes. Lymphnode
02-1441	2002	ITM	Belgium	Cervid	spleen
02-0706	2002	ITM	Belgium	bovine	faeces
02-0806	2002	ITM	Belgium	bovine	faeces
02-0702	2002	ITM	Belgium	bovine	faeces
02-0710	2002	ITM	Belgium	bovine	faeces
02-1696	2002	ITM	Belgium	bovine	faeces
02-1717	2002	ITM	Belgium	bovine	faeces

Map and Maa isolates …/…

Isolate (ITM nr)	Year of isolation	Isolated by	Origin	Source	Sample
02-0700	2002	ITM	Belgium	bovine	faeces
02-1715	2002	ITM	Belgium	bovine	faeces
02-0699	2002	ITM	Belgium	bovine	faeces
02-1680	2002	ITM	Belgium	bovine	faeces
02-0705	2002	ITM	Belgium	bovine	faeces
02-1675	2002	ITM	Belgium	bovine	faeces
02-1679	2002	ITM	Belgium	bovine	faeces
02-1673	2002	ITM	Belgium	bovine	faeces
02-0900	2002	ITM	Belgium	bovine	faeces
02-0708	2002	ITM	Belgium	bovine	faeces
02-0711	2002	ITM	Belgium	bovine	faeces
02-0697	2002	ITM	Belgium	bovine	faeces
02-1041	2002	ITM	Belgium	bovine	faeces
02-0808	2002	ITM	Belgium	bovine	faeces
02-1042	2002	ITM	Belgium	bovine	faeces
02-0698	2002	ITM	Belgium	bovine	faeces
02-1678	2002	ITM	Belgium	bovine	faeces
02-0701	2002	ITM	Belgium	bovine	faeces
02-0807	2002	ITM	Belgium	bovine	faeces
02-1040	2002	ITM	Belgium	bovine	faeces
02-0811	2002	ITM	Belgium	bovine	faeces
02-0310	2002	ITM	Belgium	bovine	faeces
02-0370	2002	ITM	Belgium	bovine	faeces
02-0369	2002	ITM	Belgium	bovine	faeces
02-0371	2002	ITM	Belgium	bovine	faeces

Table 15: Map and Maa isolates used in the molecular typing study

(ns: notspecified)

Restriction enzyme	Restriction T (°C)	Inactivation T (°C)	Recognition site	Adapter	Primer
*EcoR*I	37	70	G/AATTC	Ecoad1: 5' ctcgtagactgcgtacc 3' Ecoad2: 5' aattggtacgcagtctac 3'	EcoselG: 5' gactgcgtaccaattc***G*** 3'
*Apa*I	25	65	G/GGCC	Apaad1: 5' tcgtagactgcgtacaggcc 3' Apaad2: 5' tgtacgcagtctac 3'	ApaselG: 5' gactgcgtacaggccc***G*** 3'
*Taq*I	65	85	T/CGA	Taqad1: 5' gacgatgagtcctgac 3' Taqad2: 5' cggtcaggactcat 3'	TaqselG: 5' cgatgagtcctgaaccga***G*** 3'
*Msp*I	37	70	C/CGG	Mspad1: 5' gacgatgagtcctgag 3' Mspad2: 5' cgctcaggactcatc 3'	MspselG: 5' gatgagtcctgagccgg***G*** 3'

Table 16: Restriction enzymes, adaptors and primers used din AFLP

(T: temperature, in bold: selective nucleotide)

6.2.1.3.2 PCR Amplification

In a series of preliminary experiments, the PCR conditions were optimised. First the temperature cycling conditions were adjusted, thereby using an annealing temperature as high as possible. This would increase the reproducibility and the specificity of the assay. A temperature gradient PCR was used to find the best annealing temperatures. Best results were obtained when using an annealing temperature of 60°C. The use of a hot start PCR approach increased the specificity of the PCR reaction. The optimal amount of template DNA to add was found to be 10 ng. The following conditions were used: 10 ng DNA, 0.5 U Taq DNA polymerase (Silverstar), 200 µM dNTP's, 40 pmol primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$. The thermal cycling conditions were 4 min at 94° C, followed by 40 cycles of 94°C for 45 s, 60°C for 60 s, 72° C for 120 s and a final extension at 72°C for 10 min.

6.2.1.4 Gel electrophoresis and visualisation

The first AFLP reactions were electrophoresed using a gel mixture of Synergel (Omnilabo Int, Breda, The Netherlands) and agarose (1.5g:1.5g) in 150 ml 0.5 x TAE (20 mM Tris-acetate, 0.5 mM EDTA). Seven µl of PCR product were mixed with 3 µl loading buffer (2% glycerol, 0.25% bromophenol blue) and transferred onto the gel (size 15 x 15 cm). A DNA ladder (GeneRuler™ 100 bp DNA Ladder Plus) was included on the gels to size DNA positions. The gels were run at 100 V for 6 h. Bands were visualised by ethidium bromide staining. Due to a low resolution power, the agarose gel electrophoresis was later replaced by a polyacrylamide gel electrophoresis (PAGE) followed by silverstaining. A 10% polyacrylamide (Biorad, acrylamide/bis solution 29/1) gel (size: 30 x 40 cm spacer: 0.8 mm) was made with 1x TBE (90 mM Tris-borate, 2 mM EDTA). Seven µl PCR product were mixed with 3 µl loading buffer and transferred onto the PAGE gel. A DNA ladder (GeneRuler™ 100 bp DNA Ladder Plus) was included on the gels to size DNA positions. The PAGE gels were run on a Hoefer SQ3 Sequencer (Amersham Bioscience) at 100 V for 6 h. After electrophoresis, the gel was transferred for at least 30 min to a bath containing a 10 % acetic acid fixating solution. The gel was rinsed three times with MilliQ water to wash out the acid. The gel was then impregnated in a solution containing 0.1% $AgNO_3$ and 0.03 % formaldehyde for 20 min. After a short wash with MilliQ water, the gel was developed with a solution containing 2.5% sodium

carbonate, 0.03% formaldehyde and 0.002% sodium thiosulphate. The development was stopped with a 1.46% EDTA solution, after which the gel was soaked in a solution containing 15% ethanol and 10% glycerol. As a last step, the gel was wrapped in cellophane for further conservation.

In parallel to the PAGE gels, a new electrophoresis system was evaluated. In the SEA 2000® Electrophoresis Apparatus (Elchrom, Cham, Switzerland), the specially designed heat exchange element keeps the temperature of the running buffer constant and a circulating system guarantees a constant buffer flow. The electrodes are positioned in the gel plane, providing a uniform electric field over the gel compartment. In combination with ready-to-use gels, the SEA 2000® apparatus provides high reproducibility, high throughput and high resolution. Elchrom recommends two kinds of pre-cast gels for AFLP. The Spreadex gel can be used for applications that require a high resolution over a narrow size range. The Poly(Nat) gels give a high resolution over a wide size range. Before running a gel, the 0.5 x TAE buffer must be heated to 55°C with a thermostated circulating water bath. Five µl PCR product were mixed with 2 µl loading buffer and transferred onto the gel. A DNA ladder (GeneRuler™ 100 bp DNA Ladder Plus) was included on all gels to size DNA positions. The gels were run at 96 V for 100 min with a constantly circulating 0.5 x TAE buffer, subsequently stained with ethidium bromide for 40 min and destained in MilliQ water for 30 min.

6.2.2 Results

6.2.2.1 AFLP on synergel-agarose gel

The first results of the AFLP were promising (Figure 13) as differences in fingerprint patterns could be detected visually. The AFLP reactions were repeated and the fingerprint patterns were reproducible. However, due to the low resolution power of the gel, it was not possible to cut out AFLP fragments for further processing.

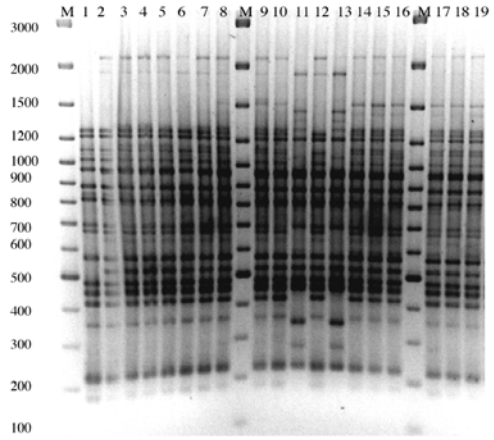


Figure 13: AFLP reaction run on synergel (15 x 15 cm)
(M: 100 bp plus ladder; 1-9, 12, 14-19: Map strains; 11 and 13: Maa strains)

6.2.2.2 AFLP on PAGE gel

PAGE gels, run on a sequence apparatus, are commonly used for the detection of AFLP fragments. The visualisation of the fragments is based on fluorescently-labelled primers. The technique has one major disadvantage, i.e. the fragments can only be detected and can not be used for further analysis. An alternative lies in the use of radioactively labelled primers but this requires radioactivity handling facilities. It also involves a complicated technique to recover fragments from the gel. Silver staining is commonly used for the coloration of small (8 x 10 cm) PAGE gels. Due to the thinness of the gel (0.8 mm), staining of big (30 x 40 cm) gels is more difficult. The gels tear easily during handling and this problem was resolved by a change in the design of the baths. All fluids used in the staining and washing were removed through a small hole in the staining bath, leaving the transfer from the glass plates to the staining bath and the cellophane wrapping process as the only critical points.
The resolution of the PAGE gel was much better than the resolution of the synergel-agarose gels (Figure 14). The fingerprint patterns were clear and fragments could be easily cut out for further analysis. The whole procedure to load, run and stain the PAGE gel takes more than 10 hours and requires skilled lab technicians. Due to the complexity of the technique, all AFLP reactions were first checked on agarose gels before running a PAGE gel.

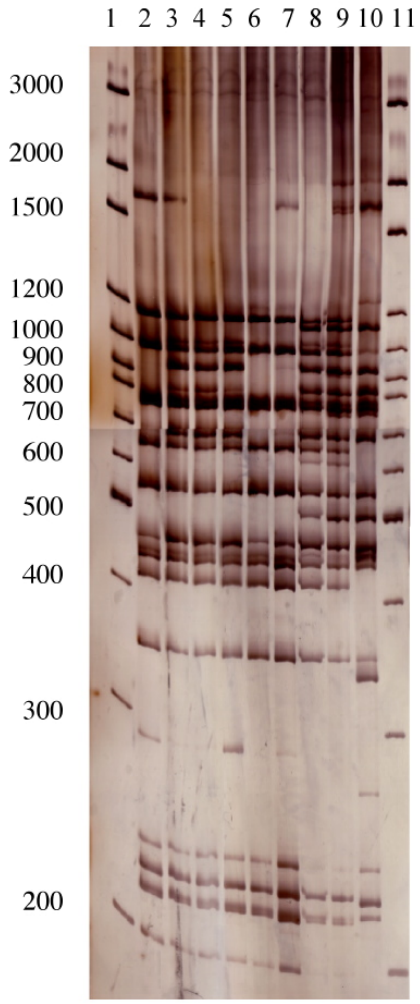


Figure 14: AFLP reaction run on PAGE gel (30 x 40 cm)
(1 and 11: 100 bp plus ladder; 2-9: Map strains; 10: Maa strain)

6.2.2.3 AFLP on Elchrom gels

The commercially available gels for the SEA 2000® Electrophoresis Apparatus were tested for their suitability in the AFLP system. The Spreadex gels gave a good resolution between 20 bp and 800 bp but bands over 800 bp were not efficiently separated. The 9 % Poly(Nat) gel (9.6 cm x 13 cm) gave a better overall resolution and, hence, was considered as the most appropriate gel for the AFLP procedure. The fingerprint patterns consisted of small, clear bands and no so-called gel smiling was

formed (Figure 15). With this technique, the AFLP results became available within 4 hours. The technique is very easy to perform and does not require special skills.

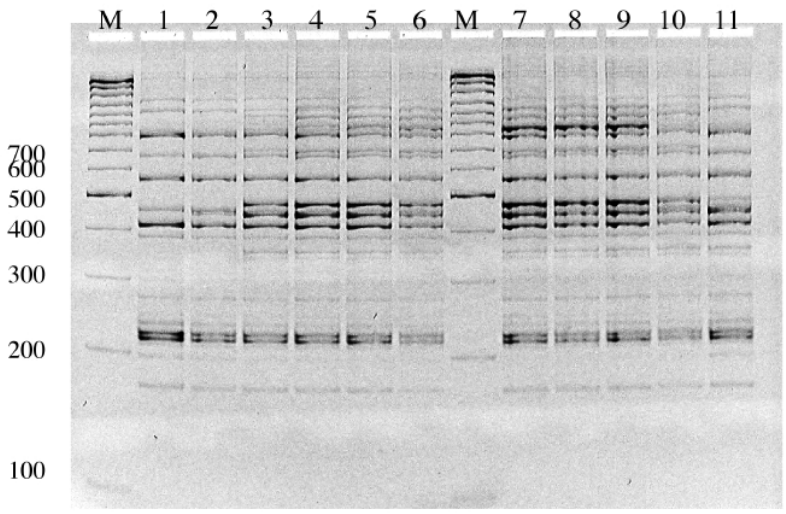


Figure 15: AFLP reaction run on ELCHROM system
(M: 100 bp plus ladder; 1-11: Map strains)

6.2.2.4 Comparison of different restriction enzymes

Four different enzymes were evaluated. The AFLP reactions were first checked on agarose gel (Figure 16). The fingerprint patterns obtained with *EcoR*I were the most useful for our purpose. The fingerprint pattern of *Taq*I and *Msp*I contained many small bands. These bands could almost not be seen on agarose but analysis on PAGE gel showed many small bands generating complex patterns unsuitable for further analysis. The fingerprint patterns obtained by restriction with *Apa*I were clear and useful but had fewer bands than the ones obtained by *EcoR*I restriction.

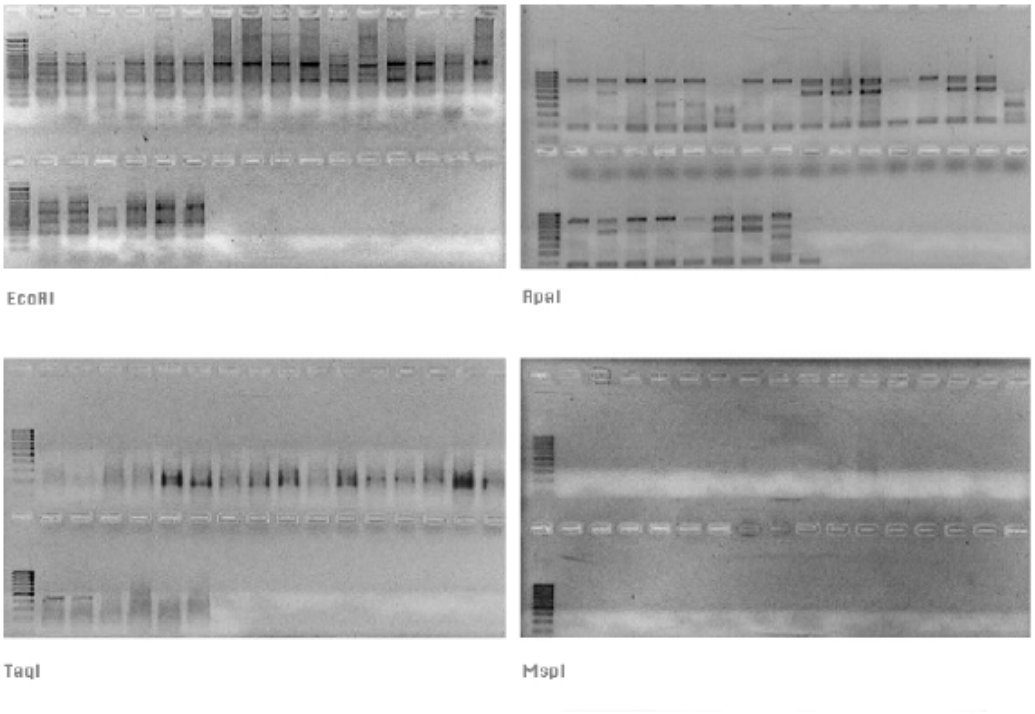


Figure 16: AFLP reaction with different enzymes as visualised on agarose gels
(Lane 1: 100 bp plus ladder, other lanes: Map strains)

6.2.2.5 Comparison of primers with different selective nucleotides

Selective primers containing the 4 possible nucleotides at their 3' end were tested. The best results were obtained with C or G as selective nucleotide. A or T as selective nucleotides generated only a few bands and gave low discriminatory power. These results can be explained by the high GC content of the Map genome. With a GC content of 69.30%, the chance of finding G or C after the restriction site is higher than finding an A or T.

6.3 Informative fragments: recovery from the gel

Each fingerprint pattern consists of a combination of fingerprint bands or fragments. The difference among various fingerprint patterns consists in the presence or absence of specific bands. Conservative bands are present in all isolates and can be considered as the basic fingerprint pattern of Map. Informative bands are only present in certain isolates and give information about nucleotide differences amongst the genomes of Map isolates. A phylogenetic analysis of the AFLP patterns was done using the computer programme Bionumerics (Applied Maths, Kortrijk). The phylogenetic tree could not subdivide our isolates in subgroups according to their geographical region or species origin. Therefore, it was decided to identify the fragments visually.
The informative bands were isolated from the gels and the DNA of these bands was amplified to have enough DNA for cloning.

6.3.1 Materials and Methods

6.3.1.1 Recovery of DNA from PAGE gels

Several protocols were tested for the recovery of DNA from the PAGE gels. Most protocols are normally used on ethidium bromide stained PAGE gels.

QIAEX II gel extraction kit (Qiagen, Hilden, Germany)
Purification of DNA fragments with the commercial available QIAEX II system is based on solubilisation of agarose or polyacrylamide and selective adsorption of nucleic acids onto QIAEX II silica-gel particles in the presence of a chaotropic salt. QIAEX II separates DNA from salts, agarose, polyacrylamide, dyes, proteins, and nucleotides without phenol extraction or ethanol precipitation. QIAEX II is effective for any type of agarose in either TAE or TBE buffer. QIAEX II particles ensure efficient DNA recovery without shearing, even for large DNA fragments. A gel slice containing the DNA band was excised with a clean and sharp scalpel. The supplied diffusion buffer was added to the gel slice and the mixture was incubated for 30 min

at 50°C. Supernatant was recovered and QIAEX II silica-gel particles were added. After incubation for 10 min at RT, the particles were collected by a brief centrifugation step. After washing, the pure DNA fragments were eluted in 20 µl of TE-buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Five µl was used in the PCR reaction.

Crush and Soak protocol (Chen et al., 1996)

The desired band was cut out with a scalpel. After crushing the acrylamide with a small (p1000) pipet tip, 500 µl of a crush and soak solution (500 mM NH_4OAc (Sigma), 0.1% SDS, 0.1 mM disodium EDTA) was added. The samples were incubated overnight at 37°C. After centrifugation for 10 min at 12,000 x *g*, the supernatant was transferred to a new 1.5 ml tube. The DNA was precipitated by adding 0.1 vol of 3 M NaOAc and 2.5 vol of 100% EtOH (0°C). The tube was centrifuged for 10 min at 12,000 x *g*, washed with 200 µl of 70% EtOH and dried after which the pellet was resuspended in 20 µl of TE buffer. Five µl was used in the PCR reaction.

MilliQ water protocol

The band of interest was excised with a scalpel and transferred to a tube. Hundred µl MilliQ water was added and the tube was incubated overnight at 60°C. The supernatant was collected after centrifugation for 10 min at 12,000 x *g*. Five µl was used in the PCR reaction.

Needle protocol (Stumm et al., 1997)

This is the only protocol described for elution of DNA from silver stained PAGE gels. The informative band was scratched with a prewetted sterile fine needle (Microlance 3, 0.3 mm, Becton Dickinson). The needle was placed for 1 min in a 25 µl PCR reaction mixture. A normal PCR reaction was performed to amplify the recovered DNA.

6.3.1.2 Recovery of DNA from ELCHROM gels

The BandPick system developed by Elchrom is an easy tool to recover DNA from gels (Figure 17). The black piston was dipped in MilliQ water to lubricate it. The

piston was then inserted in the hollow tube. The bandpick was pressed in the band of interest and turned around. The piston was lifted while the band pick was still in the gel. The whole bandpick was lifted from the gel and the gel piece was transferred to a test tube by pushing the piston. The gel piece was incubated at 70° C for 15 min in 20 µl MilliQ water containing 5 mM Mg^{2+}. Five µl was used in the PCR reaction.

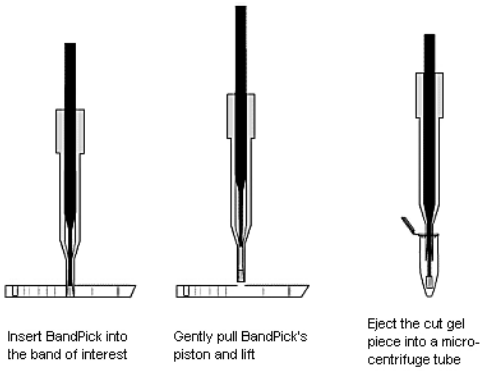


Figure 17: BandPick system of ELCHROM to recover DNA from gels

6.3.1.3 PCR reaction

To obtain sufficient DNA for cloning, the AFLP fragments were amplified in a PCR reaction with a primer complementary to the adapter sequence (same primer as used in the AFLP reaction see 6.2.1.3.2). The PCRs were performed in a final volume of 25 µl and PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: adapter primer			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	60	60	
Extension	72	120	

6.3.2 Results

The recovery of DNA from PAGE gels was difficult and gave variable results. With the commercial kit, QIAEXII, no DNA could be amplified. Only one of five bands isolated with the needle procedure could be amplified by PCR. Moreover, post-PCR processed gels had to be transferred to a pre-PCR area where the gel is scratched with the needle. Normally, no post-PCR products should enter the pre-PCR area to avoid amplicon contamination of the pre-PCR area. The performance of the MilliQ water technique was slightly better. Nine of 25 bands could be amplified in a PCR reaction. The best results were obtained with the "crush and soak" method but the results were gel dependent. All bands of some gels were easily recovered whereas the bands of other PAGE gels were impossible to recover, although the same protocol was always used for gel preparation, staining and recovery of the DNA fragments. The problem might be caused by the silver staining. The first staining step is a fixation by acetic acid and its is known that acetic acid can affect DNA. Furthermore, silver forms complexes with the DNA, making it difficult to elute the DNA from the polyacrylamide gel. Another possible reason could be an interference with the TBE used as running buffer. Due to a higher buffering capacity, TBE based buffers are used in long electrophoresis runs, although TAE is normally suggested as a buffer in case the DNA needs to be recovered (Cambrex, technical information). Elution of the DNA from the Elchrom gel was fast and easy. All bands could be amplified in the PCR reactions and could be used for further analysis. A total of 30 informative bands were recovered with the "crush and soak" and Elchrom protocol from the gels and successfully amplified in the PCR reaction.

6.4 Informative fragments: Cloning

The DNA from the eluted bands needed to be cloned before the sequence could be determined. The isolated fragments could be mixtures of different DNA fragments of identical size. DNA cloning is a general method of selective multiplication of DNA sequences to generate a homogenous DNA population.

6.4.1 Materials and methods

6.4.1.1 DNA purification

After amplification of the eluted AFLP bands, the Fast 'n Easy agarose gel DNA purification kit (Anansa, Sanvertech, Boechout) was used to recover the DNA from the agarose gel. The resulting DNA can be used for any down-stream application. The DNA was cut out from the agarose gel and placed in the spin filter basket. Three volumes of spin buffer (containing $NaClO_2$) were added to the gel slice. After 3 min incubation at 65°C, the spin filter was centrifuged for 10 s at 10,000 x *g*. The total volume of the passed liquid was reloaded on the spin filter and centrifuged for another 10 s at 10,000 x *g*. The liquid was discarded and 300 µl SpinWash buffer containing Tris/ethanol was added. After centrifugation at 10,000 x *g* for 30 s, 50 µl elution solution (10 mM Tris) was added. The DNA was collected through centrifugation at 10,000 x *g* for 30 s.

6.4.1.2 Cloning

After purification of the PCR products, the DNA was cloned in the pGem-T easy vector system II (Promega, Madison, WI, USA). The vector has a 3' T overhang at the insertion site. This improves the efficiency of ligation of PCR products into the plasmid by preventing recirculation of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. Taq Polymerase adds a single deoxyadenosine in a template-independent fashion to the 3' ends of the amplified fragments.

Three µl of the purified DNA were mixed with 50 ng of the pGem® T-easy vector (with ampicillin resistance gene), 3 units of T4 DNA ligase and 5 µl of a 2 x ligation buffer. The ligation mixture was incubated overnight at 4°C. *E. coli* high efficiency competent cells (JM 109) were transformed by mixing with the vector and subsequent incubation on ice for 20 min. Afterwards, the competent cells were subjected to a heat shock in a water bath at 42°C for 45 s. The tubes were immediately returned on ice for 2 min. Nine hundred fifty µl of Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) was added and the tubes were incubated for 1.5 h at 37°C under constant shaking (150 rpm, Thermomixer, Eppendorf). Hundred µl of each transformation culture was plated onto selective LB agar plates (1.5% agar, 1%

tryptone, 0.5% yeast extract, 1% NaCl, 100 µg/ml ampicillin (Sigma), 40 µg/ml X-Gal (MBI Fermentas), 0.5 µg/ml IPTG (inducer of the ß-galactosidase, Sigma). The plates were incubated at 37°C during 16-24 h. Successful cloning of an insert in the vector interrupts the coding sequence of ß-galactosidase. The recombinant clones can usually be identified by colour screening on the indicator plates. Blue colonies mean that the ß-galactosidase gene is not disrupted and no fragment has been inserted in the plasmid. Clones that contain PCR products produce white colonies. Of each plate, 10 white colonies were subcultured to new selective LB agar plates.

6.4.1.3 Checking clones with PCR

All subcultured clones were screened with PCR to confirm the presence of the insert in the vector. The colonies were picked up with a sterile tip and transferred to a tube with 50 µl MilliQ water. Five µl of the MilliQ mixture was used in the PCR reaction. The PCRs were performed in a final volume of 25 µl and PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: adapter primer (40 pM)			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	60	60	
Extension	72	120	

6.4.1.4 SSCP

Single-stranded DNA has a tendency to fold up and form complex structures stabilized by weak intramolecular bonds, notably base-pairing hydrogen bonds. The electrophoretic mobilities of such structures on non-denaturing gels will depend not only on their chain lengths but also on their conformations, which are dictated by the DNA sequences. For SSCP analysis, the amplified DNA samples are denatured and loaded onto a non-denaturing PAGE gel (Strachan *et al*., 1999).

All PCR positive clones of one fragment were checked with SSCP. It is possible that the clones from one fragment contain different inserts (as the band can consist of

several DNA fragments with the same bp length). Clones showing a different SSCP pattern have a different nucleotide sequence and should be sequenced.

Eight µl of D2 buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was mixed with 4 µl of PCR product. The mixture was boiled for 5 min at 95°C. The tubes were immediately cooled down on ice for 10 min. Six µl of the mix was loaded on a pre-run 10 % PAGE gel. The gels were run in a 1 x TBE buffer over 16 h at 100 V at 4°C on a Hoefer Mighty Small (Amersham Biosciences). The λ DNA/HindIII Marker (MBI Fermentas) was used as size marker on each gel. After electrophoresis, the gel was transferred to a bath containing a 10 % acetic acid solution. The gel was incubated in this fixation solution for at least 30 min. Afterwards the gel was rinsed three times with MilliQ water to wash away the acid. The gel was impregnated for 20 min in a solution containing 0.1% $AgNO_3$ and 0.03 % formaldehyde. After a short wash with MilliQ water, the gel was developed with a solution containing 2.5% sodium carbonate, 0.03% formaldehyde and 0.002% sodium thiosulphate. The development was stopped with a 1.46% EDTA solution and the gel was soaked in a solution with 15% ethanol and 10% glycerol. Finally, the gel was wrapped in cellophane to conserve the gel.

6.4.2 Results

Among the 30 fragments recovered from the AFLP patterns, 47 different clones could be detected. All these clones were sequenced by the VIB Genetic Service Facility at the University of Antwerp using the M13 forward primer.

6.5 Sequence analysis

The sequence information forms the basis for the development of PCR assays. First of all, the sequences had to be cleaned from adapter sequences. Sequences with special features were further analysed and tested for their suitability to differentiate isolates by PCR.

6.5.1 Sequence clean-up and BLAST

All sequences obtained from the 47 clones were cleaned up using the Gene Jockey II ® DNA program (Biosoft). The nucleotides belonging to the vector and the adapter were removed from the sequence. The remaining nucleotides originated from the fragment and were used for further analysis.

6.5.1.1 BLAST

Each sequence was entered in a BLAST program for homology search to find sequences similar to the query sequence. A Map nucleotide – nucleotide BLAST (BLASTN) was performed to find similarity with the Map genome. The whole sequence of Map is not yet published. An unfinished, whole genome shotgun sequence of Map strain K10 is available for BLAST on the website of NCBI (accession number NC 002944). A standard nucleotide - nucleotide BLAST on the general database was also performed to find similarities with other mycobacterial and non-mycobacterial genomes.

6.5.1.2 BLAST results (Table 17)

Six sequences showed 100% similarity with parts of the contigs of the K10 Map genome (accession number NC 002944). No similarity was found for two sequences. One sequence was the full sequence of two smaller fragments (encompassing the restriction site). Two sequences were also full sequences of two smaller fragments but without the presence of a restriction site between the two fragments. One sequence was found to have an insertion of 50 bp and could be found twice. Two sequences (fragments originating from M02-1679) showed similarity with genomes from other mycobacteria and revealed only a low homology with the Map genome. Four sequences showed only partial similarity with the Map genome, whereas one of the four was the reverse complement of one of the others. A clear restriction site change was observed in two sequences. All other sequences showed differences in only less than 6 nucleotides.

The most promising polymorphic sequences were further analysed (L17, ptb 102, K8, K13, B116). These 5 sequences showed clear polymorphisms when compared with

sequences of the K10 strain (accession number NC 002944) used as reference strain genome for Map. In the other sequences, the differences were not immediately clear.

	Clones	Length (bp)	Fragment	Elution	Strain	Origin	Remark
1	ptb121	417	F1	crush and soak	M02-1463	cervid	100% similarity with Map
2	ptb147	466	F2	crush and soak	M02-1463	cervid	1 basepair difference with Map
3	ptb120	431	F3	crush and soak	M00-04	bovine	3 basepair difference with Map
4	cl1719 (3.1)	327	F4	crush and soak	M00-04	bovine	3 basepair difference with Map
5	cl1701 (1.3)	199	F5	crush and soak	M02-0904	cervid	1 basepair difference with Map
6	cl1702 (1.4)	209	F5	crush and soak	M02-0904	cervid	1 basepair difference with Map
7	cl1710 (2.2)	250	F6	crush and soak	M02-1463	cervid	1 basepair difference with Map
8	E2	325	F7	crush and soak	M02-909	cervid	1 basepair difference with Map
9	E9	325	F7	crush and soak	M02-909	cervid	2 basepair difference with Map
10	E13	339	F8	crush and soak	M02-909	cervid	100% similarity with Map
11	E22	431	F9	crush and soak	M02-1465	cervid	3 basepair difference with Map
12	E23	463	F9	crush and soak	M02-1465	cervid	100% similarity with Map
13	E35	463	F10	crush and soak	M02-0904	cervid	3 basepair difference with Map
14	E38	499	F10	crush and soak	M02-0904	cervid	no similarity with Map or other mycobacteria
15	E44	441	F3	crush and soak	M00-04	bovine	5 basepair difference with Map
16	E45	441	F3	crush and soak	M00-04	bovine	4 basepair difference with Map
17	E54	431	F9	crush and soak	M02-1465	cervid	4 basepair difference with Map
18	E59	451	F1	crush and soak	M02-1463	cervid	6 basepair difference with Map
19	L12	441	F11	crush and soak	M02-1447	cervid	2 basepair difference with Map
20	L16	678	F12	crush and soak	M02-1458	cervid	3 basepair difference with Map
21	L16B	399	F13	crush and soak	M02-1458	cervid	insertion sequence of 50 bp (same as L17)
22	K6	424	F14	elchrom	M02-1448	cervid	1 basepair difference with Map
23	K8B	486	F15	elchrom	M02-1465	cervid	no similarity with Map or other mycobacteria
24	K27	205	F16	elchrom	M02-1465	cervid	100% similarity with Map
25	K4	597	F17	elchrom	M02-1444	cervid	1 basepair difference with Map
26	K28	597	F18	elchrom	M02-1444	cervid	1 basepair difference with Map
27	B106	771	F19	elchrom	M02-1441	cervid	4 basepair difference with Map
28	B114	771	F19	elchrom	M02-1441	cervid	4 basepair difference with Map (same as B106)
29	B104	770	F19	elchrom	M02-1441	cervid	5 basepair difference with Map

	Clones	Length (bp)	Fragment	Elution	Strain	Origin	Remark
30	B115	409	F20	elchrom	M02-1441	cervid	full sequence of 2 fragments with restriction site in the middle
31	B117	421	F20	elchrom	M02-1441	cervid	full sequence of 2 fragments without restriction site in the middle
32	B118	465	F21	elchrom	M02-1441	cervid	partial similarity with Map (reverse complement of B116)
33	B122	466	F21	elchrom	M02-1441	cervid	partial similarity with Map (same as B116)
34	B127	380	F22	elchrom	M02-1445	cervid	100% similarity with Map
35	B130	318	F22	elchrom	M02-1445	cervid	5 basepair difference with Map
36	B132	300	F22	elchrom	M02-1445	cervid	100% similarity with Map
37	B145	202	F23	elchrom	M02-1679	bovine	small similarity with Map, similarity with mycobacteria in general
38	B151	480	F23	elchrom	M02-1679	bovine	cloning vector
39	B1	449	F24	elchrom	M02-1040	bovine	2 basepair difference with Map
40	B3	365	F25	elchrom	M02-0904	cervid	1 basepair difference with Map
41	L17	398	F26	crush and soak	M02-1461	cervid	insertion sequence of 50 bp
42	E14	242	F8	crush and soak	M02-0909	bovine	full sequence of 2 fragments without restriction site in the middle
43	ptb102	421	F10	crush and soak	M02-0904	cervid	partial similarity with Map
44	B116	465	F27	elchrom	M02-1441	cervid	partial similarity with Map
45	L14	303	F28	crush and soak	M02-1679	cervid	small similarity with Map, similarity with mycobacteria in general
46	K8	301	F29	elchrom	M02-1465	cervid	change in restriction site
47	K13	338	F30	elchrom	M02-1465	cervid	change in restriction site

Table 17: Overview of all cloned fragments

6.5.2 L17 fragment

This fragment originated from an AFLP reaction with *EcoR*I and selective nucleotide G on cervid isolate M02-1461. The sequence length was 398 bp. The fragment was found in 20 isolates with 7 originating from cervids and 13 originating from cattle. No band of similar size could be observed in the other isolates.

The BLAST result is shown in Figure 18. Homology was found from nucleotide 1 to 246 and from 298 to 398 which corresponds to nucleotide 44053 to 44298 and 44310 to 44410 of Contig 234 of Map K10 (accession number NC 002944). Nucleotide 247 to 297 (51 bp) from the L17 fragment corresponds to nucleotide 44299 to 44309 (11 bp) (Figures 18 and 19). A characterisation assay could be developed amplifying the fragment encompassing the insert. A specific restriction site in the insert could be used to differentiate between isolates with and without the insert.

```
Query: 1     aattcgccggtgatgatggccgacgtcatcgtgggcagcgtcggcaagatgcgggtcaag 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 44053 aattcgccggtgatgatggccgacgtcatcgtgggcagcgtcggcaagatgcgggtcaag 44112

Query: 61    cacgatcacgccgacgtcgaggtctcggtgaaacccgatgtggtggtgcccggaaacgcg 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 44113 cacgatcacgccgacgtcgaggtctcggtgaaacccgatgtggtggtgcccggaaacgcg 44172

Query: 121   gtggccgccgtcggacagaccagcctgctgggttcgatgcacgtggagctcagtcccccg 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 44173 gtggccgccgtcggacagaccagcctgctgggttcgatgcacgtggagctcagtcccccg 44232

Query: 181   ctgggccaaccgccgcggggacggctgcagcccggggccaccatcccgctggacaggtca 240
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct: 44233 ctgggccaaccgccgcggggacggctgcagcccggggccaccatcccgctggacagggca 44292

Query: 241   tcgtcg 246
             ||||||
Sbjct: 44293 tcgtcg 44298



Query: 298   ggtggcctgggccagatcggcgacatcgtgcacaacttcagcgcggcgttgtccggccac 357
             |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 44310 ggtgtcctgggccagatcggcgacatcgtgcacaacttcagcgcggcgttgtccggccac 44369

Query: 358   gagaccgatgtgcgccaactgctgacgcgcctcgacgaatt 398
             |||||||||||||||||||||||||||||||||||||||||
Sbjct: 44370 gagaccgatgtgcgccaactgctgacgcgcctcgacgaatt 44410
```

Figure 18: BLAST results for L17 fragment against Map genome

(Query: L17, Sbjct: contig 234 of Map K10 genome)

```
43653 CGACGAACAT CATCCTGCAG CAGATCGCCA CTCCCGGAAA AATCATCTAC
43703 GCCGAACCGC GCCTGGCGCC CGGCGCCGAA GGGCCATCGC CCACGCCCCC
43753 CGACGTCCCG CCGGCGGTAT CCGCCTACAC CGGCATCAAC GGTGACTCGA
43803 CACCGCACAG CGTGCAGGAC CTGCTGCTGC CCGACGATCG CCAGCCGGCG
43853 CCGGGCGATC AGCCGCCGCC GCCGGGCCCG GGGACGCCGC CATGACGAGG
43903 GTCCGGCTTT GTATCGTAGC GTGCGCCTTC GCCGTTGGTG GCTGTTCCTT
43953 TCAGGGCGTG AACTCGTTGC CCCTGCCCGG TGCTGTCGGT CGCGGGCCAC
44003 ACGCCAGCAT CTATCACATC GAGATCGCCA ATATCGGCAC CCTGGAATCG
44053 AATTCGCCGG TGATGATGGC CGACGTCATC GTGGGCAGCG TCGGCAAGAT
44103 GCGGGTCAAG CACGATCACG CCGACGTCGA GGTCTCGGTG AAACCCGATG
44153 TGGTGGTGCC CGGAAACGCG GTGGCCGCCG TCGGACAGAC CAGCCTGCTG
44203 GGTTCGATGC ACGTGGAGCT CAGTCCCCCG CTGGGCCAAC CGCCGCGGGG
44253 ACGGCTGCAG CCCGGGGCCA CCATCCCGCT GGACAGGGCA TCGTCGGCCG
44303 CTTGCAGGGT GTCCTGGGCC AGATCGGCGA CATCGTGCAC AACTTCAGCG
44353 CGGCGTTGTC CGGCCACGAG ACCGATGTGC GCCAACTGCT GACGCGCCTC
44403 GACGAATTCG TCGGCGTCCT GGACCAACAG CGCGACAGGA TCATTGCGTC
44453 GATCGACTCA CTGAACCGGC TCGCCGGCAC ATTCGCCTCC CAGCGCGAGG
44503 TGATCACCCA AGCGCTGCGC AAGATTCCGC CCGCGCTCGA CGTGCTGATC
44553 CGCGAACGGC CACGCATCAC CGCGGCCCTG GACAAACTCC GCGTGTTCAG
44603 CAACACCGCA ACGCAATTGG TCAATGAGAC CCAGGCCGAC CTGGTGAAGA
44653 ACCTGCAAAA TCTGGAGCCG ACGATCCAGG CGCTGGCGGA CGTGGGGCCC
44703 GATCTCAGCA CCGTTTTGGG ATATGTGCCG ACCTTCCCGT TCACGCAGAA
44753 CTTCATTGAC CGGGCGGTGC GGGGTGACTA CTTCAACGTC TTCGCCGTCA
 44803 TCGACAT
```

Figure 19: Contig 234 of Map genome with in bold the sequence of the L17 fragment.
(in red: no similarity found with L17; underlined: primers L17S, L17R, L17R2)

```
1     AATTCGCCGG TGATGATGGC CGACGTCATC GTGGGCAGCG TCGGCAAGAT
51    GCGGGTCAAG CACGATCACG CCGACGTCGA GGTCTCGGTG AAACCCGATG
101   TGGTGGTGCC CGGAAACGCG GTGGCCGCCG TCGGACAGAC CAGCCTGCTG
151   GGTTCGATGC ACGTGGAGCT CAGTCCCCCG CTGGGCCAAC CGCCGCGGGG
201   ACGGCTGCAG CCCGGGGCCA CCATCCCGCT GGACAGGTCA TCGTCGTATC
251   CCTCGACCGA ACAGACCTTA TCGGCGCTGT CGGCGGTGCT CAATTCCGGT
301   GGCCTGGGCC AGATCGGCGA CATCGTGCAC AACTTCAGCG CGGCGTTGTC
 351   CGGCCACGAG ACCGATGTGC GCCAACTGCT GACGCGCCTC GACGAATT
```

Figure 20: The L17 fragment
(in red the insertion sequence that is not found in the Map K10 genome, underlined: primers L17S, L17R,)

6.5.2.1 Test 1

6.5.2.1.1 Primer design

Two primers were designed to amplify a fragment encompassing the insert. First, an alignment was made between the L17 fragment and the corresponding sequence of contig 234. This alignment was used in a primer design program PRIMER PREMIER 5 (PREMIER Biosoft International, Palo Alto, CA, USA). The obtained primers were first checked with a standard nucleotide BLAST (NCBI). All primers that could

amplify other DNA fragments were not further considered. The remaining primers were used in PCR software Amplify (Engels, 1993). Two primers (L17S: 5' GGC AAG ATG CGG GTC AAG CA 3' and L17R: 5' GCG TCA GCA GTT GGC GCA CAT 3') were selected giving amplification products of 305 bp (without insert) and 344 bp (with insert). As the differences in length were difficult to detect on agarose gel, a restriction enzyme that only restricts the fragment within the insert was identified with Gene Jockey ® DNA program (Biosoft).

The enzyme *Hae*II (5' R GCGC / Y 3') restricts the fragment at nucleotide 234 giving fragments of 234 and 109 bp long.

6.5.2.1.2 Materials and methods

PCR

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: L17S			
Primer: L17R			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	58	60	
Extension	72	90	

Restriction

Four µl PCR product was mixed with 6 U *Hae*II and the supplied buffer (New England Biolabs). The tubes were incubated overnight in a water bath at 37°C. Four µl of the mixture was loaded on a 10% PAGE gel and run at 100 V for 2 h 40. A DNA ladder (GeneRuler™ 100 bp DNA Ladder) was included in every gel. Finally, the PAGE gels were silver stained.

6.5.2.1.3 Results and conclusion

All Map isolates could be amplified with primers L17S and L17R. On agarose gel no differences between band lengths could be detected. All PCR products were used in a restriction with the *Hae*II enzyme. On PAGE gel, all isolates revealed two bands (109 bp and 234 bp) meaning all fragments were restricted and contained the insert. The amplified fragments were also analysed with SSCP and all gave the same pattern on PAGE gel. Three fragments were cloned and sequenced. The insertion could be detected in all sequences. Further analysis of the fragments showed that the two sequences maintained their open reading frame (ORF) and were not introducing a stop codon. A protein-protein BLAST revealed that both have a significant homology with pfam02470, a mycobacterial cell entry related protein. A sequence error in the K10 strain can be the cause of the absence of the insertion but the conservation of the open reading frame and the conserved coding regions indicate the opposite.

6.5.2.2 Test 2

6.5.2.2.1 Primer design

To test the hypothesis that the fragment without the insert is present in a minor fraction in the genomic DNA, a primer (L17R2 5' CAG GAC ACC CTG CAA GCG 3') was designed on the place of the insertion (Figure 19). The PCR reaction with primers L17S and L17R2 will only give amplification if a fragment without insert is present in the DNA samples. The fragments with insert cannot be amplified.

6.5.2.2.2 Materials and method

PCR

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: L17S **Primer: L17R2**			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	58	60	
Extension	72	90	

6.5.2.2.3 Results and conclusion

No amplification could be detected. The L17 without insert was definitely not present in our isolates. For the L17 fragment, no polymorphism could be demonstrated in our Map collection. We concluded that the observed polymorphisms on AFLP gels are not due to the insert but are probably due to a polymorphism in restriction sites at the 5' or 3' end of the obtained sequence. The only polymorphism that could be detected is the L17 locus of the sequenced K10 strain. It is essential to confirm this on a DNA sample of the Map K10 strain. We requested Dr. Kapur for providing us the K10 strain for further investigation but until now, we have not received the strain.

If it can be confirmed that this K10 strain does not have the insertion, it becomes important to clarify the function of this insertion. It might be that the insertion sequence could still be used in a PCR based characterisation assay.

6.5.3 Ptb 102 fragment

Fragment ptb 102 originated from an AFLP reaction with *EcoR*I restriction and a selective nucleotide G on cervid isolate M02-0904. The fragment was only observed

in this isolate and could not be detected in the other isolates. The sequence is 421 bp long. The BLAST results against the Map genome (accession number NC 002944) are shown in Figure 21. Only a part of the sequence showed homology with contig 179 of the Map genome (Figure 22). The corresponding sequence of Maa and Mtb were aligned with the sequence of contig 179 and a high degree of homology was found between these mycobacteria (Figure 23). Further analysis of ptb 102 revealed an open reading frame. The putative protein sequence was used in a protein-protein BLAST (NCBI) and homology was found with the conserved domain of the Sec A gene. Contig 179 was also translated and used in a protein-protein BLAST, giving also a good hit with the conserved domain of Sec A. SecA is part of the prokaryotic protein translocation apparatus and has a central role in coupling the hydrolysis of ATP to the transfer of pre-secretory periplasmic and outer membrane proteins across the cytoplasmic membrane.

```
Query: 4    tcgtcgacgatcaccacttcgccgtc 29
             ||||||||||||| ||| ||||||||
Sbjct: 7186 tcgtcgacgatcagcacctcgccgtc 7211


Query: 118  tcgtacaggttctcgatg 135
             |||||||||| ||||||
Sbjct: 7300 tcgtacaggttgtcgatg 7317


Query: 276  ggccgccccggagatgatcagcggcgtccgcgcttcgtcgatgaggatgctgtccacctc 335
             ||||| |||||||||||||||||| || || || |||||||| |||||   ||| |||||
Sbjct: 7458 ggccggcccggagatgatcagcggggtgcgggcctcgtcgatcaggatcgagtcgacctc
7517

Query: 336  gtcga 340
             |||||
Sbjct: 7518 gtcga 7522


Query: 389  ccatgttgtcacgcaggtagtcaaagccgaa 419
             |||||||||| |||||||||| ||||||||
Sbjct: 7571 ccatgttgtcgcgcaggtagtcgaagccgaa 7601
```

Figure 21: BLAST results for the ptb 102 fragment against the Map genome
(Query: ptb102, Sbject: contig 179 of Map genome accession number NC 002944)

Contig 179

```
6808  TACTCCGAAC GCTCGACGCT CGTGGTGCCG ATCAGCACCG GCTGGCCCTT
6858  CTGGTAGCGC TCGACCACGT CGTCGACCAC CGCGATGTAC TTGGCTTCCT
6908  CGGTCTTGTA GATCAGGTCG GACTGGTCGG TGCGGATCAT CGGCTTGTTG
6958  GTCGGGATCG GCACCACGCC GAGCTTGTAG ATCTCGTGCA GCTCGGCCGC
7008  CTCGGTCTGG GCGGTGCCGG TCATGCCGGA CAGCTTGTCG TAGAGCCGGA
7058  AGTAGTTCTG CAGCGTGATG GTGGCCAGCG TCTGGTTCTC AGCCTTGATC
7108  TCGACGTGCT CCTTGGCCTC GATGGCCTGG TGCATGCCCT CGTTGTAGCG
7158  GCGACCGTAC AGGACGCGAC CGGTGAACTC GTCGACGATC AGCACCTCGC
7208  CGTCGCGCAC GATGTAGTCC TTGTCGCGGT GGAACAGCTC CTTGGCCTTC
7258  AGCGCGTTGT TGAGGTAGCT GACCAGCGGC GAGTTGGCCG CCTCGTACAG
7308  GTTGTCGATG CCGAGCTGGT CCTCGACGAA CTCCACCCCC AGCTCGTGCA
7358  CGCCGACGGT GCGCTTGCGC AGATCCACCT CGTAGTGGAC GTCCTTTTCC
7408  ATCAGCGGGG CCAGCCGGGC GAATTCGAGG TACCAGTTGG AGGCGCCGTC
7458  GGCCGGCCCG GAGATGATCA GCGGGGTGCG GGCCTCGTCG ATCAGGATCG
7508  AGTCGACCTC GTCGACGATC GCGAAGTTGT GCCCGCGCTG CACCAGGTCG
7558  TCGAGCGAGT GCGCCATGTT GTCGCGCAGG TAGTCGAAGC CGAACTCGTT
7608  GTTGGTGCCG TAGGTGATGT CGGCGTTGTA GGCGACCCGG CGCTCGTCGG
7658  GCGTCATCTG GGCGAGAATC ACCCCGACGT CCAGGCCCAG GAAGCGGTGC
7708  ACCCGGCCCA TCCACTCGCT GTCGCGTTTG GCCAGGTAGT CGTTGACGGT
7758  GACGACGTGC ACGCCCTTGC CGCCGATGCC GTTGAGGTAG GCGGGCAGCA
7808  CCGAGGTCAG GGTCTTGCCC TCACCGGTCT TCATCTCGGC GACGTTGCCG
7858  AAGTGCAGCG CCGCCGCGCC CATCACCTGC ACGTCGAACG GGCGCTGGTC
7908  GAGCACCCGC CAGGCCGCCT CCCGGGCCAC CGCGAACGCC TCGGGCAGCA
7958  GGTCGTCGAG GCTCTCGCCG TCGGCGTGCC GCTTCTTGAA CTCGTCGGTC
8008  TTGGCCCGCA ACTCGGCGTC GGTGAGCTTT TCGACCTCGT CGGACAAGGT
8058  GTTGACGTAG TCAGCCACCC GCTTGAGACG CTTGAGCATG CGACCTTCGC
8108  CAAGGCGCAG CAACTTCGAC AGCACGGTGT TGTCCTCTAC GGTCTGATTG
```

Ptb 102

```
1     AATTCGTCGA CGATCACCAC TTCGCCGTCC TGCACCACGT AATCCACGTC
51    CCGTTTGAAC AGGACTTGCG CTTTGAGCGC AATGTTGATA TGGTGGTTCA
101   GGGTCATGTG GGCGGTGTCG TACAGGTTCT CGATGTTGAA CGCCTGCTCC
151   ACCTTGGCCA CACCCTCGTC GGTCAGGGTG ACGATTTTCA GCTTTTCATC
201   AACGGTGAAG TCCTTTTCCG GCTCCAGCCG CTTTACAAAA TGAGCACAGA
251   TGTAGTACAG CTCCGTGGAA CGGTTGGCCG CCCCGGAGAT GATCAGCGGC
301   GTCCGCGCTT CGTCGATGAG GATGCTGTCC ACCTCGTCGA TGATGGCAAA
351   GAACAGAGGG CGCTGCACCA TTTGTTCCTT GTAGAGCACC ATGTTGTCAC
401   GCAGGTAGTC AAAGCCGAAT T
```

Figure 22: Comparison between the sequence of contig 179 and ptb 102 fragment

(Bold: similarity between fragment and contig; Underlined primers: ptb102S, ptb102R, ptb102R2)

```
Map          AGTAGTTCTGCAGCGTGATGGTGGCCAGCGTCTGGTTCTCAGCCTTGATCTCGACGTGCT 7118
Maa          AGTAGTTCTGCAGCGTGATGGTGGCCAGCGTCTGGTTCTCGGCCTTGATCTCGACGTGCT 660
Mtb          AGTAGTTCTGCAGCGTGATGGTGGCCAGCGTCTGGTTCTCGGCCTTGATCTCGACGTGCT 653
             ************************************* ******************

Map          CCTTGGCCTCGATGGCCTGGTGCATGCCCTCGTTGTAGCGGCGACCGTACAGGACGCGAC 7178
Maa          CCTTGGCCTCGATGGCCTGGTGCATGCCCTCGTTGTAGCGGCGACCGTAGAGCACGCGAC 720
Mtb          CCTTGGCCTCGATGGCCTGGTGCATGCCCTCGTTGTAGCGGCGGCCGATCAGCACCCGGC 713
             ********************************************* ***   ** ** ** *

Map          CGGTGAACTCGTCGACGATCAGCACCTCGCCGTCGCGCACGATGTAGTCCTTGTCGCGGT 7238
Maa          CGGTGAACTCGTCGACGATGAGCACCTCGCCGTCGCGCACGATGTAGTCCTTGTCGCGGT 780
Mtb          CGGTGAACTCGTCGACGATGAGCACCTCACCATCGCGGACGATGTAGTCCTTGTCGCGGC 773
             ****************** ******** ** ***** ********************

Map          GGAACAGCTCCTTGGCCTTCAGCGCGTTGTTGAGGTAGCTGACCAGCGGCGAGTTGGCCG 7298
Maa          GGAACAGCTCCTTGGCCTTCAGCGCGTTGTTGAGGTAGCTGACCAGCGGCGAGTTGGCCG 840
Mtb          TGAACAGCTCTTTGGCCTTCAGAGCGTTGTTGAGATAGCTGACCAACGGCGAGTTGGCGG 833
              ********* ********** ********** ********** ************ *

Map          CCTCGTACAGGTTGTCGATGCCGAGCTGGTCCTCGACGAACTCCACCCCCAGCTCGTGCA 7358
Maa          CCTCGTACAGGTTGTCGATGCCGAGCTGGTCCTCGACGAACTCCACACCCAGCTCGTGCA 900
Mtb          CCTCGTACAGGTTGTCGATGCCGAGCTGGTCTTCGACGAATTCCACACCCTTCTCGTGCA 893
             ******************************** ******** ***** ***  ********

Map          CGCCGACGGTGCGCTTGCGCAGATCCACCTCGTAGTGGACGTCCTTTTCCATCAGCGGGG 7418
Maa          CGCCGACGGTGCGCTTGCGCAGATCCACCTCGTAGTGGACGTCCTTTTCCATCAGCGGGG 960
Mtb          CGCCGACGGTGCGTTTGCGTAGATCGACCTCGTAGTGGACGTCCTTTTCCATCAGCGGCG 953
             ************* ***** ***** ********************************* *

Map          CCAGCCGGGCGAATTCGAGGTACCAGTTGGAGGCGCCGTCGGCCGGCCCGGAGATGATCA 7478
Maa          CCAGCCGGGCGAACTCGAGGTACCAGTTGGAGGCGCCGTCGGCCGGCCCGGAGATGATCA 1020
Mtb          CCAACCGGGCGAACTCGGTGTACCAGTTGGAGGCGCCGTCGGCGGGACCGGAGATGATCA 1013
             *** ********* ***  ************************ ** **************

Map          GCGGGGTGCGGGCCTCGTCGATCAGGATCGAGTCGACCTCGTCGACGATCGCGAAGTTGT 7538
Maa          GCGGGGTGCGGGCCTCGTCGATCAGGATCGAGTCGACCTCGTCGACGATGGCGAAGTTGT 1080
Mtb          GCGGGGTGCGGGCCTCGTCGATCAGGATGGAATCGACCTCGTCGACAATGGCGTAATGGT 1073
             **************************** ** ************** ** *** * * **

Map          GCCCGCGCTGCACCAGGTCGTCGAGCGAGTGCGCCATGTTGTCGCGCAGGTAGTCGAAGC 7598
Maa          GCCCGCGCTGCACCAGGTCGTCGAGCGAGTGCGCCATGTTGTCGCGCAGGTAGTCGAAGC 1140
Mtb          GCCCGCGCTGCACCAGATCATCCAGTGAGTGCGCCATGTTGTCGCGCAGGTAGTCGAACC 1133
             ***************** ** ** ** ********************************* *

Map          CGAACTCGTTGTTGGTGCCGTAGGTGATGTCGGCGTTGTAGGCGACCCGGCGCTCGTCGG 7658
Maa          CGAACTCGTTGTTGGTGCCGTAGGTGATGTCGGCGTTGTAGGCGACCCGGCGCTCGTCGG 1200
Mtb          CAAACTCGTTATTGGTGCCGTAGGTGATGTCGGCGTTATAGGCCACCCGGCGTTCATCGG 1193
             * ******** ************************** ***** ******** ** ****
```

Figure 23: Alignment between corresponding sequences of Maa and Mtb

(In red: ptb 102 analogue in Map K10 genome. Similarity between the nucleotides is indicated with *)

6.5.3.1 Test 1

6.5.3.1.1 Primer design

Two primers were designed to amplify a part of the open reading frame containing the fragment. Ptb102S (5' CTT GGC TTC CTC GGT CTT GTA GA 3') and Ptb102R (5' GTG GCT GAC TAC GTC AAC ACC T 3') are complementary to nucleotide 6897 to 6919 and 8054 to 8074, respectively. The length of the amplicon is 1178 bp.

6.5.3.1.2 Materials and methods

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: ptb102S			
Primer: ptb102R			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	60	40
Annealing	54	90	
Extension	72	150	

No specific results were obtained and another Taq polymerase (Bioline Bio-X-act short DNA polymerase, Luckenwalde, Germany) was used in the PCR reaction. In a final volume of 25 µl, 9.25 µl MilliQ, 2.5 µl 10 x Optibuffer, 5 µl HiSpec additive, 0.5 µl of 100 mM dNTP's, 20 pM of each primer and 0.5 µl Bio-X-act short DNA polymerase (4U/µl) were used. All reagents except the primers were from the Bio-X-act kit and cycling conditions were according to the manufacturer's protocol: one cycle of denaturation at 94°C for 4 min followed by 1 cycle of annealing at 55°C for 60 s, 30 cycles of extension at 70°C for 60 s, denaturation at 94°C for 30 s and annealing at 55°C for 30 s and a final elongation step at 72°C for 10 min.

6.5.3.1.3 Results and conclusion

No PCR amplification was detected when using primers ptb102S and ptb102R. Adjustment of cycling conditions and PCR conditions yielded no improvement. New primers were designed to amplify a smaller part of the ptb102 fragment.

6.5.3.2 Test 2

6.5.3.2.1 Primer design

A new primer was developed to amplify a smaller fragment of 701 bp. Primer ptb102R2 (5' TTC GAC TAC CTG CGC GAC AAC AT 3') can anneal to nucleotide 7573 to

7595 of contig 179 and is also complementary with nucleotide 391 to 412 of the ptb102 fragment.

6.5.3.2.2 Materials and methods

PCR

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: ptb102S **Primer: ptb102R2**			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	54	60	
Extension	72	90	

SSCP

Eight µl of D2 buffer was mixed with 4 µl of PCR product. The mixture was boiled for 5 min at 95°C. The tubes were immediately cooled on ice for 10 min. Six µl of the mix was loaded on a precasted GMA® gel (Elchrom) and run for 16 h at 100 V on the SEA2000 electrophoresis apparatus. The 0.5 x TAE running buffer was cooled at 4°C. The gel was stained with Sybr Green II (Biowittakker, Vallensbaek, Denmark) for 40 min and destained for 40 minutes with MilliQ water.

6.5.3.2.3 Results and conclusion

Four Map isolates (including M02-0904) gave no amplification for ptb102 with the new primers, but amplification was positive for all other isolates. An SSCP was performed on these amplicons but no difference could be detected. Seven amplicons, randomly chosen, were sent for sequencing to the VIB genetic facility.

The sequence results from six amplicons (M02-0710, M02-0370, M02-0900, M02-1696, M02-0909, M02-0806) did not reveal major differences with the Map K10

genome. The sequence of M02-0369 showed only 89% similarity with the Map K 10 genome and the open reading frame and the conserved region for SecA protein were also conserved.

Further analysis of the ptb102 fragment revealed a GC content of 54.9%, whereas the GC content of Map contig 179 is 64.8%. The low GC content for ptb102 suggests that this fragment is not originating from Map (overall GC content of 69.3%) but from another organism. It can not be excluded that through the amplification step in AFLP, a DNA contamination could have become visible on the gels. Normally, MilliQ water, molecular biology grade reagents and sterile plastic ware are free of DNA, but a contamination can not be ruled out. This highlights the sensitive nature of an AFLP reaction and the special care the technique requires in avoiding contamination.

No difference between M02-0369 and the other isolates was detected on SSCP, although there was a sequence difference of 11%. It can be concluded that SSCP could not reveal differences in fragments of 700 bp. It is well known that the optimal length for SSCP is 200 bp (Strachan *et al.*, 1999). The ptb102 fragments of Map isolates from different geographical region were sequenced and compared with the K10 Map genome. The ptb102 fragments of these isolates revealed no major difference with the K10 Map genome.

The amplified fragment of M02-369 was found to be polymorphic. To find fragments similar to M02-369, a RFLP with *Alw*I was developed in order to avoid further sequencing. Only one fragment had the same RFLP pattern as M02-369 i.e. M02-371 (both Belgian bovine samples). Sequencing of all fragments would be the most appropriate method to analyse further the polymorphisms and will be done in the near future.

6.5.4 K13 fragment

The K13 fragment originated from an AFLP reaction with *EcoR*I restriction using selective nucleotide G on cervid isolate M02-1465. The sequence length of the fragment was 338 bp. The whole sequence showed 100% similarity with contig 161 of the Map genome and is situated in an open reading frame. Analysis of contig 161 showed a nucleotide T after the fragment where this should be a C for a restriction site

of *Eco*RI (Figure 24). The K13 fragment was obtained due to a restriction site that does not occur in the contig 161.

```
 501  CTGATTACCG CAGCGTCATC GCTGACAGCT TTGAGGTATT GGTGGACTCC
 551  AATCCGAAGC ATCCGGCTAA GCCGGTAATC GGACTCAACG TTGATCCGAT
 601  TCTCGGCTAT GGAGAATCCT TCATCATGCC GATTGAATTC GAGGCGGCAC
 651  GCGATCTCGC GATGTTGATG ATGAAGACAT TGATGGTTGA AGCACCCGAG
 701  CTATTCACCG GGAAGTTTTG ATGACCGACG TCGTAATCGT GTCGGACCCG
 751  CTAAGGGTTC CGATTACCGC CGAACGCCGT ACTGATGGCG GGGTGGCAGT
 801  GCGCGGACAT TTTCGCGGCG TCCTCGTACT CAGTGATACC GAGCTTGATC
 851  GCCTTATCGC CTTCGCCCGC GATGAACCGC CAAGGGCACG GATACAGCGT
 901  TTCGTGATGA CACCTGGGCA TGGAAGCACC GGATGCCTCG CTTAGACTAA
 951  CCGCGACTAC TTCTCACCGA ATTTCGAAAG AATATCCAGA GCCTTCGACA
1001  GCAGCAGCGC AACTTCTCCC GAACTAGGCT CAGACTCTGC CTTGGCGCCG
1051  GAATCAAGGT CAGGGGTTGA AAACGCTCGT TTGCCCAGAA TCACCTTGAG
1101  CTCATTTTTT GATTCGGCGG GTAGATCAGT TACGTAAGCA CTCACTGAGT
1151  CAATTTGTGT CACCAAGATG TTCATGTGCC GAGCCGTGTC TCGGTGCTGG
1201  GACGCAATTT TGCCACAGTA GGCAGCGAAT GCAAGTAGCG GAATCCCAAT
1251  GAATGCCTTA ATCAGCGTCG CGGACAAGCC GGTAACTGCG GGAAACTGTA
1301  CGCCCCGTGA AAACAGTACG ACGGGGATCG CGATAGCAGT GCATGTCAAC
1351  AAGAACGCTG TTACCGTCCA CCCTGCCGCC CACCAAGTCT CACGGTTGAC
```

Figure 24: The sequence of contig 161 in comparison with the K13 fragment
(in bold K13 and in red the changed restriction site, underlined: primer K13S, K13R)

6.5.4.1 Test 1

6.5.4.1.1 Primer design

Two primers were designed with Primer PREMIER. The sense primer (K13S 5' ACG CGA TCT CGC GAT GTT 3') was chosen in the beginning of the K13 fragment (from nucleotide 649-666 of contig161). The restriction site is situated at nucleotide 973 of the contig 161. The second primer (K13R: 5' TAC AGT TTC CCG CAG TTA CCG 3') was designed to be complementary with nucleotide 1280 to 1300 of contig 161. The two primers (K13S and K13R) amplified a fragment of 655 bp with in the middle the restriction site for *Eco*RI. This restriction site will be used after PCR reaction to differentiate between the amplicons with and without the restriction site.

6.5.4.1.2 Materials and methods

PCR

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: K13S			
Primer: K13R			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	58	60	
Extension	72	90	

Restriction

Four µl PCR product was mixed with 3 U *EcoR*I and the supplied buffer (MBI fermentas). The tubes were incubated overnight in a water bath at 37°C. Four µl of the mixture was loaded on a 10 % PAGE gel and run at 100 V for 2 h 40. A DNA ladder (GeneRuler™ 100 bp DNA Ladder) was included on every gel. Afterwards the PAGE gel was silver stained.

6.5.4.1.3 Results and conclusion

All Map isolates were amplified and the amplicons were of the expected size. All the amplicons were tested with the restriction enzyme *EcoR*I. None of the amplicons were restricted. Only one band of 650 bp could be detected on the PAGE gel. No restriction site could be found.

The fact that these fragments (with the ligated adaptor) were found in AFLP means that there should be a restriction site. Because of the amplification step in the AFLP, sequences present in small quantities could be detected (i.e. replication errors). Every 10^{10} nucleotide, a mistake is made in the incorporation of a nucleotide in the bacterial genome during replication (Snyder *et al.,* 2003). This means that 1 in every 2000 progeny bacteria will carry a mutation.

It could be that we picked up such a mutation in the AFLP reaction. Therefore, a new PCR assay was developed to detect isolates where the restriction site was present in a minor fraction in the genomic DNA.

6.5.4.2 Test 2

6.5.4.2.1 Primer design

New primers were developed annealing on the restriction site of the K13 fragment. K13R3 (5' TCT GGA TAT TCT TTC GAA T 3') in combination with K13S was expected to give an amplicon of 344 bp.

6.5.4.2.2 Materials and methods

A PCR was performed on all Map isolates in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: K13S			
Primer: K13R3			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	54	60	
Extension	72	90	

6.5.4.2.3 Results and conclusion

Seventeen (3 cervid and 14 bovine) isolates showed an amplification with the primers K13S and K13R3. The amplicons were of the expected size (344 bp). All other isolates gave no amplification. It was shown that the restriction site was present in the DNA of some extracts.

The fact that we found amplification for K13 in 17 isolates when using primers complementary to the restriction site, ruled out that the restriction site was a replication error.

Mixed infections or cultures are the most likely explanation for these observations. We could not detect an isolate showing a pure K13 restriction site. More isolates should be tested to find a "mono"-culture.

These results show the suitability of the used AFLP technique, revealing polymorphism in the mixed cultures.

6.5.5 K8 fragment

The K8 fragment originated from an AFLP with *EcoR*I restriction using selective nucleotide G on cervid isolates M02-1465. The length of the sequence was 301 bp. 297 bp were similar with contig 205 of the Map genome. At the 3' end of the fragment, no *EcoR*I restriction site is found in the contig (Figure 25). Therefore, K8 has been generated by a restriction site at the 3' end that does not occur in the contig 205.

```
2973  ATGGCTGGCA CCTTCATATC CATGCCCTCG TGTTCTCAGT GACCAGCTTG
3023  TCGAGCGGTC TGATTGAGGG CATTGAGCGG ACTCTGGGCC GCGGAGTTAA
3073  TCATGATTGG TTGGCGCGCA ACGTCTTTGC TGCTCGAATA CATCAGCGCT
3123  GGTCGCAGGG ATTGGCCAAG GCTGGCTGTC AGATGCCGGG GTCGGTCGCC
3173  GTTGATGTCC GAGAGATTGA CGACGAGGGC GCTGAATACG TTGGCCGATA
3223  CCTGTCCAAG GCTACATATG ATGTCGCGGC ACGCATAGGT CTTGAGGTGG
3273  GAGCTGGCGT TTCAACGAAA GACGCTCGGG CAGAACGTAA TCAGACTCCG
3323  TTCGAAGTGC TTGCGAATCT CGCTGAGTCG GTGGATGCTC GCGGGTTCGG
3373  AATTCGGACC CCGCGTCATT GGGCTGTTCT CCCTGCAGGA AACGGAGATT
3423  GGGCTGTAAT CGACAGTGAT ACAGGCGAAG TCGCGAGCAT CACGGCGCCT
3473  GGACAGTGGA AGGTATGGCA TGAGTGGGAG CAAGCGTCGT GTGGTCGTCG
3523  TCAGATTACT TGGTCTCGTC GACGGTCAAA CCCTGAATCG GGCCGCGAGA
3573  TGCTGTGGAA TGACTTGTTG GATAGTCGTG GACGGTCAGC AGAAGCATCG
3623  GACGAAGAAA TTGCGGTCGA TGAGGTCGAT GCCGAGTCGG TCGGCGTTAT
3673  TAGTCGACAG GTTTGGTACC AGGTGTTTGC CTGGCGTCCA GGGTTGATTG
3723  TGGATCTTCT GGAGGCGGCC GAGCGGTGTG GTGTGGCGGC TGTTGGTGTG
3773  TTATCGCAAT CTGCTGGTTG TGACGTCGCC GGGTGGCCAC CAGGCGGGTA
3823  GCACCAACAT TTACGCGGCA GAAATGTGTT CCAACCTTGA CCCCCTACGG
3873  ATTGTCGGTG GCTCGTGCGA GCCTATGTAT CGATAACTTT GAGGAGGATG
3923  GGATGTCGTA TGTTGTGCGC CGAATTACTC TGGACGAGTT ATCTGCTCCT
3973  CGCCCAACGA AGCGGATATC GGTTGGTTGG GCCGATGGGC TACTTAACGG
4023  CGGTATGCCC GCACGGAAGT TCGCGATGAT CTACGGACAA CCTGGTGTCG
```

Figure 25: The sequence of contig 205 in comparison with the K8 fragment
(in bold: K8 fragment and in red the changed restriction site, underlined primers: K8S, K8R)

6.5.5.1 Test 1

6.5.5.1.1 Primer design.

Two primers were designed to amplify a fragment of 368 bp. Primer K8S (5' GGG CTG TAA TCG ACA GTG ATA CAG G 3') is complementary to nucleotide 3423 to 3448 of contig 205. The putative restriction site of *EcoR*I is situated at nucleotide 3670. The reverse primer K8R (5' CAG CAG ATT GCG ATA ACA CAC C 3') is complementary with nucleotide 3767 to 3788 of contig 205. The PCR will produce an amplicon of 368 bp. The restriction site will be used after the PCR reaction to differentiate between the amplicons with and without the restriction site.

6.5.5.1.2 Materials and methods

PCR

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: K8S			
Primer: K8R			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	56	60	
Extension	72	90	

Restriction

Four µl PCR product was mixed with 6 U *EcoR*I and the supplied buffer (MBI fermentas). The tubes were incubated overnight in a water bath at 37°C. Four µl of the mixture was loaded on a 10% PAGE gel and run at 100 V for 2 h 40. A DNA ladder (GeneRuler™ 100 bp DNA Ladder) was included in every gel. Finally the PAGE gel was silver stained.

6.5.5.1.3 Results and conclusion

All Map isolates were amplified using the PCR assay for K8 fragment, with amplicons of the expected size (368 bp). All amplicons were evaluated with a restriction assay for *EcoR*I but none of the amplicons could be restricted. Only one band could be seen on the PAGE gel. The same approach as for the K13 locus was used to further investigate these results.

6.5.5.2 Test 2

6.5.5.2.1 Primer design

As for K13, a second primer is designed complementary to the restriction site of K8. With this primer, it should be possible to amplify those sequences which are present in a minority of the extract and contain the restriction site. K8R3 (5' CCA AAC CTG TCG ACG AAT TC 3') in combination with K8S should produce amplicons of 268 bp length.

6.5.5.2.2 Materials and methods

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: K8S			
Primer: K8R3			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	56	60	
Extension	72	90	

6.5.5.2.3 Results and conclusion

Only 15 (2 cervid and 13 bovine isolates) Map isolates showed amplification with the primers K8S and K8R3.

The same conclusions as for K13 could be made. More cultures should be tested to find a pure culture and to evaluate this polymorphism for its suitability for typing Map strains using a PCR assay.

6.5.6 B116 fragment

The B116 fragment originated from an AFLP reaction with *Apa*I restriction using a selective nucleotide C on cervid isolate M02-1441. The fragment is 465 bp long. The result of a BLAST search against the Map K 10 genome is seen in Figure 26. Only nucleotides 193-419 gave a hit with nucleotides 1-229 of contig 152 of the Map genome, but contig 152 is a short contig of only 230 nucleotides. A standard BLAST resulted in a hit of nucleotides 8-188 of the B116 fragment with nucleotide 1509 to 1689 of a Map sequence, ISMav2, coding for a putative transposase gene (accession number AF286339) (Figure 27). A BLAST search against the Map genome with the total ISMav2 sequence did not reveal any similarity. This is probably due to gaps in the incomplete genome sequence of the Map K10 strain. Alignment of B116 and IsMav2 is shown is Figure 28.

An analogue of ISMav2 could be found in Maa. Figure 29 shows an alignment between ISMav2 and the Maa analogue. The first 799 nucleotides are very similar. From nucleotide 800, the two sequences are completely different. The open reading frame of ISMav2 is situated from nucleotide 911 to 2098. ISMav2 has a low similarity with its Maa analogue. The B116 fragment is situated in the open reading frame of ISMav2.

```
Query: 193 aacacaagggctttctcaaatctgaagacggctcggcaagccctcg-acgaakgggtgca 251
           |||||||||||||||||||||||||||||||||||| |||||||||| |||||| |||||||
Sbjct: 1   aacacaagggctttctcaaatctgaagacggctcagcaagccctcgcacgaatgggtgca 60


Query: 252 ctactacaacaccgcccgtccgcatcaatcgctgaacatgacc-accccagccgaacgg- 309
           |||||||||||||||||| |||||||||||||||||||||| |||||   |||||||
Sbjct: 61  ctactacaacaccgcccgtc-gcatcaatcgctgaacatgacccacccccagcgaacggg 119


Query: 310 ttcaccgccaccgcctcgccggtgagccctggcgacgacgtacccgccagcatcgaccga 369
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 120 ttcaccgccaccgcctcgccggtgagccctggcgacgacgtacccgccagcatcgaccga 179


Query: 370 gacagccaggackgggtcagccgccgggtaacgaccaacggggtggtcag 419
           ||| |||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 180 gacggccaggactgggtcagccgccgggtaacgaccaacggggtggtcag 229
```

Figure 26: BLAST results for the B116 fragment against Map genome

(Query B116, Subject: contig 152 of Map genome. Homology with the 5' region of B116)

```
Query: 8    agcagattttgactgataacggcaaggtgttcaccggccggtttaaccatcccccggkgg 67
           ||||||| ||||| ||||||||||||||||||||||  ||||||   ||||| ||||  |
Sbjct: 1509 agcagatcttgaccgataacggcaaggtgttcaccgagcggttttgtcatccaccggtcg
1568


Query: 68   aggtgctcttcgacgcgatctgccggcaaaacggcatcgaccacctgctcacccagccgc 127
           |||||||||| || |||||||||||  |  |||||||||| || ||| | ||||||||||
Sbjct: 1569 aggtgctctttgatgcgatctgccgcgagcacggcatcgaacatctgttgacccagccgc
1628


Query: 128  gtagcccgacgacgacaggcaagattgagcggttccaccgcagtctgcgcgccgaattcc 187
           | |||||||| ||||| ||||| || |||| ||| ||||||||||||||||| || ||||
Sbjct: 1629 gcagcccgaccacgaccggcaaaatcgagcagtttcaccgcagtctgcgcgctgagttcc
1688


Query: 188  t 188
           |
Sbjct: 1689 t 1689
```

Figure 27: Results of standard BLAST for B116

(Query: B116, Sbjct ISMav2. Homology with the 3' region of B116)

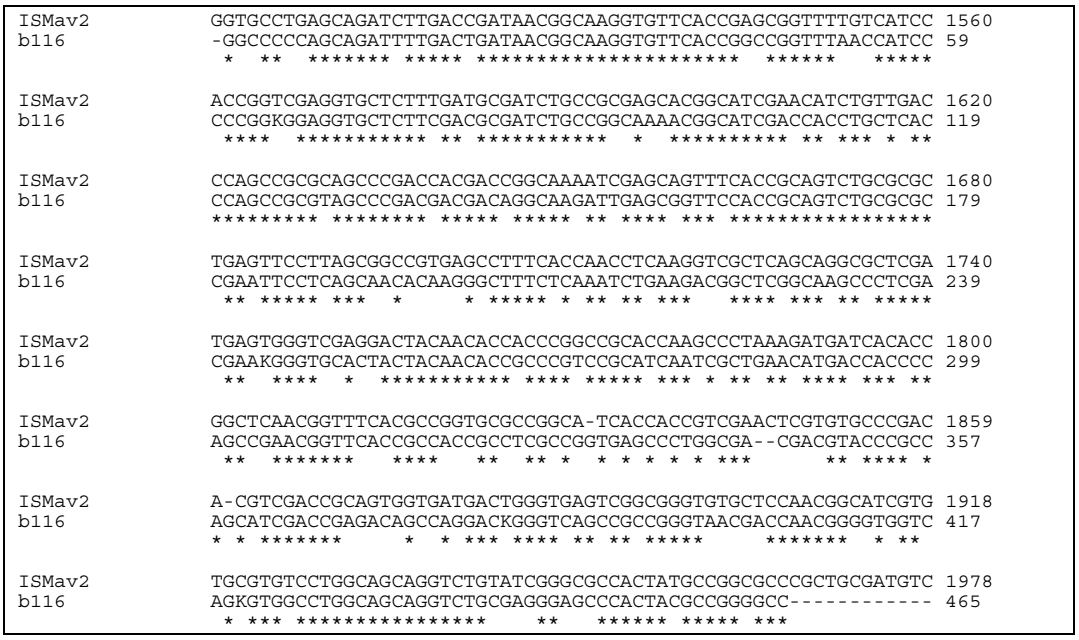
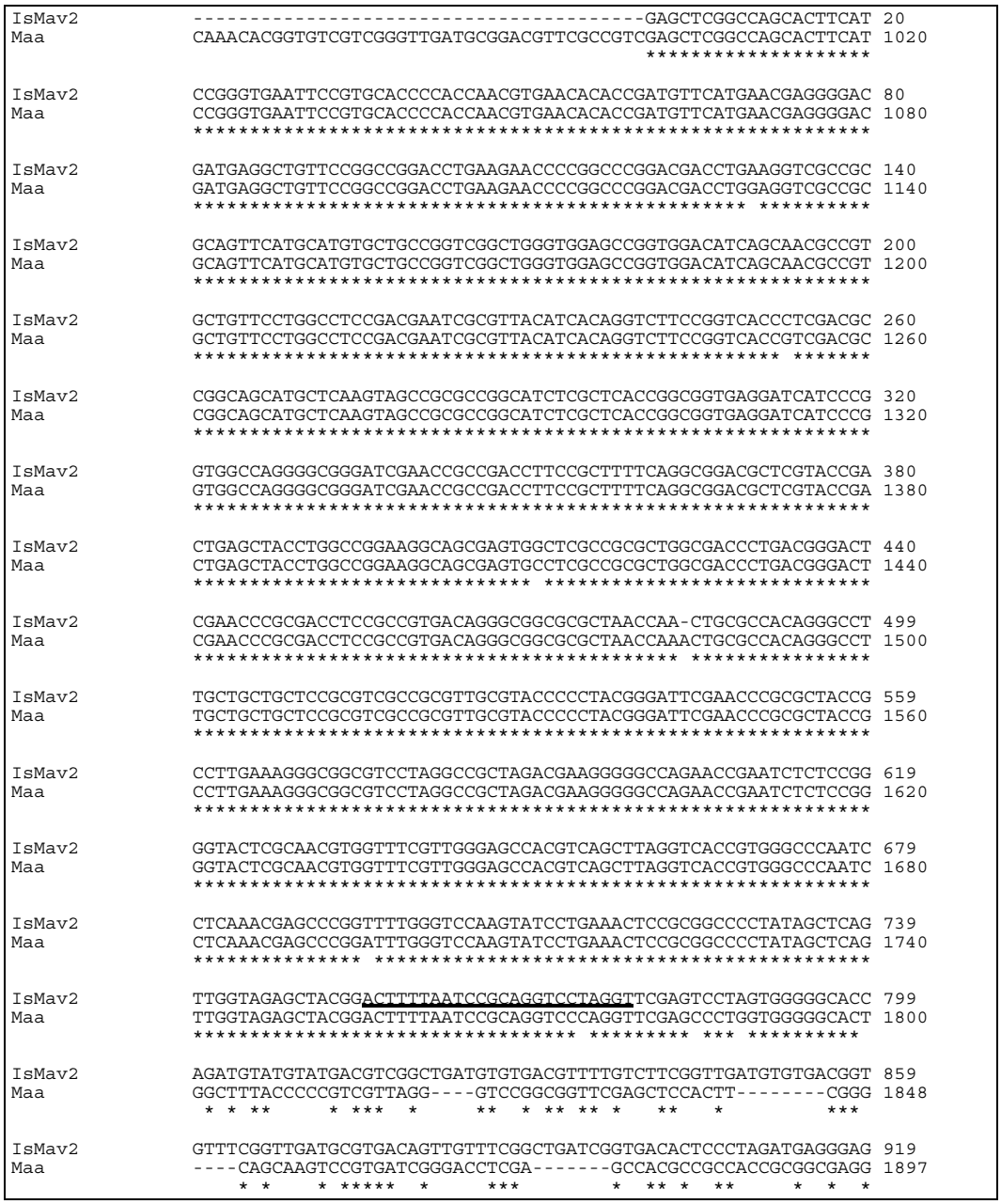
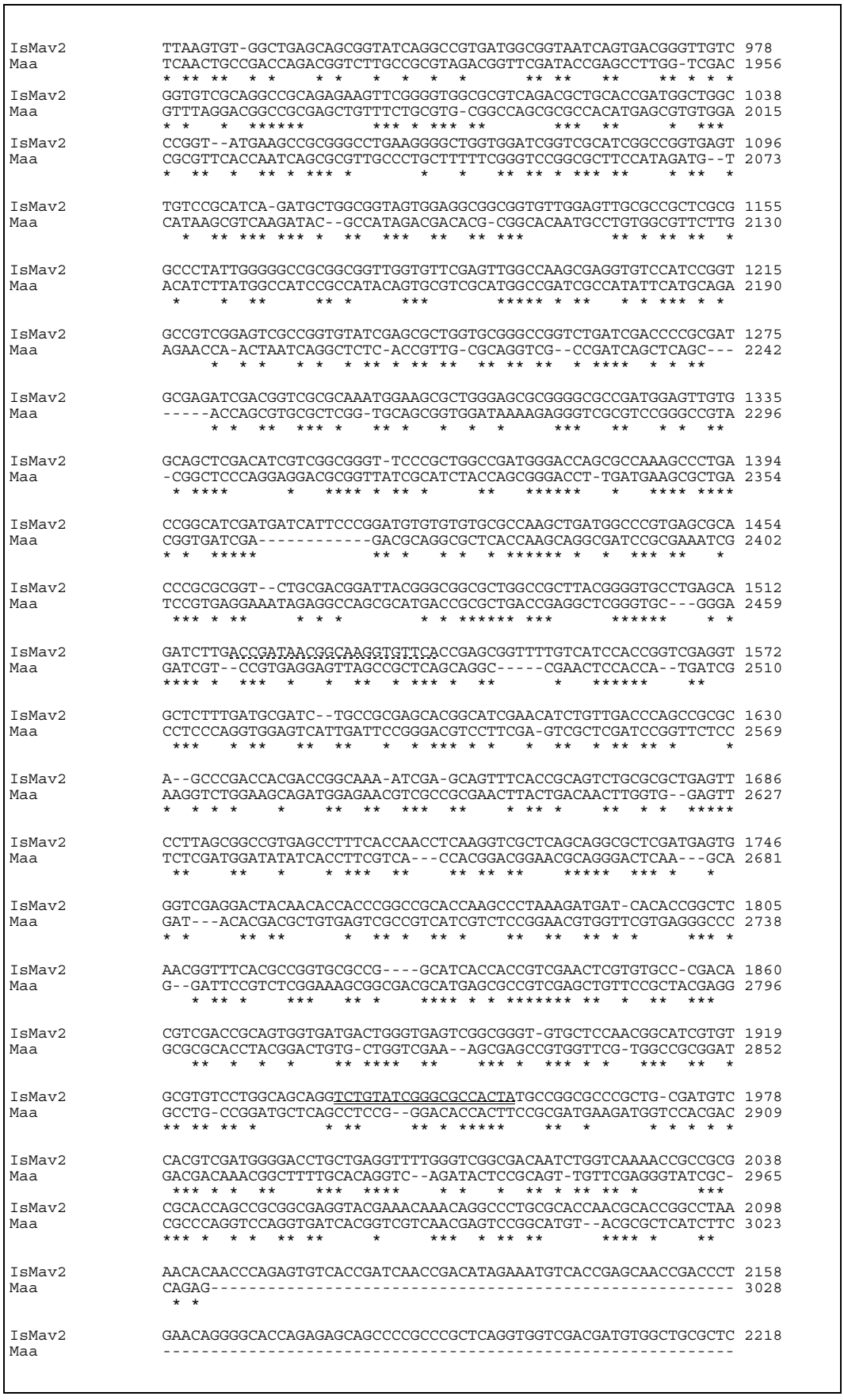


Figure 28: Alignment between the sequences of ISMav 2 and B116


IsMav2 ----------GAGCTCGGCCAGCACTTCAT 20
Maa CAAACACGGTGTCGTCGGGTTGATGCGGACGTTCGCCGTCGAGCTCGGCCAGCACTTCAT 1020
IsMav2 CCGGGTGAATTCCGTGCACCCCACCAACGTGAACACACCGATGTTCATGAACGAGGGGAC 80
Maa CCGGGTGAATTCCGTGCACCCCACCAACGTGAACACACCGATGTTCATGAACGAGGGGAC 1080
IsMav2 GATGAGGCTGTTCCGGCCGGACCTGAAGAACCCCGGCCCGGACGACCTGAAGGTCGCCGC 140
Maa GATGAGGCTGTTCCGGCCGGACCTGAAGAACCCCGGCCCGGACGACCTGGAGGTCGCCGC 1140
IsMav2 GCAGTTCATGCATGTGCTGCCGGTCGGCTGGGTGGAGCCGGTGGACATCAGCAACGCCGT 200
Maa GCAGTTCATGCATGTGCTGCCGGTCGGCTGGGTGGAGCCGGTGGACATCAGCAACGCCGT 1200
IsMav2 GCTGTTCCTGGCCTCCGACGAATCGCGTTACATCACAGGTCTTCCGGTCACCCTCGACGC 260
Maa GCTGTTCCTGGCCTCCGACGAATCGCGTTACATCACAGGTCTTCCGGTCACCGTCGACGC 1260
IsMav2 CGGCAGCATGCTCAAGTAGCCGCGCCGGCATCTCGCTCACCGGCGGTGAGGATCATCCCG 320
Maa CGGCAGCATGCTCAAGTAGCCGCGCCGGCATCTCGCTCACCGGCGGTGAGGATCATCCCG 1320
IsMav2 GTGGCCAGGGGCGGGATCGAACCGCCGACCTTCCGCTTTTCAGGCGGACGCTCGTACCGA 380
Maa GTGGCCAGGGGCGGGATCGAACCGCCGACCTTCCGCTTTTCAGGCGGACGCTCGTACCGA 1380
IsMav2 CTGAGCTACCTGGCCGGAAGGCAGCGAGTGGCTCGCCGCGCTGGCGACCCTGACGGGACT 440
Maa CTGAGCTACCTGGCCGGAAGGCAGCGAGTGCCTCGCCGCGCTGGCGACCCTGACGGGACT 1440
IsMav2 CGAACCCGCGACCTCCGCCGTGACAGGGCGGCGCGCTAACCAA-CTGCGCCACAGGGCCT 499
Maa CGAACCCGCGACCTCCGCCGTGACAGGGCGGCGCGCTAACCAAACTGCGCCACAGGGCCT 1500
IsMav2 TGCTGCTGCTCCGCGTCGCCGCGTTGCGTACCCCCTACGGGATTCGAACCCGCGCTACCG 559
Maa TGCTGCTGCTCCGCGTCGCCGCGTTGCGTACCCCCTACGGGATTCGAACCCGCGCTACCG 1560
IsMav2 CCTTGAAAGGGCGGCGTCCTAGGCCGCTAGACGAAGGGGGCCAGAACCGAATCTCTCCGG 619
Maa CCTTGAAAGGGCGGCGTCCTAGGCCGCTAGACGAAGGGGGCCAGAACCGAATCTCTCCGG 1620
IsMav2 GGTACTCGCAACGTGGTTTCGTTGGGAGCCACGTCAGCTTAGGTCACCGTGGGCCCAATC 679
Maa GGTACTCGCAACGTGGTTTCGTTGGGAGCCACGTCAGCTTAGGTCACCGTGGGCCCAATC 1680
IsMav2 CTCAAACGAGCCCGGTTTTGGGTCCAAGTATCCTGAAACTCCGCGGCCCCTATAGCTCAG 739
Maa CTCAAACGAGCCCGGATTTGGGTCCAAGTATCCTGAAACTCCGCGGCCCCTATAGCTCAG 1740
IsMav2 TTGGTAGAGCTACGGACTTTTAATCCGCAGGTCCTAGGTTCGAGTCCTAGTGGGGGCACC 799
Maa TTGGTAGAGCTACGGACTTTTAATCCGCAGGTCCCAGGTTCGAGCCCTGGTGGGGGCACT 1800
IsMav2 AGATGTATGTATGACGTCGGCTGATGTGTGACGTTTTGTCTTCGGTTGATGTGTGACGGT 859
Maa GGCTTTACCCCCGTCGTTAGG----GTCCGGCGGTTCGAGCTCCACTT--------CGGG 1848
IsMav2 GTTTCGGTTGATGCGTGACAGTTGTTTCGGCTGATCGGTGACACTCCCTAGATGAGGGAG 919
Maa ----CAGCAAGTCCGTGATCGGGACCTCGA-------GCCACGCCGCCACCGCGGCGAGG 1897


IsMav2 TTAAGTGT-GGCTGAGCAGCGGTATCAGGCCGTGATGGCGGTAATCAGTGACGGGTTGTC 978
Maa TCAACTGCCGACCAGACGGTCTTGCCGCGTAGACGGTTCGATACCGAGCCTTGG-TCGAC 1956
IsMav2 GGTGTCGCAGGCCGCAGAGAAGTTCGGGGTGGCGCGTCAGACGCTGCACCGATGGCTGGC 1038
Maa GTTTAGGACGGCCGCGAGCTGTTTCTGCGTG-CGGCCAGCGCGCCACATGAGCGTGTGGA 2015
IsMav2 CCGGT--ATGAAGCCGCGGGCCTGAAGGGGCTGGTGGATCGGTCGCATCGGCCGGTGAGT 1096
Maa CGCGTTCACCAATCAGCGCGTTGCCCTGCTTTTTCGGGTCCGGCGCTTCCATAGATG--T 2073
IsMav2 TGTCCGCATCA-GATGCTGGCGGTAGTGGAGGCGGCGGTGTTGGAGTTGCGCCGCTCGCG 1155
Maa CATAAGCGTCAAGATAC--GCCATAGACGACACG-CGGCACAATGCCTGTGGCGTTCTTG 2130
IsMav2 GCCCTATTGGGGGCCGCGGCGGTTGGTGTTCGAGTTGGCCAAGCGAGGTGTCCATCCGGT 1215
Maa ACATCTTATGGCCATCCGCCATACAGTGCGTCGCATGGCCGATCGCCATATTCATGCAGA 2190
IsMav2 GCCGTCGGAGTCGCCGGTGTATCGAGCGCTGGTGCGGGCCGGTCTGATCGACCCCGCGAT 1275
Maa AGAACCA-ACTAATCAGGCTCTC-ACCGTTG-CGCAGGTCG--CCGATCAGCTCAGC--- 2242
IsMav2 GCGAGATCGACGGTCGCGCAAATGGAAGCGCTGGGAGCGCGGGGCGCCGATGGAGTTGTG 1335
Maa -----ACCAGCGTGCGCTCGG-TGCAGCGGTGGATAAAAGAGGGTCGCGTCCGGGCCGTA 2296
IsMav2 GCAGCTCGACATCGTCGGCGGGT-TCCCGCTGGCCGATGGGACCAGCGCCAAAGCCCTGA 1394
Maa -CGGCTCCCAGGAGGACGCGGTTATCGCATCTACCAGCGGGACCT-TGATGAAGCGCTGA 2354
IsMav2 CCGGCATCGATGATCATTCCCGGATGTGTGTGTGCGCCAAGCTGATGGCCCGTGAGCGCA 1454
Maa CGGTGATCGA------------GACGCAGGCGCTCACCAAGCAGGCGATCCGCGAAATCG 2402
IsMav2 CCCGCGCGGT--CTGCGACGGATTACGGGCGGCGCTGGCCGCTTACGGGGTGCCTGAGCA 1512
Maa TCCGTGAGGAAATAGAGGCCAGCGCATGACCGCGCTGACCGAGGCTCGGGTGC---GGGA 2459
IsMav2 GATCTTGACCGATAACGGCAAGGTGTTCACCGAGCGGTTTTGTCATCCACCGGTCGAGGT 1572
Maa GATCGT--CCGTGAGGAGTTAGCCGCTCAGCAGGC-----CGAACTCCACCA--TGATCG 2510
IsMav2 GCTCTTTGATGCGATC--TGCCGCGAGCACGGCATCGAACATCTGTTGACCCAGCCGCGC 1630
Maa CCTCCCAGGTGGAGTCATTGATTCCGGGACGTCCTTCGA-GTCGCTCGATCCGGTTCTCC 2569
IsMav2 A--GCCCGACCACGACCGGCAAA-ATCGA-GCAGTTTCACCGCAGTCTGCGCGCTGAGTT 1686
Maa AAGGTCTGGAAGCAGATGGAGAACGTCGCCGCGAACTTACTGACAACTTGGTG--GAGTT 2627
IsMav2 CCTTAGCGGCCGTGAGCCTTTCACCAACCTCAAGGTCGCTCAGCAGGCGCTCGATGAGTG 1746
Maa TCTCGATGGATATATCACCTTCGTCA---CCACGGACGGAACGCAGGGACTCAA---GCA 2681
IsMav2 GGTCGAGGACTACAACACCACCCGGCCGCACCAAGCCCTAAAGATGAT-CACACCGGCTC 1805
Maa GAT---ACACGACGCTGTGAGTCGCCGTCATCGTCTCCGGAACGTGGTTCGTGAGGGCCC 2738
IsMav2 AACGGTTTCACGCCGGTGCGCCG----GCATCACCACCGTCGAACTCGTGTGCC-CGACA 1860
Maa G--GATTCCGTCTCGGAAAGCGGCGACGCATGAGCGCCGTCGAGCTGTTCCGCTACGAGG 2796
IsMav2 CGTCGACCGCAGTGGTGATGACTGGGTGAGTCGGCGGGT-GTGCTCCAACGGCATCGTGT 1919
Maa GCGCGCACCTACGGACTGTG-CTGGTCGAA--AGCGAGCCGTGGTTCG-TGGCCGCGGAT 2852
IsMav2 GCGTGTCCTGGCAGCAGGTCTGTATCGGGCGCCACTATGCCGGCGCCCGCTG-CGATGTC 1978
Maa GCCTG-CCGGATGCTCAGCCTCCG--GGACACCACTTCCGCGATGAAGATGGTCCACGAC 2909
IsMav2 CACGTCGATGGGGACCTGCTGAGGTTTTGGGTCGGCGACAATCTGGTCAAAACCGCCGCG 2038
Maa GACGACAAACGGCTTTTGCACAGGTC--AGATACTCCGCAGT-TGTTCGAGGGTATCGC- 2965
IsMav2 CGCACCAGCCGCGGCGAGGTACGAAACAAACAGGCCCTGCGCACCAACGCACCGGCCTAA 2098
Maa CGCCCAGGTCCAGGTGATCACGGTCGTCAACGAGTCCGGCATGT--ACGCGCTCATCTTC 3023
IsMav2 AACACAACCCAGAGTGTCACCGATCAACCGACATAGAAATGTCACCGAGCAACCGACCCT 2158
Maa CAGAG---------- 3028
IsMav2 GAACAGGGGCACCAGAGAGCAGCCCCGCCCGCTCAGGTGGTCGACGATGTGGCTGCGCTC 2218
Maa ----------

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IsMav2          GCCGTGGTAGACCAGGTCATTGAAATCGACCTCGAACCCGAGCTC 2263
Maa             ---------------------------------------------
```

Figure 29: Alignment between the sequences of ISMav 2 and the Maa analogue (underlined primers: B116S, B116R4, B116S3)

6.5.6.1 Test 1

6.5.6.1.1 Primer design

First, a primer pair was designed to amplify a fragment of 1201 bp. The sense primer B116S (3' ACT TTT AAT CCG CAG GTC CTA GGT 5') is situated in the sequences where there is similarity between Maa and ISMav2 (nucleotides 755-777 of ISMav2). The reverse primer B116R4 (5' TAG TGG CGC CCG ATA CAG A 3') was complementary with nucleotide 1937 to 1956 of ISMav2. Because of no PCR amplification, a new sense primer B116S3 (5' ACC GAT AAC GGC AAG GTG TTC A 3') was developed. The amplified fragment is 437 bp. The new primer is situated at nucleotides 1520-1541 of ISMav2.

6.5.6.1.2 Materials and methods

PCR

A PCR was performed on all Map isolates (Table 15) in a final volume of 25 µl with 1 µl DNA extract. PCR conditions were as described in 3.2.1.4 with following variables:

PCR			
Primer: B116S3			
Primer: B116R4			
	T (°C)	**Time (s)**	**Cycles**
Denaturation	94	45	40
Annealing	55	60	
Extension	72	90	

SSCP

Eight µl of D2 buffer was mixed with 4 µl of PCR product. The mixture was boiled for 5 min at 95°C. The tubes were immediately cooled on ice for 10 min. Six µl of the mix was loaded on a precasted GMA® gel (Elchrom) and run for 16 h at 100 V. The 0.5 x TAE running buffer was cooled at 4°C. The gel was stained with Sybr Green II for 40 min and destained for 40 min with MilliQ water.

6.5.6.1.3 Results and conclusion

Eleven isolates could not be amplified (M99-2678, M02-466, M02-0909, M02-1463, M02-0700, M02-1679, M02-1673, M02-0900, M02-0701, M02-0369, M02-0371). All other isolates (including M02-1441) gave amplicons of the expected size (437 bp). These amplicons were tested in an SSCP assay. All amplicons had the same SSCP profile. The sequence of three amplicons was determined in the VIB genetic facility. All amplicons showed a sequence identical to ISMav2.

The reverse primer is situated in a sequence that shows little similarity with the corresponding sequence of Maa. Normally, the Maa and Map genomes show more than 98 % similarity. The lack of good homology between the ISMav2 sequences of Maa and Map was further investigated by dividing the ISMav2 sequence in two parts: nucleotide 1 to 800 (high homology with Maa) and nucleotide 801 to 2263 (low homology).

A standard BLASTN of part one indicated a homology with tRNA genes of Mtb, *Mycobacterium bovis* and *Mycobacterium leprae.* A BLAST against the Map K10 genome showed a 99% similarity with contig 250. Contig 250 stops just before the open reading frame of ISMav. The standard BLASTN of part two revealed 83% homology with a putative tranposase in Mtb and *Mycobacterium bovis.* The putative

transposase gene is normally located 70,000 nucleotides further in the genome of Mtb and *Mycobacterium bovis* than the tRNA genes. The fact that ISMav2 is a putative transposon and can integrate in the genome on different places could explain its different location in Map. Our PCR assay with the sense primer (B116S) in part one gave no amplification. The reverse primer was situated in part two and gave amplification when used with another sense primer (B116S3). The fact that no amplification could be obtained with the B116S primer suggests that the ISMav2 open reading frame in some of our isolates is integrated at another place in the genome than with the Map isolate of accession number AF286339. This would also explain why part two of ISMav2 shows no homology with the second part of the Maa analogue.

A new reverse primer was developed based on the B116 sequences to try to amplify the 11 isolates that could not be amplified with the previous PCR. The primer B116R5 (5' TAG TGG GCT CCC TCG CAG A 3') is complementary with nucleotides 437-455 of the B116 fragment and is not complementary to ISMav2. With the B116R5 primer, 4 more isolates could be amplified.

This shows that the ISMav2 region is aparrently a problem region. The Map genome project has only a contig of 230 nucleotides. No flanking sequences could be found in the K10 Map genome. The whole genome is not yet fully available and it looks as if the Map sequence project had sequence problems in the ISMav2 region.

If the Map K10 genome is finalised, more information will become available to solve the place of ISMav2 insertion in the Map K10 strain. Our results indicate that ISMav2 is inserted at a different place in the genome, meaning that the place of insertion could be a useful locus in the Map characterisation.

6.5.7 General discussion

We selected 5 of the 47 sequences derived from 30 informative bands for further analysis and development towards a PCR based characterisation method. These 5 sequences showed clear polymorphisms when compared with sequences of the K10 strain, the reference strain used in the Map genome sequencing project. The other sequences showed less obvious differences.

It was found difficult to develop PCR assays based on polymorphic fragments generated by AFLP techniques. Mixed infections and DNA contaminations can make interpretation difficult.

Many more sequences need to be analysed to guarantee the selection of informative target sequences for characterisation of Map isolates. A totally different approach will be necessary. First, the polymorphism needs to be determined. To this end, PCR assays need to be developed to amplify approximately 1,000 bp of the flanking sequences of the fragments. The obtained amplicons of all isolates have to be sequenced because SSCP was shown not to be sensitive for large amplicons. This approach would imply a lot of expensive and time-consuming sequence work. We suggest to use a panel of all non-Belgian Map isolates in combination with two Belgian bovine and 2 Belgian cervid Map isolates to keep down the cost. All obtained sequences should be compared and the polymorphism should be determined. With this information new PCRs can be developed and used on all isolates.

We still believe it will be beneficial to have a PCR based assay for the typing of Map and we will use the new approach for detecting polymorphism.

Although we could not develop PCR assay for Map typing, the described AFLP study revealed a lot of useful information and made it possible to adjust protocols and techniques for further use in the lab. Our SE-AFLP technique as developed during the study is now used with great success to study genetic variations in sympatric and allopatric trypanosome isolates at the veterinary department of the ITM.

7 General conclusions

The overall objective of this research work was to conduct a survey for the occurrence of Map in the animal collection in the RZSA. In the past, the RZSA had occasionally some problems with Map in their animals. The existing diagnostic and typing methods were not suitable for a large-scale survey and new more specific, sensitive and faster methods had to be developed. To avoid the long incubation for the culture of Map, a molecular based test was chosen as diagnostic method. Faeces were chosen as a matrix because faeces are much easier to sample in a zoo environment than blood or biopsies.

The following conclusions were drawn from our study:

1) New specific primers targeting IS*900* were designed and evaluated with a large panel of Map and non-Map isolates. The primers gave a good detection limit (1 CFU per PCR volume) and were specific. Computer simulations were performed to evaluate the specificity of the new IS*900* primers and showed a higher specificity for the newly developed primers.

2) A second nested PCR assay targeting the f57 sequence was developed for the confirmation of IS*900* positive results. The f57 assay gave also a detection limit of 1 CFU per PCR and was highly specific. The f57 was a good alternative for IS*900* and could replace the IS*900* assay.

3) In a third part, the possibility of a multiplex PCR targeting two Maps specific sequences (IS*900* and f57) was tested. Amplification of the two targets was shown for all Map isolates. There was no amplification for the non-Map isolates. The multiplex PCR is very promising and should be evaluated on clinical samples.

4) The development of a specific PCR assay was followed by the exploration of a new faecal DNA extraction method. Of several methods that were tested, an approach including in a homogenisation with Tween-20 in combination with a

sequence-capture procedure for DNA isolation (reported by Mangiapan *et al*., 1996) was chosen. This faecal extraction method had a detection limit of 100 Map per g faeces, which is comparable to the detection limit described for faecal culture. The method was evaluated and compared with culture on clinical faecal samples. The benefit of the PCR assay was demonstrated in that more positive samples were detected with PCR (33.0%) than with culture (17.1%).

5) Owing to the good performance of the sequence-capture procedure for DNA isolation, the technique could be adapted to tissue samples. Also here more positive samples were found by PCR (42.2%) than with culture (31.8%).

6) Using the newly developed primers and the DNA faecal extraction method as well as culture and an ELISA method for detection of anti-Map antibodies, the occurrence of Map in the zoos of the RZSA was studied. With culture (conventional and radiometric culture) no Map cells were isolated from animals of the RZSA. However, three tissue samples and one faecal sample were positive on PCR for IS*900*. To have an overall view on the occurence of Map in the RZSA, a commercially available ELISA test was evaluated. This non-species dependent ELISA was for the first time tested in zoo animals. The ELISA proved to be useful to obtain an idea of the herds infected with Map. The only problem was the determination of cut-off values for the different animals species.

7) A modified single-enzyme AFLP was applied to Map isolates and was shown to be suitable to detect polymorphism among the Map isolates. Several visualisation techniques for the AFLP were evaluated. The SEA 2000® Electrophoresis Apparatus (Elchrom) was shown to be the easiest and fastest method. Moreover, the elution of specific DNA bands from the Elchrom gels was easy to perform.

8) Forty-seven clones were sequenced and further analysed. Five polymorfic sequences were selected for the development of PCR assays. No ready-to use PCR assay emerged. Additional research is necessary to confirm potential

loci. All other cloned sequences should be analysed before some polymorphic sequences can be selected for use in a PCR based typing method.

In general, we have not detected an extensive Map problem in the RZSA. However, we found Map in the animal collection and this finding warrants further investigation. The new specific PCR assay in combination with the newly developed faecal DNA extraction procedure was shown to be useful in the zoo environment. Our method can be further used in prevalence studies in the RZSA and in other zoos and animal parks. In combination with the prevalence study, the spread of the Map strains can be further investigated. However, the development of a PCR based typing method is still in progress.

8 Summary

Johne's disease or paratuberculosis is an incurable chronic enteritis mainly affecting ruminants. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map), an organism of the same genus as the bacteria causing tuberculosis. Paratuberculosis occurs throughout the world and is responsible for enormous financial losses. Map has been associated with Crohn's disease in human but this link has not been proven yet.

Paratuberculosis is also a problem in zoo animals. Up to one third of zoos accredited by the American Zoo and Aquarium Association have reported a minimum of one infected animal since 1995. After the positive faecal culture from an okapi in 1999, the Antwerp zoo had some serious concerns about the prevalence of paratuberculosis in their animal collection. The existing diagnostic methods were not sufficiently specific, sensitive and rapid to be used in a survey of the occurrence of Map.

In *Chapter 1*, the biology of Map is reviewed and a description of the different diagnostic methods is given. The existing molecular typing methods are outlined.

The objectives of the study are formulated in *Chapter 2*. The overall objective was the study of the occurrence of Map in zoo animals. To this end new specific and rapid tests had to be developed. Specific objectives included the design of a more specific PCR assay, the development of a faecal DNA extraction method and the development of a PCR based typing method.

In *Chapter 3* the problem of non-specific IS*900* primers is discussed and solutions are offered. New specific primers for IS*900* were designed and evaluated with a panel of mycobacterial and non-mycobacterial isolates, resulting in a detection limit of 1 CFU per PCR. The higher specificity of the new primers was confirmed with computer simulation.

Additional to the new IS*900* primers, new primers targeting another Map specific fragment i.e. f57 were designed. The detection limit of this new PCR assay was 1 CFU per PCR and the primers showed good specificity. A multiplex PCR combining the two Map specific sequences, i.e. IS*900* and f57 was developed and evaluated. The

two fragments were amplified in one PCR reaction and the multiplex approach was found to be promising.

In *Chapter 4*, the development of a new faecal DNA extraction method was described. To this end, several homogenisation and DNA isolation methods were compared using spiked faecal samples. The best method was a combination of homogenisation with Tween-20 and a DNA isolation based on a sequence-capture technique according to Mangiapan *et al*. (1996). A detection limit of 100 Map cells per g faeces was obtained.
The new faecal extraction method was evaluated on clinical faecal samples and was found to be a good alternative for the time-consuming culture techniques. More positives were found with PCR than with culture. The sequence-capture DNA isolation was also evaluated on tissue samples and performed better than the culture method.

The newly designed IS*900* primers in combination with the faecal DNA extraction were used in a survey for the occurrence of Map in the RZSA (*Chapter 5*). Faecal and tissue samples of zoo ruminants were used in a comparitive study between PCR detection and conventional and radiometric culture. For the first time, a commercial non-species dependent ELISA test for the detection of anti-Map antibodies was also used in zoo animals. No Map cells were isolated using culture. However, three tissue samples and one faecal sample were PCR positive. The ELISA results indicated the occurrence of Map in several zoo species. In combination with the history of Map in the RSZA, we conclude that there is evidence for the presence of Map in the animal collection, but no high faecal shedders could be identified.

In *Chapter 6*, the attempts to develop a PCR based typing method directly applicable to clinical samples were described. As the conventional fingerprint method, i.e. RFLP is time-consuming and is culture dependent, another approach was desirable.
A modified AFLP was used to determine polymorphisms between 74 isolates. The observed polymorphic bands were cloned and sequenced. Five polymorphic sequences were further analysed and PCRs were designed for the amplification of these fragments. It proved difficult to convert the AFLP polymorphism in PCR

detectable fragments. Many more sequences need to be analysed to allow the selection of some valuable target sequences for typing of Map isolates.

Chapter 7 summarises the conclusions of the study.

9 Samenvatting

De ziekte van Johne of paratuberculose is een ongeneeslijke chronische darmontsteking die vooral voorkomt bij herkauwers. Deze ziekte wordt veroorzaakt door *Mycobacterium avium* subspecies *paratuberculosis* (Map), een organisme van hetzelfde genus als de veroorzaker van tuberculosis.
Paratuberculose komt wereldwijd voor en is verantwoordelijk voor enorme financiële verliezen. Map wordt ook geassocieerd met de ziekte van Crohn bij de mens maar deze link is nog niet bewezen. Paratuberculose is ook een probleem in zoo-dieren. Eén derde van de zoo's aangesloten bij de American Zoo and Aquarium Association meldt minstens 1 met Map geïnfecteerd dier sinds 1995. Na een positieve fecescultuur voor een okapi in 1999 had de Antwerpse zoo serieuze vragen over de prevalentie van Map in hun dierencollectie. De huidige diagnostische methoden waren niet specifiek, gevoelig en vlug genoeg om gebruikt te worden in een onderzoek naar Map.

In Hoofdstuk 1 wordt de biologie van Map besproken en wordt een opsomming van de huidige diagnostica gegeven. De bestaande moleculaire karakterisatietechnieken worden beschreven.

De objectieven van dit doctoraraatsonderzoek worden beschreven in Hoofdstuk 2. Het algemeen objectief was een studie over de verspreiding van Map in zoo-dieren. Daarvoor moet een nieuwe specifieke, gevoelige en vlugge test ontworpen worden. De specifieke doelstellingen waren het ontwerp van een specifieke PCR, het op punt stellen van een fecale DNA extractie methode en de ontwikkeling van een PCR gebaseerde karakterisatiemethode.

In Hoofdstuk 3 wordt het probleem rond de huidige aspecifieke IS*900* primers bestudeerd. Nieuwe specifieke primers voor IS*900* werden ontworpen en geëvalueerd met een panel van Map en niet-Map isolaten. Er werd een detectielimiet bekomen van 1 CFU per PCR. De betere specificiteit werd ook bewezen met computersimulaties van de PCR. Naast de IS*900* primers werden ook andere primers ontworpen. Deze nieuwe primers amplificeren een ander specifiek Map fragment

namelijk f57. Ook hier werd een detectielimiet van 1 CFU per PCR bekomen en vertoonden de primers een goede specificiteit. Een multiplex PCR voor de simultane amplificatie van het IS*900* en het f57 fragment werd ontwikkeld. Deze multiplex PCR was veelbelovend.

In Hoofdstuk 4 wordt de ontwikkeling van een fecale DNA extractie methode beschreven. Hiervoor werden verschillende homogenisatie-en DNA isolatieprotocols getest op kunstmatig belaste positieve stalen. De beste methode was een combinatie van een Tween-20 homogenisatie met een DNA isolatie gebaseerd op sequence-capture beschreven door Mangiapan *et al.,* 1996. Een detectielimiet van 100 Map cellen per g feces werd bekomen. De nieuwe fecale extractie methode werd geëvalueerd op "veldstalen" en bleek een goed alternatief voor de tijdrovende cultuur methoden. Er werden meer positieve stalen met PCR dan met cultuur gevonden. De sequence-capture techniek werd ook uitgetest op weefsel stalen. Ook hier werd een beter resultaat bekomen met PCR dan met cultuur.

In Hoofdstuk 5 wordt het onderzoek naar Map in de KMDA (Koninklijke Maatschappij voor Dierkunde, Antwerpen) beschreven. Hiervoor werden de nieuw ontworpen IS*900* primers in combinatie met de nieuwe fecale DNA extractie gebruikt. PCR detectie werd vergeleken met conventionele en radiometrische cultuurmethoden op weefselstalen en feces van herkauwers in de zoo. Er werd voor de eerste keer een commerciële Map ELISA kit gebruikt bij zoo-dieren. Drie weefselstalen en 1 feces staal werden positief bevonden met PCR. De ELISA resultaten toonden de aanwezigheid van Map aan in verschillende diersoorten. Als men rekening houdt met de historiek van Map in de Zoo en onze resultaten, kan men besluiten dat er inderdaad Map aanwezig is in hun dierencollectie maar e'r konden echter geen Map cellen teruggevonden worden met kweek.

In Hoofdstuk 6 worden de pogingen voor de ontwikkeling van een PCR gebaseerde karakterisatiemethode beschreven. De huidige gebruikte methode (RFLP) is tijdsrovend en is afhankelijk van cultuur. Een aangepaste AFLP werd gebruikt om polymorfismen tussen 74 Map isolaten op te sporen. De polymorfe fragmenten werden gecloneerd en hun sequentie werd bepaald. Vijf fragmenten werden verder bestudeerd en PCRs werden ontwikkeld om deze fragmenten te amplificeren. De

omzetting naar kant en klare PCRs voor de detectie van deze fragmenten bleek niet eenvoudig te zijn. Veel meer sequenties moeten geanalyseerd worden om een goede selectie te kunnen maken van waardvolle targets voor de karakterisatie van de Map isolaten.

Hoofdstuk 7 vat de conclusie's van deze studie samen.

10 References

Alhaji I, Johnson DW, Muscoplat CC, Thoen CO. (1974) Diagnosis of *Mycobacterium bovis* and *Mycobacterium paratuberculosis* infections in cattle by in vitro lymphocyte immunostimulation. Am J Vet Res. 35:725-7

Amonsin A, Li LL, Zhang Q, Bannantine JP, Motiwala AS, Sreevatsan S, Kapur V. (2004) Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis* strains. J Clin Microbiol. 42:1694-702

Bannantine JP, Baechler E, Zhang Q, Li L, Kapur V. (2002) Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. J Clin Microbiol. 40:1303-10

BANR, ***Board on Agriculture and Natural Resources, committee on diagnosis and control of Johne's disease, National Academy of Sciences***. (2003) Diagnosis and Control of Johne's disease. The National Academies Press ISBN 0 309 08611 6

Barclay R, Ewing DF, Ratledge C. (1985) Isolation, identification, and structural analysis of the mycobactins of *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, and *Mycobacterium paratuberculosis*. J Bacteriol. 164:896-903

Barrington GM, Gay JM, Eriks IS, Davis WC, Evermann JF, Emerson C, O'Rourke JL, Hamilton MJ, Bradway DS. (2003) Temporal patterns of diagnostic results in serial samples from cattle with advanced paratuberculosis infections. J Vet Diagn Invest. 15:195-200

Bauerfeind R, Benazzi S, Weiss R, Schliesser T, Willems H, Baljer G. (1996) Molecular characterization of *Mycobacterium paratuberculosis* isolates from sheep,

goats, and cattle by hybridization with a DNA probe to insertion element IS*900*. J Clin Microbiol. 34:1617-21

Beard PM, Daniels MJ, Henderson D, Pirie A, Rudge K, Buxton D, Rhind S, Greig A, Hutchings MR, McKendrick I, Stevenson K, Sharp JM. (2001) Paratuberculosis infection of nonruminant wildlife in Scotland. J Clin Microbiol. 39:1517-21

Bech-Nielsen S, Jorgensen JB, Ahrens P, Feld NC. (1992) Diagnostic accuracy of a *Mycobacterium phlei*-absorbed serum enzyme-linked immunosorbent assay for diagnosis of bovine paratuberculosis in dairy cows. J Clin Microbiol. 30:613-8

Beerwerth W, Schurmann J. (1969) Zur Okologie der Mycobakterien. Zentralbl Bakteriol Mikrobiol. 211:58-69

Belak S, Ballagi-Pordany A. (1993) Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. Mol Cell Probes. 7:241-8

Biospec technical information (2003) http://www.biospec.com/beads.htm

Bleumink-Pluym NM, Houwers DJ, Peeters-van Gent SD. (1994) Sample preparation for *M. paratuberculosis* polymerase chain reaction in faeces and tissues. Vet Rec. 135:120

Boelaert F, Walravens K, Biront P, Vermeersch JP, Berkvens D, Godfroid J. (2000) Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population. Vet Microbiol. 77:269-81

Buergelt CD, Layton AW, Ginn PE, Taylor M, King JM, Habecker PL, Mauldin E, Whitlock R, Rossiter C, Collins MT. (2000) The pathology of spontaneous paratuberculosis in the North American bison (*Bison bison*). Vet Pathol. 37:428-38

Bull TJ, Hermon-Taylor J, Pavlik I, El-Zaatari F, Tizard M. (2000) Characterization of IS*900* loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. Microbiology. 146:2185-97

Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J. (2003a) Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. J Clin Microbiol. 41:2915-23

Bull TJ, Sidi-Boumedine K, McMinn EJ, Stevenson K, Pickup R, Hermon-Taylor J. (2003b) Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. Mol Cell Probes. 17:157-64

Chan MS, Maiden MC, Spratt BG. (2001) Database-driven multi locus sequence typing (MLST) of bacterial pathogens.
Bioinformatics. 17:1077-83

Chemlal K, Portaels F. (2003) Molecular diagnosis of nontuberculous mycobacteria. Curr Opin Infect Dis. 16:77-83

Chen Z, Ruffner DE. (1996) Modified crush-and-soak method for recovering oligodeoxynucleotides from polyacrylamide gel. Biotechniques. 21:820-2

Choy E, Whittington RJ, Marsh I, Marshall J, Campbell MT. (1998) A method for purification and characterisation of *Mycobacterium avium* subsp. *paratuberculosis* from the intestinal mucosa of sheep with Johne's disease. Vet Microbiol. 64:51-60

Christopher-Hennings J, Dammen MA, Weeks SR, Epperson WB, Singh SN, Steinlicht GL, Fang Y, Skaare JL, Larsen JL, Payeur JB, Nelson EA. (2003) Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. J Vet Diagn Invest. 15:87-93

Cocito C, Gilot P, Coene M, de Kesel M, Poupart P, Vannuffel P. (1994) Paratuberculosis. Clin Microbiol Rev. 7:328-45

Coetsier C, Vannuffel P, Blondeel N, Denef JF, Cocito C, Gala JL. (2000) Duplex PCR for differential identification of *Mycobacterium bovis, M. avium*, and *M. avium* subsp. *paratuberculosis* in formalin- fixed paraffin-embedded tissues from cattle. J Clin Microbiol. 38:3048-54

Colgrove GS, Thoen CO, Blackburn BO, Murphy CD. (1989) Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture. Vet Microbiol. 19:183-7

Collins DM, Gabric DM, De Lisle GW. (1989) Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol Lett. 51:175-8

Collins DM, Stephens DM, de Lisle GW. (1993) Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. Vet Microbiol. 36:289-99

Collins MT, Kenefick KB, Sockett DC, Lambrecht RS, McDonald J, Jorgensen JB. (1990) Enhanced radiometric detection of *Mycobacterium paratuberculosis* by using filter-concentrated bovine fecal specimens. J Clin Microbiol. 28:2514-9

Collins MT. (1994) Clinical approach to control of bovine paratuberculosis. J Am Vet Med Assoc. 204:208-10

Collins MT. (1996) Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract. 12:357-71

Collins M, Lisby G, Moser C, Chicks D, Christensen S, Reichelderfer M, Hoiby N, Harms B, Thomsen O, Skibsted U, Binder V. (2000) Results of multiple diagnostic test for *Mycobacterium avium* subsp. *paratuberculosis* in patients with inflammatory bowel disease and in controls. J. Clin. Microbiol. 38: 4373-81

Cousins DV, Evans RJ, Francis BR. (1995) Use of BACTEC radiometric culture method and polymerase chain reaction for the rapid screening of faeces and tissues for *Mycobacterium paratuberculosis*. Aust Vet J. 37:1077-83

Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P. (1999) Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS*900*-like sequences detectable IS*900* polymerase chain reaction: implications for diagnosis. Mol Cell Probes.13:431-42

Coussens PM, Colvin CJ, Wiersma K, Abouzied A, Sipkovsky S. (2002) Gene expression profiling of peripheral blood mononuclear cells from cattle infected with *Mycobacterium paratuberculosis*. Infect Immun. 70:5494-502

De Meurichy W, Portaels F, Hoorens J, Bauwens L. (1985) Outbreak of a paratuberculosis-like disease in a captive Pudu Herd (*Pudu pudu*). Verhandlungsbericht des 27 internationalen Symposium über die Erkrankungen der Zootiere, St.Vincent/ Torino, 9 Juni bis 13 Juni 1985, pp 469-480

D'Haese E, Dumon I, Werbrouck H, Wiszniewska A, Herman L, Nelis HJ. (2003) Rapid enumeration of viable *Mycobacterium paratuberculosis* in milk. Proceedings of the 7th international colloquium of paratuberculosis, 11-14th june, Bilbao, Spain, pp 213-217

D'Haese E, Nelis HJ. (2002) Rapid detection of single cell bacteria as a novel approach in food microbiology. J AOAC Int. 85:979-83

Donaghy JA, Totton NL, Rowe MT. (2003) Evaluation of culture media for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from Cheddar cheese. Lett Appl Microbiol. 37:285-9

Ellingson JL, Bolin CA, Stabel JR. (1998) Identification of a gene unique to *Mycobacterium avium* subspecies *paratuberculosis* and application to diagnosis of paratuberculosis. Mol Cell Probes. 12:133-42

EMBL-EBI: http://www.ebi.ac.uk/clustalw/

Engels WR. (1993) Contributing software to the internet: The Amplify program. Trends Biochem Sci. 18:448-50

Englund S, Bolske G, Ballagi-Pordany A, Johansson KE. (2001) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples by single, fluorescent and nested PCR based on the IS*900* gene. Vet Microbiol. 81:257-71

Englund S, Bolske G, Johansson KE. (2002) IS*900*-like sequence found in a *Mycobacterium sp*. other than *Mycobacterium avium* subsp. *paratuberculosis*. FEMS Microbiol Lett. 209:267-71

Fang Y, Wu WH, Pepper JL, Larsen JL, Marras SA, Nelson EA, Epperson WB, Christopher-Hennings J. (2002) Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. J Clin Microbiol. 40:287-91

Ferreira R, Fonseca LS, Lilenbaum W. (2002) Agar gel immunodiffusion test (AGID) evaluation for detection of bovine paratuberculosis in Rio de Janeiro, Brazil. Lett Appl Microbiol. 35:173-5

Frothingham R, Meeker-O'Connell WA. (1998) Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology. 144:1189-96

Gaafar A, Unzaga MJ, Cisterna R, Clavo FE, Urra E, Ayarza R, Martin G. (2003) Evaluation of a modified single-enzyme amplified-fragment length polymorphism technique for fingerprinting and differentiating of *Mycobacterium kansasii* type I isolates. J Clin Microbiol. 41:3846-50

Garrido JM, Cortabarria N, Oguiza JA, Aduriz G, Juste RA. (2000) Use of a PCR method on fecal samples for diagnosis of sheep paratuberculosis. Vet Microbiol. 77:379-86

Gascoyne-Binzi DM, Barlow RE, Frothingham R, Robinson G, Collyns TA, Gelletlie R, Hawkey PM. (2001) Rapid identification of laboratory contamination with Mycobacterium tuberculosis using variable number tandem repeat analysis. J Clin Microbiol. 39:69-74

Grange JM. (1996) Mycobacteria and human disease 2nd ed. Arnold ISBN 0 340 64563 6

Grant IR, Hitchings EI, McCartney A, Ferguson F, Rowe MT. (2002) Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. Appl Environ Microbiol. 68:602-7

Grant IR, Kirk RB, Hitchings E, Rowe MT. (2003) Comparative evaluation of the MGIT and BACTEC culture systems for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from milk. J Appl Microbiol. 95:196-201

Grant IR, O'Riordan LM, Ball HJ, Rowe MT. (2001) Incidence of *Mycobacterium paratuberculosis* in raw sheep and goats' milk in England, Wales and Northern Ireland. Vet Microbiol. 79:123-31

Grant IR, Pope CM, O'Riordan LM, Ball HJ, Rowe MT. (2000) Improved detection of *Mycobacterium avium* subspecies *paratuberculosis* in milk by immunomagnetic PCR. Vet Microbiol. 77: 369-378

Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J. (1989) Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. 17:9063-73

Greenstein RJ. (2003) Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. Lancet Infect Dis. 3:507-14

Halldorsdottir S, Englund S, Nilsen SF, Olsaker I. (2002) Detection of *Mycobacterium avium* subsp. *paratuberculosis* by buoyant density centrifugation, sequence capture PCR and dot blot hybridisation. Vet Microbiol.87:327-40

Harris NB, Barletta RG. (2001) *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. Clin Microbiol Rev. 14:489-512

Hines ME 2nd, Styer EL. (2003) Preliminary characterization of chemically generated *Mycobacterium avium* subsp. *paratuberculosis* cell wall deficient forms (spheroplasts). Vet Microbiol. 95:247-58

Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411:599-603

Hulten K, Karttunen TJ, El-Zimaity HM, Naser SA, Collins MT, Graham DY, El-Zaatari FA. (2000) Identification of cell wall deficient forms of *M. avium* subsp. *paratuberculosis* in paraffin embedded tissues from animals with Johne's disease by in situ hybridization. J Microbiol Methods. 42:185-95

Huys G, Rigouts L, Chemlal K, Portaels F, Swings J. (2000) Evaluation of amplified fragment length polymorphism analysis for inter- and intraspecific differentiation of *Mycobacterium bovis, M. tuberculosis, and M. ulcerans*. J Clin Microbiol. 38:3675-80

Jiang X, Wang J, Graham DY, Estes MK. (1992) Detection of Norwalk virus in stool by polymerase chain reaction. J Clin Microbiol. 30:2529-34

Jorgensen JB. (1982) An improved medium for culture of *Mycobacterium paratuberculosis* from bovine faeces. Acta Vet. Scand. 23:325-335

Juste RA, Marco JC, Saez de Ocariz C, Aduriz JJ. (1991) Comparison of different media for the isolation of small ruminant strains of *Mycobacterium paratuberculosis*. Vet Microbiol. 28:385-90

Kalis CH, Barkema HW, Hesselink JW, van Maanen C, Collins MT. (2002) Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding *Mycobacterium avium* subspecies *paratuberculosis*. J Vet Diagn Invest. 14:219-24

Kapur (2003) website genoom: http://pathogenomics.ahc.umn.edu/map_index.htm

Kennedy DJ, Benedictus G. (2001) Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species. Rev Sci Tech. 20:151-79

Kormendy B. (1994) The effect of vaccination on the prevalence of paratuberculosis in large dairy herds. Vet Microbiol. 41:117-25

Kramsky JA, Manning EJ, Collins MT. (2003) Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. J Vet Diagn Invest. 15:253-61

Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PW, Martin C, Palittapongarnpim P, Plikaytis BB, Riley LW, Yakrus MA, Musser JM, van Embden JD. (1999) Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol. 37:2607-18

Kristensen VN, Kelefiotis D, Kristensen T, Borresen-Dale AL. (2001) High-throughput methods for detection of genetic variation. Biotechniques. 30:318-22, 324, 326

Lambrecht RS, Collins MT. (1992) *Mycobacterium paratuberculosis*. Factors that influence mycobactin dependence. Diagn Microbiol Infect Dis. 15:239-46

Larsen AB, Moon HW, Merkal RS. (1972) Susceptibility of horses to *Mycobacterium paratuberculosis*. Am J Vet Res. 33:2185-9

Linden A, Canivet P, Mousset B, de Rijk P, Rigouts L, Portaels F. (2003) Evidence of paratuberculosis in wild red deer (Cervus elaphus) in Belgium. Proceedings of the 7th international colloquium of paratuberculosis, 11-14th june, Bilbao, Spain, p523

Mahillon J, Chandler M. (1998) Insertion sequences. Microbiol Mol Biol Rev. 62:725-74

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 95:3140-5

Mangiapan G, Vokurka M, Schouls L, Cadranel J, Lecossier D, van Embden J, Hance AJ. (1996) Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. J Clin Microbiol. 34:1209-15

Manning EJB, Kramsky JA and Collins MT. (1999) Johne's disease diagnosis for non-domestic hoofstock. Proceedings of the sixth international colloquium on paratuberculosis, Melbourne, Australia, february, 14-18, 1999, pp458-461

Manning EJ, Collins MT. (2001a) *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. Rev Sci Tech. 20:133-50

Manning EJ. (2001b) *Mycobacterium avium* subspecies *paratuberculosis*: a review of current knowledge. J Zoo Wildl Med. 32:293-304

Markesich DC, Graham DY, Yoshimura HH. (1988) Progress in culture and subculture of spheroplasts and fastidious acid-fast bacilli isolated from intestinal tissues. J Clin Microbiol. 26:1600-3

Marsh I, Whittington R, Cousins D. (1999) PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* based on polymorphisms in IS1311. Mol Cell Probes. 13:115-26

Marsh I, Whittington R, Millar D. (2000) Quality control and optimized procedure of hybridization capture-PCR for the identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces. Mol Cell Probes. 14:219-32

Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, Gicquel B, Tibayrenc M, Locht C, Supply P. (2001) High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc Natl Acad Sci U S A. 98:1901-6

McClure HM, Chiodini RJ, Anderson DC, Swenson RB, Thayer WR, Coutu JA. (1987). *Mycobacterium paratuberculosis* infection in a colony of stumptail macaques (*Macaca arctoides*). J Infect Dis. 155:1011-9

McDonald WL, Ridge SE, Hope AF, Condron RJ. (1999) Evaluation of diagnostic tests for johne's disease in young cattle. Aust Vet J. 77:113-119

Merkal RS. (1973) Laboratory diagnosis of bovine paratuberculosis. J Am Vet Med Assoc. 163:1100-2

Mijs W, de Haas P, Rossau R, Van der Laan T, Rigouts L, Portaels F, van Soolingen D. (2002) Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and *'M. avium* subsp. *hominissuis*' for the human/porcine type of *M. avium*. Int J Syst Evol Microbiol. 52:1505-18

Millar DS, Withey SJ, Tizard ML, Ford JG, Hermon-Taylor J. (1995) Solid-phase hybridization capture of low-abundance target DNA sequences: application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*. Anal Biochem. 226:325-30

Monaghan ML, Doherty ML, Collins JD, Kazda JF, Quinn PJ. (1994) The tuberculin test. Vet Microbiol. 40:111-24

Moreno AM, Baccaro MR, Ferreira AJ, Pestana De Castro AF. (2003) Use of single-enzyme amplified fragment length polymorphism for typing *Pasteurella multocida* subsp. *multocida* isolates from pigs. J Clin Microbiol. 41:1743-6

Motiwala AS, Strother M, Amonsin A, Byrum B, Naser SA, Stabel JR, Shulaw WP, Bannantine JP, Kapur V, Sreevatsan S. (2003) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis. J Clin Microbiol. 41:2015-26

Motiwala AS, Amonsin A, Strother M, Manning EJ, Kapur V, Sreevatsan S. (2004) Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis isolates recovered from wild animal species.J Clin Microbiol. 42:1703-12

Muskens J, van Zijderveld F, Eger A, Bakker D. (2002) Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. Vet Microbiol. 86:269-78

Mve-Obiang A, Mestdagh M, Portaels F. (2001) DNA isolation from chloroform/methanol-treated mycobacterial cells without lysozyme and proteinase K. Biotechniques. 30:272-4, 276

Naser SA, Felix J, Liping H, Romero C, Naser N, Walsh A, Safranek W. (1999) Occurrence of the IS*900* gene in *Mycobacterium avium* complex derived from HIV patients. Mol Cell Probes. 13:367-72

NCBI: http://www.ncbi.nlm.nih.gov/

Nielsen SS, Nielsen KK, Huda A, Condron R, Collins MT. (2001) Diagnostic techniques for paratuberculosis. Bulletin of the international Diary Federation 362:5-15

Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 411:603-6

Olive DM, Bean P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol. 37:1661-9

Olsen I, Sigurgardottir G, Djonne B. (2002) Paratuberculosis with special reference to cattle. A review. Vet Q. 2002 24:12-28

Ott SL, Wells SJ, Wagner BA. (1999) Herd-level economic losses associated with Johne's disease on US dairy operations. Prev Vet Med. 40:179-92

Paul J. (1965) Cell and tissue culture 5th ed. Churchill Livingstone ISBN 0 443 01298 9

Pavlik I, Bejckova L, Pavlas M, Rozsypalova Z, Koskova S. (1995) Characterization by restriction endonuclease analysis and DNA hybridization using IS*900* of bovine, ovine, caprine and human dependent strains of *Mycobacterium paratuberculosis* isolated in various localities. Vet Microbiol. 45:311-8

Pavlik I, Horvathova A, Dvorska L, Bartl J, Svastova P, du Maine R, Rychlik I. (1999) Standardisation of restriction fragment length polymorphism analysis for *Mycobacterium avium* subspecies *paratuberculosis*. J Microbiol Methods. 38:155-67

Pavlik I, Matlova L, Bartl J, Svastova P, Dvorka L, Whitlock R. (2000) Parallel faecal and organ *Mycobacterium avium* subsp. *paratuberculosis* culture of different productivity types of cattle. Vet Microbiol 77:309-324

Payeur JB. (1998) Overview of Johne's disease diagnostics. Proceedings of the workshop on diagnosis, prevention and control of Johne's disease in non-domestic hoofstock, 26-28th juneYulee, Florida, USA, pp 21-26

Pence M, Baldwin C, Black CC (2003) The seroprevalence of Johne's disease in Georgia beef and dairy cull cattle. J Vet Diagn Invest. 15:475-7

Peters TM, Threlfall EJ. (2001) Single-enzyme amplified fragment length polymorphism and its applicability for *Salmonella* epidemiology. Syst Appl Microbiol. 24:400-4

Petroff SA. (1915) A new and rapid method for the isolation and cultivation of tubercle bacilli directly from sputum and feces. J Exp Med. 21:38-42

Pillai SR, Jayarao BM, Gummo JD, Hue EC, Tiwari D, Stabel JR, Whitlock RH. (2001) Identification and sub-typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* by randomly amplified polymorphic DNA. Vet Microbiol. 79:275-84

Poupart P, Coene M, Van Heuverswyn H, Cocito C. (1993) Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. J Clin Microbiol. 31:1601-5

Purohit HJ, Kapley A, Moharikar AA, Narde G. (2003) A novel approach for extraction of PCR-compatible DNA from activated sludge samples collected from different biological effluent treatment plants. J Microbiol Methods. 52:315-23

Puyang X, Lee K, Pawlichuk C, Kunimoto DY. (1999) IS*1626*, a new IS*900* related *Mycobacterium avium* insertion sequence. Microbiology. 145:3163-3168

Rajya BS, Singh CM. (1961) Studies on the pathology of Johne's disease in sheep. III. Pathologic changes in sheep with naturally occurring infections. Am J Vet Res. 22:189-203

Ranamukhaarachchi DG, Kane ME, Guy CL, Li QB. (2000) Modified AFLP technique for rapid genetic characterization in plants. Biotechniques. 29:858-9, 862-6

Rastogi N, Legrand E, Sola C. (2001) The mycobacteria: an introduction to nomenclature and pathogenesis. Rev Sci Tech. 20:21-54

Reddacliff LA, Vadali A, Whittington RJ. (2003) The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces.Vet Microbiol. 95:271-82

Richter E, Wessling J, Lugering N, Domschke W, Rusch-Gerdes (2002) *Mycobacterium avium* subsp. *paratuberculosis* infection in a patient with HIV, Germany. Emerg Infect Dis. 8:729-31

Ridge SE, Morgan IR, Sockett DC, Collins MT, Condron RJ, Skilbeck NW, Webber JJ. (1991) Comparison of the Johne's absorbed EIA and the complement-fixation test for the diagnosis of Johne's disease in cattle. Aust Vet J. 8:253-7

Robino P, Nebbia P, Meneguz PG, De Meneghi D. (2003) Survey on paratuberculosis in roe deer (*Capreolus capreolus*) and small ruminants in North-Western Italy . Proceedings of the 7th international colloquium of paratuberculosis, 11-14th june, Bilbao, Spain, p 472

Schroen C, Gwozdz J, Roche M, Bradley T, McDonald W, Condron R. (2002) Evaluation of diagnostic tests for the diagnosis of Johne's disease in deer. Proceedings of the 7th international colloquium of paratuberculosis, 11-14th june, Bilbao, Spain, pp 154-161

Sechi LA, Mura M, Tanda F, Lissia A, Solinas A, Fadda G, Zanetti S, Manuela M, Francesco T, Amelia L, Antonello S, Giovanni F, Stefania Z. (2001) Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by in situ hybridization. J Clin Microbiol. 39:4514-7

Sergeant ES, Baldock FC. (2002) The estimated prevalence of Johne's disease infected sheep flocks in Australia. Aust Vet J. 80:762-8

Shames B, Fox JG, Dewhirst F, Yan L, Shen Z, Taylor NS. (1995) Identification of widespread *Helicobacter hepaticus* infection in feces in commercial mouse colonies by culture and PCR assay. J Clin Microbiol. 33:2968-72.

Singh SN. (1998) *Mycobacterium paratuberculosis* PCR assay of feces. In L.H. Lauerman (ed.), Nucleic acid amplification assays for diagnosis of animal disease. American association of veterinary laboratory diagnosticians, Davis, Calif., p27

Snyder L, Champness W. (2003) Genes: replication and expression *In* :Molecular genetics of bacteria 2nd Edition ISBN 1 55581 204 X pp 24-28

Sockett DC, Carr DJ, Collins MT. (1992) Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of *Mycobacterium paratuberculosis* infections in cattle. Can J Vet Res. 56:148-53

Stevenson K, Sharp JM. (1997) The contribution of molecular biology to *Mycobacterium avium* subspecies *paratuberculosis* research. Vet J. 153:269-86

Stevenson K, Hughes VM, de Juan L, Inglis NF, Wright F, Sharp JM. (2002) Molecular characterization of pigmented and nonpigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis*. J Clin Microbiol. 40:1798-804

Stöbel K, Schönberg A, Staal C. (2002) A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s.l. in zoo animals. Int J Med Microbiol. 291:88-99

Storset AK, Hasvold HJ, Valheim M, Brun-Hansen H, Berntsen G, Whist SK, Djonne B, Press CM, Holstad G, Larsen HJ. (2001) Subclinical paratuberculosis in goats following experimental infection. An immunological and microbiological study. Vet Immunol Immunopathol. 80:271-87

Strachan T, Read A. (1999) Human Molecular Genetics 2nd ed. BIOS Scientific publishers ISBN 1 85996 202 5

Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP, Rings DM. (1995) Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am J Vet Res. 56:1322-4

Stumm G, Vedder H, Schlegel J. (1997) A simple method for isolation of PCR fragments from silverstained polyacrylamide gels by scratching with a fine needle. Elsevier trends journals technical tips online. http://tto.biometnet.com

Sweeney RW, Whitlock RH, Rosenberger AE. (1992a) *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J Clin Microbiol. 30:166-71

Sweeney RW, Whitlock RH, Rosenberger AE. (1992b) *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. Am J Vet Res. 53:477-80

Temple RM, Muscoplat CC, Thoen CO, Himes EM, Johnson DW. (1979) Observations on diagnostic tests for paratuberculosis in a deer herd. J Am Vet Med Assoc. 175:914-5

Tessema MZ, Koets AP, Rutten VP, Gruys E. (2001) How does *Mycobacterium avium* subsp. *paratuberculosis* resist intracellular degradation? Vet Q. 23:153-62

Thomas GW. (1983) Paratuberculosis in a large goat herd. Vet Rec. 113:464-6

Uhlen M. (1989) Magnetic separation of DNA. Nature. 340:733-4

van Boxtel RM, Lambrecht RS, Collins MT. (1990) Effect of polyoxyethylene sorbate compounds (Tweens) on colonial morphology, growth, and ultrastructure of *Mycobacterium paratuberculosis*. APMIS. 98:901-8

van der Giessen JW, Eger A, Haagsma J, Haring RM, Gaastra W, van der Zeijst BA. (1992a) Amplification of 16S rRNA sequences to detect *Mycobacterium paratuberculosis*. J Med Microbiol. 36:255-63

van der Giessen JW, Haring RM, Vauclare E, Eger A, Haagsma J, van der Zeijst BA. (1992b) Evaluation of the abilities of three diagnostic tests based on the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in cattle: application in a control program. J Clin Microbiol. 30:1216-9

van der Hoek L, Boom R, Goudsmit J, Snijders F, Sol CJ. (1995) Isolation of human immunodeficiency virus type 1 (HIV-1) RNA from feces by a simple method and difference between HIV-1 subpopulations in feces and serum. J Clin Microbiol. 33:581-8

van Der Wurff AW, Chan YL, van Straalen NM, Schouten J. (2000) TE-AFLP: combining rapidity and robustness in DNA fingerprinting. Nucleic Acids Res. 28:E105

van der Zee A, Steer N, Thijssen E, Nelson J, van't Veen A, Buiting A. (2003) Use of multienzyme multiplex PCR amplified fragment length polymorphism typing in analysis of outbreaks of multiresistant *Klebsiella pneumoniae* in an intensive care unit. J Clin Microbiol. 41:798-802

van Schaik G, Kalis CH, Benedictus G, Dijkhuizen AA, Huirne RB. (1996) Cost-benefit analysis of vaccination against paratuberculosis in dairy cattle. Vet Rec. 139:624-7

van Zwet AA, Thijs JC, Kooistra-Smid AM, Schirm J, Snijder JA. (1994) Use of PCR with feces for detection of *Helicobacter pylori* infections in patients. J Clin Microbiol. 32:1346-8

Vary PH, Andersen PR, Green E, Hermon-Taylor J, McFadden JJ. (1990) Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J Clin Microbiol. 28:933-7

Volk,W (1992) Basic microbiology 7th ed. Harper Collins ISBN 0 06 046849 1

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M Zabeau M. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-14

Wall S, Kunze ZM, Saboor S, Soufleri I, Seechurn P, Chiodini R, McFadden JJ. (1993) Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. J Clin Microbiol. 31:1241-5

Walsh PS, Metzger DA, Higuchi R. (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 10:506-13

Weber A, Gurke R, Bauer K, Schreyer K. (1992) Bakteriologische Untersuchungen zum Vorkommen von *Mycobacterium paratuberculosis* in Kotproben van Zoowiederkäuern. Berl Munch Tierarztl Wochenschr. 105:161-4

Whan LB, Grant IR, Ball HJ, Scott R, Rowe MT. (2001) Bactericidal effect of chlorine on *Mycobacterium paratuberculosis* in drinking water. Lett Appl Microbiol. 33:227-31

Whitlock R and Rosenberger A. (1990) A fecal culture protocol for *Mycobacterium paratuberculosis*. A recommended protocol. Proceedings of the 94th Annual Meeting of the U.S. Animal Health Association 1990; pp280-285

Whittington R, Marsh I, Choy E, Cousins D. (1998a) Polymorphisms in IS*1311*, an insertion sequence common to *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species. Mol Cell Probes. 12:349-58

Whittington RJ, Marsh I, Turner MJ, McAllister S, Choy E, Eamens GJ, Marshall DJ, Ottaway S. (1998b) Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS*900* PCR. J Clin Microbiol. 36:701-7

Whittington RJ, Marsh I, McAllister S, Turner MJ, Marshall DJ, Fraser CA. (1999a) Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. J Clin Microbiol. 37:1077-83

Whittington RJ, Reddacliff L, Marsh I, Saunders V. (1999b) Detection of *Mycobacterium avium* subspecies *paratuberculosi*s in formalin-fixed paraffin-embedded intestinal tissue by IS*900* polymerase chain reaction. Aust Vet J. 77:392-397

Whittington RJ, Hope AF, Marshall DJ, Taragel CA, Marsh. (2000) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS*900* restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. J Clin Microbiol. 38:3240-8

Widjojoatmodjo MN, Fluit AC, Torensma R, Verdonk GP, Verhoef J. (1992) The magnetic immuno polymerase chain reaction assay for direct detection of *salmonellae* in fecal samples.J Clin Microbiol. 30:3195-9

Wilson IG. (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol. 63:3741-51

Yeboah-manu D, Yates MD, Wilson SM (2001) Application of a simple multiplex PCR to aid in routine work of the Mycobacterium reference laboratory. J Clin Microbiol. 39:4166-68

Zwick LS, Walsh TF, Barbiers R, Collins MT, Kinsel MJ, Murnane RD. (2002) Paratuberculosis in a mandrill (Papio sphinx). J Vet Diagn Invest. 14:326-8