Molecular Diagnostic Methods for *Mycobacterium avium* subsp. *paratuberculosis*

More Than a Gut Feeling

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Cover: Free-ranging cow outside Pushkar, India, and an alignment of the *Mycobacterium avium* subsp. *paratuberculosis* specific IS*900* element with some related insertion sequence elements. One newly developed confirmative PCR system is shown.

(photo: D. Herthnek)

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Molecular Diagnostic Methods for *Mycobacterium avium* subsp. *paratuberculosis*. More Than a Gut Feeling

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of the chronic enteric disease paratuberculosis in ruminants that causes considerable economic losses worldwide. Due to rigorous control measures, paratuberculosis is rare or absent in Sweden. However, import-related outbreaks have occurred. Diagnostic surveillance and outbreak investigations are mainly carried out by very slow culture methods. Faster and equally reliable molecular methods are needed for detection of MAP in several clinical matrixes. MAP is primarily shed in faeces, the most important testing material. The abundance of PCR inhibitory substances in faeces constitutes a diagnostic challenge. Semen, imported for breeding purposes, may contain MAP if the donor bulls are asymptomatic carriers. MAP has been suggested as the causative agent of Crohn's disease and its presence in consumers' milk might be of concern. In this thesis, the development and sensitivity assessment of protocols for detection of MAP in ruminant faeces, semen and milk by real-time PCR are described. The analytical sensitivities were assessed to 10^4 MAP/g faeces, 10 MAP/100 µl semen and 100 MAP/ml milk. The faeces direct PCR was validated on 202 proficiency test samples. MAP was detected in 97% of previously frozen positive samples - better than culture. Pellet and cream fractions of milk were pooled before cell lysis and DNA extraction by automated magnetic bead separation. In a study of 56 dairy herds, tank milk PCR was compared to culture of environmental faecal samples for herd prevalence testing. By the latter, 68% of the herds were positive, while 30% were positive by PCR. Due to the concluded low abundance of MAP in milk tanks, milk PCR would be more useful for testing of MAP in consumers' milk, than for herd prevalence testing. Three real-time PCR systems were designed for confirmation of PCR positives and validated on 267 strains and 58 positive faecal and tissue samples. The system based on the gene F57 was the most specific. A faecal culture screening of 501 wild guanacos in Chile yielded MAP colonies from 21 guanacos (4.2%), representing the first isolation of MAP from wild animals in the Chilean Patagonia. Confirmation was done by PCR and typing was performed by PCR-REA. All strains proved to be of C type.

Keywords: cattle, confirmation, detection, diagnosis, F57, IS*900*, Johne's disease, microbiology, *Mycobacterium avium* subsp. *paratuberculosis*, real-time PCR.

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There is a theory which states that if ever anybody discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.

Douglas Adams (1952-2001)

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Herthnek, D., Englund, S., Willemsen, P.T.J. & Bölske, G. (2006). Sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine semen by real-time PCR. *J Appl Microbiol* 100, 1095-102.

II Herthnek, D. & Bölske, G. (2006). New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*. *BMC Microbiol* 6, 87

III Herthnek, D., Nielsen, S.S., Lindberg, A., Bölske, G. (2008). A robust method for bacterial lysis and DNA purification to be used with real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *J Microbiol Methods* 75, 335-40

IV Salgado, M., Herthnek, D. Bölske, G., Leiva, S., Kruze, J. (2009). First isolation of *Mycobacterium avium* subsp. *paratuberculosis* from wild guanacos (*Lama guanicoe*) in Tierra del Fuego island (Accepted for publication in *Journal of Wildlife Diseases*)

V Herthnek, D. & Bölske, G. Validation of a new detection method for *Mycobacterium avium* subsp. *paratuberculosis* in faeces by real-time PCR (manuscript)

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A witty saying proves nothing.

Voltaire

Abbreviations

Listed if used more than once or if not explained in the text.

AGID	agar gel immunodiffusion
CD	Crohn's disease
CFU	colony forming units
Ct	cycle threshold value
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxyuracil triphosphate
ELISA	enzyme-linked immunosorbent assay
HEYM	Herrolds egg yolk medium
IAC	internal amplification control
IFN-γ	interferon gamma
IMS	immunomagnetic separation
IS	insertion sequence
IS*900*	insertion sequence *900*
IL2	interleukin 2
MAA	*Mycobacterium avium* subsp. *avium*
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
P	probability that an observation occurs by chance
PCR	polymerase chain reaction
PCR-REA	PCR – restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
TNF-β	tumour necrosis factor beta
UNG	Uracil N-glycosylase
ΔCt	delta cycle threshold, i.e. difference in the no. of cycles
σ	standard deviation

1 Background

Ever since 1895, when Jöhne and Frothingham described the slowly progressing enteric disease of ruminants (Jöhne & Frothingham, 1895) that would later be known as paratuberculosis, or Johne's disease, the cattle industry in the world has been trying to stop the spread of its causative agent, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), also known as *Mycobacterium paratuberculosis*. No effective treatment of this serious infectious disease is available and the clinically ill animal, suffering from chronic or intermittent diarrhoea, inevitably emaciates, weakens and dies. The long-term effect of vaccination, although it reduces the incidence of clinical disease and bacterial shedding, is controversial and fails to eradicate the disease in a herd (Rosseels & Huygen, 2008). Therefore, the spread of the disease can only be stopped by rigorous control programmes, in which animals are tested for paratuberculosis and kept separate from other animals or even culled, as is the practice in Sweden. This country is virtually free from paratuberculosis, although limited import-related outbreaks have occurred (Lewerin *et al.*, 2007; Holmström *et al.*, 2003).

1.1 The pathogen

MAP is a gram-positive, acid-fast, fastidious and extremely slow growing bacillus (Lilenbaum *et al.*, 2007). Culture of visible colonies can take more than 16 weeks (Collins, 1996) and practically all strains require supplement of mycobactin (Cocito *et al.*, 1994). Mycobactin is a so called siderophore, an iron-chelating agent that most other mycobacteria produce endogenously to assist the uptake of iron, thereby circumventing the active removal of intracellular iron by MAP-infected macrophages (Harris & Barletta, 2001).

The organism has a thick, waxy and lipid-rich cell wall, giving it a general survival advantage and increased resistance to high temperatures when pasteurising milk (Grant *et al.*, 2005; Grant *et al.*, 2002b), low pH, salt and chemicals, such as chlorine (Rowe & Grant, 2006; Donaghy *et al.*, 2004). Although it has been claimed that proper pasteurisation should kill all MAP (Rademaker *et al.*, 2007; Stabel, 1997), viable MAP has been reported in retail milk (Ayele *et al.*, 2005; Ellingson *et al.*, 2005a; Grant *et al.*, 2002a). MAP has been shown to be able to survive for long periods of time in the environment, complicating eradication of the disease (Whittington *et al.*, 2005). Probably, the thickness of the cell wall is also contributing to its slow growth, due to restricted uptake of nutrients, although MAP's inability to produce mycobactin is thought to be one of the main constraints.

Mycobacteria belong to the phylum *Actinobacteria* and are characterized by rod shape, acid-alcohol fastness (distinguishable by Ziehl Neelsen staining), high genomic content of guanine and cytosine (61-71%) and the presence of long and complex mycolic acids in the cell wall (Shinnick & Good, 1994). At the time of writing, there were 133 recognized and proposed mycobacterial species, and several subspecies, (Euzéby, 2009; http://www.bacterio.cict.fr/m/mycobacterium.html; 21-Jan-2009), taxonomically grouped primarily according to speed of growth and temperature requirements, pigmentation and resistance to antibiotics.

Two important mycobacteria other than MAP are *Mycobacterium tuberculosis*, the most common causal agent of tuberculosis in animals and humans, *Mycobacterium bovis,* the causal agent of bovine tuberculosis and *Mycobacterium leprae*, the cause of leprosy. The phylogenetic relationship between these and some other mycobacteria is shown in the evolutionary tree in Fig. 1, based on the 16S rRNA sequences of the respective species. MAP was suggested to be classified as a subspecies of *Mycobacterium avium* together with *Mycobacterium avium* subsp. *avium* (MAA) and *Mycobacterium avium* subsp. *silvaticum* by Thorel and colleagues (1990). The proposed change in nomenclature was based on the results of 22 characteristics, such as growth preferences and antibiotic tolerance, of 38 strains. *"Mycobacterium avium* subsp. *hominissuis"* is a newly suggested subspecies designation for some of the serogroups of MAA, for isolates from humans and pigs. Together with *Mycobacterium intracellulare*, these *Mycobacterium avium* subspecies form the *Mycobacterium avium* complex (MAC). In human medicine, this group of mycobacteria is known to be implicated in opportunistic infection of AIDS patients (Horsburgh, 1999).

One characteristic of MAP that is important for its molecular detection is the presence of 15-20 copies of the 1451 base pair long insertion sequence

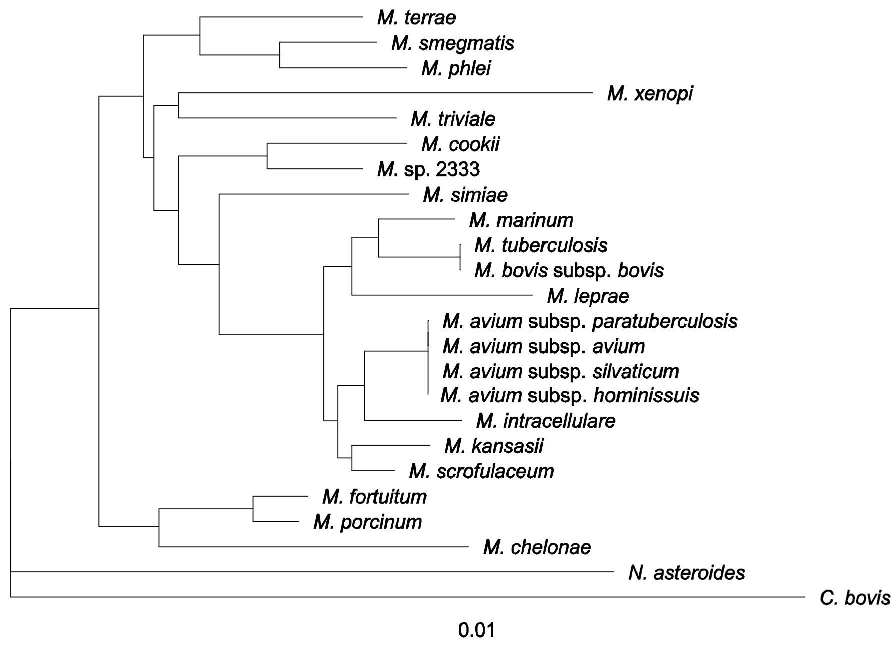

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences of selected mycobacteria. The scale bar shows a distance equivalent to 1 substitution per 100 nucleotide positions.

(IS) element IS*900* (Moss *et al.*, 1991; Green *et al.*, 1989), a member of the IS*110* family (Mahillon & Chandler, 1998). Insertion sequences are a type of small (<2500 base pairs) transposable elements, common in prokaryotes, which only carry the genes necessary for transposition, i.e. the occasional moving or duplication of itself to a new location in the genome. Although there are sequentially similar insertion elements in other mycobacteria, the sequence as a whole is considered specific for MAP and has been widely used in molecular diagnosis of paratuberculosis (Schönenbrücher *et al.*, 2008; Slana *et al.*, 2008b; Kawaji *et al.*, 2007; Ellingson *et al.*, 2005b; Pillai & Jayarao, 2002; Grant *et al.*, 1998; Millar *et al.*, 1996; van der Giessen *et al.*, 1992; Vary *et al.*, 1990)

Different strains of MAP were grouped into cattle (C) and sheep (S) genotypes by Collins and colleagues (1990), by using restriction fragment length polymorphism analysis (RFLP) to differentiate between strains isolated from cattle, sheep and goats from various countries. Characterization of strains by molecular methods is referred to as molecular typing and is useful for epidemiological purposes, such as tracing of outbreaks and understanding of disease transmission. Two sets of RFLP

types are used to characterize a strain (briefly described in the section "Molecular typing"), giving the most common strain in cattle in Western Europe the designation B-C1. MAP strains of type C predominantly infect cattle, but also other animals, while S strains appear to only infect sheep under normal circumstances. A simpler and faster method, PCR-REA, was later developed to discriminate between C and S strains only (Marsh *et al.*, 1999; Whittington *et al.*, 1998), and will be further described below. With this method, a new basic type of MAP was found and designated Bison strain (B), as it was first found in American bison (Whittington *et al.*, 2001).

1.2 Prevalence

Surveys undertaken during the latter part of the 20th century show that paratuberculosis is spread worldwide, causing significant economic losses in the affected countries (Kennedy & Benedictus, 2001; Johnson-Ifearulundu & Kaneene, 1997). In these surveys, estimates of herd prevalence in Europe range from 7% in Austria to 55% in Denmark (Kennedy *et al.*, 2001). Nielsen & Toft (2009) critically reviewed reported prevalence in Europe and pointed out flaws in the design of many studies, but estimated the overall prevalence to 20%. In the United States, average herd prevalence in dairy herds was 22% in 1996, while it differed from 20% to 40% depending on herd size (Wells & Wagner, 2000). The true prevalence is difficult to assess, as methods to diagnose the disease have generally not been sufficiently sensitive, and tests and sampling methods differ between countries. Local assays sometimes result in very high rates of infected herds, as 97.2% in Rio Grande de Sul (south Brazil) (Gomes *et al.*, 2005) and 80% in Minnesota, US (Raizman & Wells, 2005). A few countries, including Sweden, have very low prevalence. For three centuries, there were no reports of paratuberculosis in Sweden, until four clinical cases were found in beef cattle in 1993. A total of 53 infected cattle herds were identified – all linked to animal imports. All animals in the herds were culled, in accord with Sweden's stamping-out policy. Since then, paratuberculosis has been found in imported cattle and contact herds on two occasions in 2000 and 2005. To reduce the risk of importing the disease, the Swedish Animal Health Service advises farmers to import semen or embryos, instead of live animals.

MAP has also been shown to reside in nature, both in the environment (Manning & Collins, 2001) and in wildlife, acting as reservoirs for the bacteria. The organism has been isolated from deer, wild sheep and goats, elk and bison in North America, and in deer and rabbits in Italy and

Scotland, respectively (Judge *et al.*, 2005; Harris & Barletta, 2001; Greig *et al.*, 1999). Corn *et al.* isolated MAP from various wild monogastric mammals and birds, such as raccoons, opossums and starlings (2005). The risk of transmission of paratuberculosis from wild animals to domestic livestock appears to be real and should not be neglected (Daniels *et al.*, 2003). It has also been shown that MAP can survive in protozoa, insects and biofilms in the environment and has been reported in rivers and catchment areas (Rowe & Grant, 2006; Pickup *et al.*, 2005; Whan *et al.*, 2005). In the present study, a screening for MAP in guanacos (*Lama guanicoe)* was performed. The guanaco is a wild camelid of South America, and is abundant on the Tierra del Fuego Island ("Land of Fire") in Patagonian Chile, where the present screening was carried out.

One of the outcomes of several investigations of the potential relationship between MAP and Crohn's disease (CD) in humans (further discussed below) was that MAP was found in biopsies and blood of a relatively high percentage of the healthy control patients (Naser *et al.*, 2004; Bull *et al.*, 2003). These findings together with the reports on MAP in wildlife and in the environment suggest that MAP may be more abundant in the environment and/or in foods than what is presently known. The presence of MAP in consumers' milk is discussed below.

1.3 Pathogenesis

Faecal-oral transmission of MAP is the most common way for the disease to spread among cattle (Stabel, 1998; Chiodini *et al.*, 1984). The animals are most susceptible when younger than 30 days and usually ingest the bacteria when suckling from an infected dam with faecally contaminated teats or, probably less likely, from one that sheds MAP directly into her milk and colostrum (Streeter *et al.*, 1995; Sweeney *et al.*, 1992). Heavily infected dams may also convey the organisms to foetuses *in utero* (Sweeney, 1996; Lawrence, 1956). Both the susceptibility of infection and the risk of progress to severe disease decrease with age. Adult cows may, however, ingest bacteria that were shed into the environment and if the ingested dose is large, they too can develop clinical disease. Some animals resist the infection but become asymptomatic carriers, intermittently shedding the bacteria without showing any signs of infection.

MAP has been isolated from the reproductive organs and semen of infected bulls (Khol *et al.*, 2007; Ayele *et al.*, 2004; Buergelt *et al.*, 2004; Glawischnig *et al.*, 2004; Eppleston & Whittington, 2001; Larsen *et al.*, 1981), which poses the possibility of venereal transmission of the disease.

The magnitude of this risk is not sufficiently investigated, but is expected to be low. However, in a country like Sweden, where prevalence is kept extremely low by strict control programs, imported semen from an asymptomatic carrier would be a way for the disease to circumvent surveillance – if the semen is not tested.

All ruminants are susceptible to the disease. Most common hosts are cattle, goats and sheep, but less common domestic stock, such as camels and alpaca, and exotic animals and wildlife, such as moose and buffalos, are also reported to have contracted the disease. Monogastric animals, such as horses and poultry, have been shown to be susceptible when experimentally infected, while usually not developing clinical disease (Corn *et al.*, 2005; Chiodini *et al.*, 1984). The possible relevance of MAP in the human inflammatory bowel disease CD is discussed in the section "Zoonotic aspects".

When MAP is ingested, it penetrates the intestinal mucosa via the lymphoid follicles in the Peyer's patches of the ileum, and is soon phagocytosed by macrophages, its target cells (Sigurethardottir *et al.*, 2004; Cocito *et al.*, 1994). Characteristically to MAP, it is able to survive and replicate within macrophages in the intestinal wall and in lymph nodes. Possibly, MAP blocks the phagosome-lysosome fusion or disturbs the production of oxygen radicals, necessary for destruction of internalised bacteria. The TH1 lymphocyte population must produce cytokines (IL2, TNF-β and most importantly IFN-γ) to activate cell-mediated immune function and prepare the macrophages for digestion of MAP (Sigurethardottir *et al.*, 2004; Stabel, 2000b). As breakdown and presentation of MAP-antigen by the macrophage to T-lymphocytes is prevented, much of the subsequent immune process is also slowed or stopped. As the disease progresses, the humoral immune response usually takes over. This response has, however, little effect on the intracellularly protected bacteria. How MAP manages to endure the active reduction of iron levels by the macrophage without the assistance of mycobactin is unclear.

The progress of the disease can be divided in three clinical stages (Cocito *et al.*, 1994). In the subclinical stage I, the disease develops silently, with no symptoms or bacterial shedding. In stage II, the animal still shows no symptoms, but may shed intermediate amounts of bacteria in the faeces, as the concentration of MAP in the intestinal mucosa and lumen increases. In an attempt to contain the infection and to recruit inflammatory cells, cytokines are released that forms granuloma (Chiodini, 1996), aggregates of macrophages and lymphocytes. These do, however, not form distinct and functional granuloma, but distorted lesions that allow the mycobacteria to

multiply. As a result, inflammatory recruitment continues, causing the granulomas to swell and coalesce, which soon causes clinical disease, stage III. The incubation period before reaching this stage may range from less than 6 months to over 15 years (Chiodini *et al.*, 1984). Tissues in the terminal small intestine have become distorted, swollen and leaks plasma, causing continuous or intermittent diarrhoea, protein-loss and malabsorption (Stabel, 1998; Chiodini, 1996; Chiodini *et al.*, 1984). Shedding of bacteria in stage III can exceed 10^8 colony forming units (CFU) per gram faeces (Chiodini *et al.*, 1984), and diagnosis by detection of MAP is usually not a problem. Despite remaining a good appetite, the affected animal emaciates and produces less milk. Its hair coat becomes rough, sometimes with fading colour. Death is often preceded by bloody diarrhoea, debilitation, loss of appetite and severe emaciation. Animals are usually culled before this advanced clinical stage.

1.4 Zoonotic aspects

An association between MAP and the human enteritis Crohn's disease (CD) was first suggested by Dalziel (1913), as he noted its clinical and histopathological similarities with Johne's disease. CD is a rare chronic inflammatory disease that mainly affects the last part of the small intestine, but can be manifested in any part of the gastrointestinal tract (Chamberlin & Naser, 2006; Grant, 2005). The illness is incurable and severe, causing abdominal pain, diarrhoea, weight loss and weakness, as the intestines of the patient becomes thickened and corrugated. Most patients require surgery, once or more. Some assays by culture and PCR from blood and intestinal tissue biopsies from CD patients, patients with other bowel diseases and healthy control patients, have shown that MAP is more abundant in the former, thus supporting previous suppositions about an association between MAP and CD (Singh *et al.*, 2008; Scanu *et al.*, 2007; Sechi *et al.*, 2005; Naser *et al.*, 2004; Bull *et al.*, 2003; Schwartz *et al.*, 2000). In contrast, others report findings of more or roughly equal occurrence of MAP in patients with ulcerative colitis (Collins *et al.*, 2000) and yet other authors failed to find MAP in any patients or controls (Parrish *et al.*, 2008; Ellingson *et al.*, 2003; Kanazawa *et al.*, 1999). Most importantly, and independently of the ratios of occurrence of MAP in patients; the presence of MAP in patients does not mean that it is causative of the disease, but may instead be a secondary opportunistic invader of the already clinically ill patient.

Several arguments support or oppose causation in the ongoing debate of the role of MAP in the aetiology of CD, as reviewed by several authors

(Mendoza *et al.*, 2009; Abubakar *et al.*, 2008; Behr & Kapur, 2008; Feller *et al.*, 2007; Sartor, 2005). A recent study reports the finding of a wide range of pathogens in CD patients, among which mycobacteria were only the third most commonly occurring (Knösel *et al.*, 2009). There is also support for theories combining the potential role of MAP with other theories concerning genetically predisposed immune dysregulation or deficiency (Chamberlin & Naser, 2006), involving, among other newly discovered susceptibility genes for CD, the Nucleotide-binding Oligomerization Domain 2 (*NOD2*) (Ferwerda *et al.*, 2007; Hugot *et al.*, 2001). Currently, there is no evidence for neither independent causation by MAP nor independence of MAP and therefore, one should consider MAP a potential health hazard for people.

In one of the surveys, biopsies from 26% of the controls without any inflammatory bowel disease contained MAP (Bull *et al.*, 2003), and in other studies, the equivalent values were also remarkably high (Singh *et al.*, 2008; Sechi *et al.*, 2005; Naser *et al.*, 2004; Stabel, 2000a). This raises a question: How and from where did these patients acquire MAP? As mentioned above, MAP has been found in the environment and may be more widespread than we know, also in food.

1.4.1 MAP in milk

Dairy cattle with Johne's disease do to some extent shed MAP in milk (Giese & Ahrens, 2000; Sweeney *et al.*, 1992). In addition, faecal contamination is a likely source of MAP transmission to milk. It has been shown that small amounts of MAP can remain viable after pasteurisation, both when performed in experimental setups (Grant *et al.*, 2005; Chiodini & Hermon-Taylor, 1993), on-farm facilities (Ruzante *et al.*, 2008) and in retail milk (Ayele *et al.*, 2005; Ellingson *et al.*, 2005a). MAP also survived the ripening process of Cheddar cheese, experimentally manufactured from artificially contaminated milk (Donaghy *et al.*, 2004). Considering the possible zoonotic potential of MAP, there is a need for simple and sensitive methods for monitoring the amount of MAP in the milk of dairy farms. Would MAP be found in Swedish milk, it would add interesting substance to the debate on the etiology of Crohn's disease, since it is present with relatively high incidence in Sweden (Lapidus, 2006; Lindberg & Jornerot, 1991), while paratuberculosis is not. If shedding or contamination of MAP from infected animals into milk was evenly distributed over animals and time, screening of bulk tank milk would also be an alternative for testing herd prevalence of paratuberculosis.

1.5 Diagnostic methods

Several methods have been suggested and used for diagnosis of Johne's disease, but many of them suffer from inferior specificity and sensitivity (Whittington, 2002). In fact, because of the pathobiology of paratuberculosis; the slow progress of infection and the inappropriate immune response, it is impossible for any method to perform well during all stages of the disease.

1.5.1 Immunology

During the early cell-mediated immune response, detection of cytokines, such as IFN-γ, a product of T lymphocytes, can be done *in vitro* by enzyme-linked immunosorbent assay (ELISA) (Harris & Barletta, 2001; Collins, 1996). *In vivo* skin testing with the antigen Johnin has also been employed (de Lisle *et al.*, 1980). Neither of these tests is specific for MAP, and it is known that MAA can cause false positive results. In stage II and III, when the concentration of antibodies increases, serological tests can be useful in some circumstances. Complement Fixation (CF), however, lacks in both sensitivity and specificity. The sensitivity performance of agar gel immunodiffusion (AGID) is better, but not as good as that of antibody ELISA which, however, also suffers from insufficient specificity (Stabel, 1998; Collins, 1996).

1.5.2 Histopathology

Greater specificity is achieved when the presence of the aetiological agent, MAP, can be demonstrated and confirmed with bacteriological or molecular methods when the animal sheds bacteria in faeces, milk or occasionally in semen. From post mortem or slaughtered animals, gross lesions of corrugated ileum, as well as typical histopathological lesions and the presence of acid-fast bacilli, are suggestive of paratuberculosis, but for specific diagnosis, culture or PCR is necessary. Likewise, characteristic acid-fast bacilli in faecal samples are suggestive of paratuberculosis, but culture or PCR is necessary for a reliable diagnosis.

1.5.3 Bacteriology

MAP is a fastidious organism and has a generation time of 1.5 to 4 days when cultured in liquid media (Lambrecht *et al.*, 1988). As mentioned above, culture of clinical material, usually faeces, may sometimes take more than 16 weeks to yield visible colonies on solid media, such as Herrolds egg yolk medium (HEYM) supplemented with mycobactin. Solid medium is cast in glass tubes, slanting to increase the contact surface and therefore

Figure 2. MAP colonies on a slope of Löwenstein-Jensen medium, supplemented with mycobactin.

referred to as “slopes”. Optimisation of the sensitivity is difficult due to the chemicals used for decontamination of the faster growing sample microflora that also kill or decrease the viability of MAP by 70-99% (Whittington, 2002; Grant & Rowe, 2001). If the animal is an intermittent or low shedder, false negatives can therefore occur. Although culture is problematic, isolation of the bacteria makes the method 100% specific, as the colony material can be further tested for confirmation of the result by molecular methods or by the classical methods to examine the characteristics of growth and morphology. Colonies should be small, raised, firm, glistening and or pale yellow (Merkal, 1970), although some sheep strains are strongly pigmented. When significant growth is observed, lack of growth in a control tube without mycobactin indicates that the organism is mycobactin-dependent and thus, represents MAP.

Because of the specificity and high sensitivity of culture compared to other methods, it is since long considered the “gold standard” for diagnosis of paratuberculosis. However, for subclinical infection, the sensitivity of culture and other agent detection methods on faecal samples is very poor, and culture could therefore only be regarded as “gold standard” on faecal samples in clinical cases where shedding of MAP is very frequent. To be regarded as a general “gold standard” for paratuberculosis, it has to be applied on suitable lymph nodes and intestinal samples (preferably, last part of ileum and adjacent lymph node), thereby maximising the chance of finding MAP.

1.5.4 PCR

The invention of the polymerase chain reaction (PCR) earned Kary Mullis a shared Nobel Prize in Chemistry 1993, as the technique soon became

immensely important in the field of molecular biology (Mullis & Faloona, 1987). There is a vast range of research and clinical applications for PCR in biology and medicine. Molecular detection is one of them, gaining ground over the last two decades as a sensitive, specific and fast method to diagnose paratuberculosis by detection of MAP DNA. The principle of PCR is the enzymatically driven and temperature controlled exponential amplification of a specific target sequence in the template DNA extracted from the sample. Two target-specific oligonucleotides (DNA fragments), called primers, are designed to confine the range of the sequence elongation by acting as extension points for a DNA polymerase, often Taq polymerase (originally isolated from the thermophilic bacterium *Thermus aquaticus*). The temperature of the reaction is cycled through DNA denaturing heat, a low annealing temperature and an intermediate elongation temperature, optimal for the polymerase. Every cycle, the polymerase synthesizes a copy of each strand of the target DNA.

Under optimal conditions, one single copy of a target gene, extracted from clinical sample material, should be enough to start a chain reaction. The resulting product of millions of identical DNA fragments can be separated by gel electrophoresis and visualized by UV light, after which the positions of the gel bands reveal if the amplicons were of expected size. The method of DNA extraction and purification is very important for successful detection. Some sample materials, such as faeces, contain high amounts of substances inhibitory to the PCR reaction; complex polysaccharides, certain salts and ions, urea and proteinases. As these are removed, one must make certain that introduced PCR inhibitory extraction reagents, for example phenol and ethanol, are also removed from the sample.

Real-time PCR

For identity confirmation of cultured colonies, the sensitivity of conventional PCR is sufficient (Manning & Collins, 2001). For direct PCR on clinical samples, however, higher sensitivity is usually required. Improvements of the conventional PCR include nested PCR and TaqMan real-time PCR. In the former, the product from one PCR reaction serves as template in a second reaction with fresh reagents, thus diluting any PCR inhibiting substances and increasing the sensitivity. In TaqMan real-time PCR, a specific probe sequence, with attached fluorescent reporter dye and quencher dye, hybridises to the target sequence near one of the primers (Fig. 3). The presence of the quencher prevents the reporter to emit light, but as the PCR reaction proceeds, the 5' to 3' exonuclease activity of the Taq polymerase allows for digestion of the probe. The reporter is then

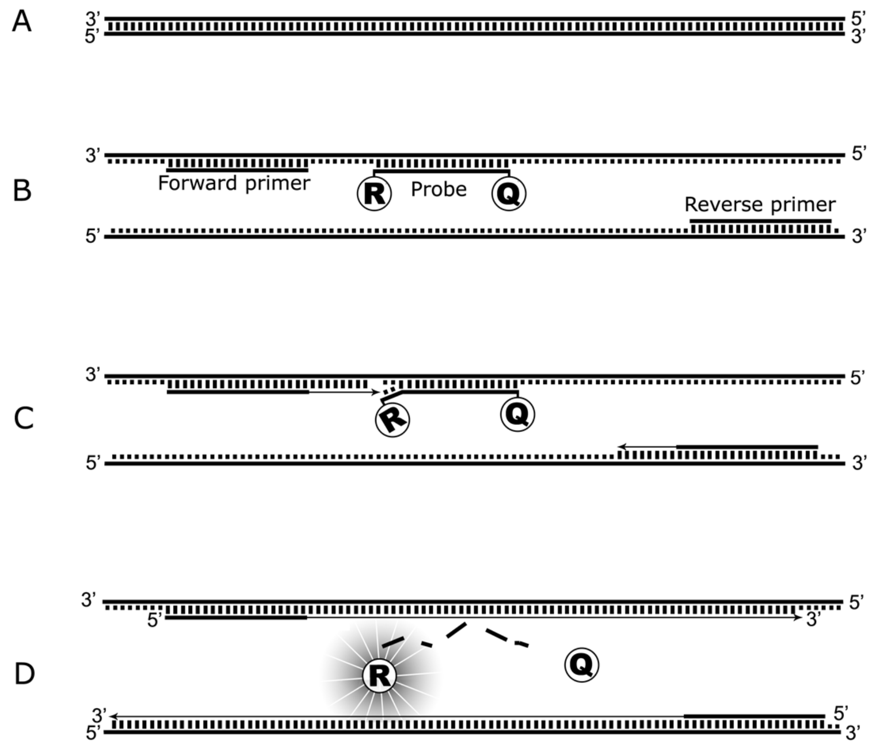

Figure 3. The principle of TaqMan real-time PCR. Double-stranded template DNA (A) is denatured by high temperature. At low temperature, primers and probe anneal to the template (B). When elongation of the primer reaches the probe, labeled with a fluorescent reporter (R) and a quencher (Q), it is digested (C). In the absence of the quencher, the reporter dye emits light, detected by the instrument and indicative of amplification (D). Narrow lines represent the newly extended strand.

released and can emit light with a wavelength determined by the choice of reporter fluorophore.

Henceforth, this technique will be referred to as only real-time PCR (although there are other real-time PCR techniques, such as SYBR Green PCR, named after the non-specific double-stranded DNA-intercalating dye it utilizes instead of a probe). The amplification reaction takes place in a machine that cycles the temperature, emits light that excites the fluorophore and measures the light emitted from the freed fluorophore, thereby monitoring the progress of amplification.

Depending on the amount of starting target DNA, it will take a certain number of cycles before the fluorescent signal reaches a defined threshold level. This cycle number (Ct) can be used to compare and quantify amounts of starting DNA in the samples. Because the sensor can measure weak

fluorescence signals, resulting from a PCR product that might not be visible on a gel to the naked eye, the method is very sensitive. The probe can be chosen to make the method more specific than conventional PCR.

The most widely used target gene for detection of MAP is IS*900*, first described Greet *et al.* (1989), and presently considered specific for MAP. The MAP genome is reported to have 15 to 20 copies of the insertion element, and the sequenced strain K-10 has 17 copies (Li *et al.*, 2005). This high target copy number gives an increased sensitivity compared to systems targeting single copy genes, which makes it popular in molecular diagnostic methods for paratuberculosis. However, successful detection of IS*900* is not necessarily definitive for identification of MAP, as initially presumed. It has many similarities with IS elements of other mycobacteria, which means that detection with PCR systems located in a conserved area may cause false positives in some strains, as previously reported (Taddei *et al.*, 2005; Tasara *et al.*, 2005; Harris & Barletta, 2001; Cousins *et al.*, 1999; Naser *et al.*, 1999). This is especially true for an IS element in strain 2333, with 94% sequence similarity to IS*900* (Englund *et al.*, 2002). This mycobacterial strain, as well as the species that gave rise to the false positives in the references above that were identified, can be viewed in the evolutionary tree in Fig. 1 (*Mycobacterium porcinum, Mycobacterium scrofulaceum, Mycobacterium xenopi, Mycobacterium chelonae, Mycobacterium terrae*). The suggested close relation between IS*900* and the equivalent IS-element in these species highlights the transpositional activity of the gene. Notably for the study in paper III is the fact that *Mycobacterium porcinum* was found in milk.

Suggested measures to increase PCR specificity for IS*900* include the use of annealing temperatures higher than 60°C (Whittington, 2002). A temperature increase will, however, reduce false positives in an arbitrary manner and compromise the sensitivity. Hence, there is instead a need for confirmative methods for PCR positives. Sometimes, when PCR is performed in parallel with culture, growth characteristics (as described above) can confirm the PCR result, but when fast results are needed or when culture fails because of contamination or poor growth, molecular methods for confirmation should be used. One approach for this is to perform a confirmatory PCR test, based on another independent and at least equally specific gene.

1.6 Methods of DNA extraction

One of the challenges with molecular detection of MAP is to get the genomic DNA out of the bacteria, which is protected by its thick and waxy

cell wall. In this thesis, mechanical disruption by the use of bead beating is applied. Bead beating is a general term for using small beads mixed with the sample, usually in the presence of a proteolytic enzyme and lysis buffer, to break tissues or tough cell walls and spores by forceful shaking in a cell disrupter, or "bead beater". It is one of several suggested methods to lyse MAP (Lanigan *et al.*, 2004; Grant & Rowe, 2001; Odumeru *et al.*, 2001; Hermon-Taylor *et al.*, 2000; Hurley *et al.*, 1987). Others include combinations of enzymatic treatment, freeze-thaw/boiling and kits for plant DNA purification (Chui *et al.*, 2004; Zecconi *et al.*, 2002; Odumeru *et al.*, 2001) or even sole boiling (Pinedo *et al.*, 2008; Grant *et al.*, 2000).

After lysis, the bacterial DNA has to be extracted from the sample matrix and purified from lipids, proteins and PCR-inhibiting substances. This step is crucial for successful PCR and may involve solvent extraction with phenol and chloroform or separation by binding of DNA to a solid phase, such as magnetic beads or a spin-column, before washing away impurities and eluting the DNA with a buffer.

Prior to lysis, steps may also be taken to separate the bacteria from the bulk of the material, e.g. by centrifugation, sedimentation or immunomagnetic separation (IMS). IMS involves the capture of bacteria by magnetic beads coated with specific antibodies and separation from the sample material by magnets.

1.7 Molecular typing

The previously mentioned technique RFLP is widely used for molecular subtyping of MAP (Djønne *et al.*, 2005; Pavlik *et al.*, 2000; Greig *et al.*, 1999). Briefly, RFLP is performed by digestion of the chromosomal DNA with a specific restriction enzyme, separation of the resulting fragments by agarose gel electrophoresis, southern blotting and hybridization of a labelled probe to the membrane. The restriction enzymes are chosen to obtain a clear discrimination between subtypes of a species. Depending on the type of strain, visualization of the probe will yield different characteristic patterns of bands. Except for differentiating MAP strains into C and S strains, RFLP with either restriction endonuclease *Pst*I or *BstE*II divides the strains into two suggested standard sets of subtypes; [A-M] and [C1-C20, S1-S3, I1-I2], respectively (Pavlik *et al.*, 1999; Collins *et al.*, 1990). The latter set indicates type C or S, but also type I, which corresponds to an intermediate pattern.

Another technique that has been used for discriminative molecular typing of MAP is pulsed field gel electrophoresis (PFGE)(de Juan *et al.*, 2005) where the genome is digested to larger fragments than by RFLP. Larger

fragments than around 20 kb cannot normally be separated by gel electrophoresis, but by frequently alternating the angle of the field, maintaining a net forward movement of the fragments, the resolution is dramatically increased.

A simpler method for a more basic subtyping, PCR-restriction endonuclease analysis (PCR-REA), was previously applied to type isolates of MAP from various hosts and regions (Sevilla *et al.*, 2005; Motiwala *et al.*, 2004; Stevenson *et al.*, 2002; Whittington *et al.*, 2001; Whittington *et al.*, 2000) and was also used in the present study. It takes advantage of a single point mutation polymorphism in the multicopy insertion sequence IS*1311* (Whittington *et al.*, 1998). A specific part of the sequence is amplified by conventional PCR (system IS*1311*-2)(Marsh *et al.*, 1999) and the product is incubated with the endonuclease *Hinf*I. In sheep strains, the polymorphic base at position 223 is a C in all copies of IS*1311* and does not allow digestion by the enzyme, yielding a single band by agarose gel electrophoresis analysis. In cattle strains, the base T at position 223 in some copies of IS*1311* grants digestion and yields three sizes of digested and undigested fragments on the agarose gel, where the two largest are visible as bands. In a B strain, position 223 is a T in all copies of IS*1311* and complete digestion yields two fragments, one of which is visible as a band on the agarose gel. If primers for amplification of a larger sequence of IS*1311* are used (system IS*1311*-1), discrimination between MAP and MAA can also be made. The larger sequence includes one more *Hinf*I site and, only on IS*1311* in MAA, one *Mse*I site, resulting in a different pattern after digestion. A schematic diagram of the typical gel patterns with these two PCR-REA systems is shown in Fig. 4.

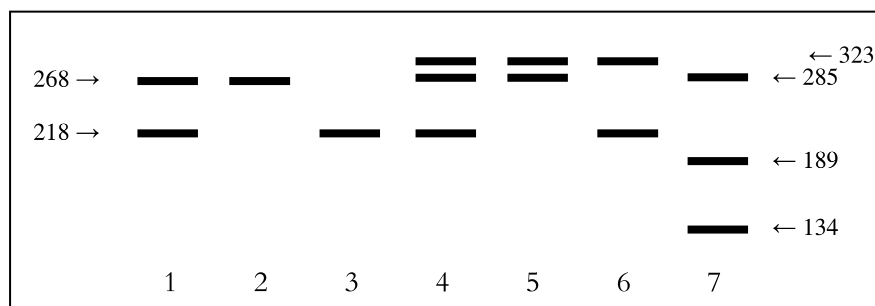

Figure 4. Schematic diagram of IS*1311* PCR-REA patterns yielded by digested PCR products visualized by agarose gel electrophoresis. Lane 1-3, digested products of system IS*1311*-2, indicating C, S and B strain. Lane 4-7, digested products of system IS*1311*-1, indicating C, S and B strain and MAA. The numbers by the arrows indicate the size of the fragments.

2 Aims and outline of the present study

All studies concerned molecular methods for detection and confirmation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in various types of clinical specimens, as well as in colonies of cultures. In order to assess the sensitivities in a fair and reproducible manner, effort was made to imitate clinical samples accurately by precise quantification of the bacteria used for spiking, and to identify associated problems. Each method should be as fast, economical, sensitive, specific and robust as possible. Specifically, the aims were:

- To develop a fast and sensitive protocol for detection of MAP in bovine semen.
- To develop a robust protocol for detection of MAP in bovine faeces and to validate the method on clinical samples.
- To develop a fast and sensitive protocol for detection of MAP in milk and to validate the method on milk samples from farm bulk tanks.
- To establish an accurate and reproducible method for assessment of the analytical sensitivity of molecular detection methods for MAP.
- To make critical evaluation of the analytical sensitivities of the developed methods.
- To design and validate alternative real-time PCR systems for confirmation of a positive PCR test for MAP.
- To use the alternative PCR systems to confirm the isolation of MAP in Chilean guanacos, and to perform PCR-REA typing of the isolates.

3 Methodological considerations

3.1 Real-time PCR

Conventional PCR with visualization of the product by agarose gel electrophoreses is probably the cheapest and most basic molecular diagnostic method, but it is not sensitive enough for samples containing very few infectious organisms. Post-PCR steps are both laborious and a source for cross contamination, which is the reason why electrophoresis should be performed in a separate laboratory. The same applies for nested PCR, but here, the risk of cross contamination is even higher, since any contamination occurring during the opening of the PCR-tubes after the first reaction will be amplified in the second reaction. In Sweden, where the prevalence of paratuberculosis is very low, a false positive caused by cross contamination from, for instance, a positive control, would have very drastic consequences.

With real-time PCR however, there is virtually never a need for opening of the tubes after amplification. The one step approach allows for the use of carry-over prevention strategies with Uracil N-glycosylase (UNG), which further diminishes the risk of cross contamination. In this strategy, the dNTP-mix used in the PCR contains the nucleotide base uracil, which replaces thymine in the extension process. With the addition of the UNG enzyme in the PCR mixture and a temperature hold at 50°C, all previous PCR amplicons containing uracil are digested. When the temperature program continues, only indigenous template without uracil remains.

The representation of the amplification process as curves is very helpful when analysing the results. PCR inhibition, laboratory mistakes or any other artefacts upstream may sometimes be identified or at least hinted by atypical curves. The possibility to quantify the starting material, relatively or absolutely (by the use of standard curves) allows for comparative studies and

optimisation. These features, together with the reported high sensitivity (Patel *et al.*, 2003; Fang *et al.*, 2002; Larsen *et al.*, 2002; O'Mahony & Hill, 2002; Greiner *et al.*, 2001; Jauregui *et al.*, 2001), made real-time PCR the preferred tool for detection.

Depending on the clinical material and the purification method, PCR inhibition may or may not be a problem. Even a method that usually performs well can occasionally fail to sufficiently purify a highly PCR inhibitory sample and can therefore lead to false negative results. Monitoring of PCR inhibition is therefore critical for direct PCR methods, and in our PCR setup it is done by the addition of an internal amplification control (IAC) to all samples. Our IAC is a custom made plasmid that contains binding sites for the primers of our primary IS*900* system MP, but with a unique probe with a different fluorophore that is measured by the instrument in a separate channel. When testing a sample for the presence of MAP, absence of signal from both IS*900* and the IAC indicates PCR inhibition. PCR on several parallel targets is known as multiplex PCR, and can also be used to target several different pathogens.

In early experiments and diagnostic testing, glycerol was included in the PCR mixture, advisable for freezing and storing of the mixture once or twice. While it initially seemed to have no negative effect on the PCR reaction, it was later shown to decrease the sensitivity of the PCR and was therefore omitted. It was not found out why the effect of glycerol seemed to have changed over time.

3.2 Specificity

Although PCR, with certain primers and probes for IS*900*, may cause false positive results with some strains, the insertion element as a whole is, to current knowledge, specific to MAP. Because of the high copy number of IS*900*, it is still of interest to design PCR systems for the gene. However, care should be taken to make systems as specific as possible, i.e. to avoid targeting conserved areas of the sequence. Therefore, as a framework for the design, IS*900* was aligned with five long and two short sequences from other bacteria, possessing high degrees of similarity to IS*900*. The long sequences were IS*1613* (GenBank accession no. AJ011837), IS*901*, IS*902* and ORF 1179 and 930 of IS*1626*, present among some *Mycobacterium avium* subsp. but not in MAP (Puyang *et al.*, 1999; Ahrens *et al.*, 1995; Moss *et al.*, 1992; Kunze *et al.*, 1991), and the equivalent to IS*900* in the mycobacterial strain 2333 (Englund *et al.*, 2002). The latter was virtually identical to IS*900* among the first 450 base pairs. The short sequences with

similarity to IS*900* were found in the genomes of *Streptomyces avermitilis* and *Rhodococcus erythropolis*. By studying this alignment, oligonucleotides could be chosen so that the new PCR systems would contain at least a few base pairs that differ in respect to all other known similar genes. A small part of the alignment is shown in the cover illustration of this thesis.

The gene F57 (Poupart *et al.*, 1993) is hitherto considered specific for MAP and has no known similarities with genes of other bacteria (Tasara & Stephan, 2005; Vansnick *et al.*, 2004; Coetsier *et al.*, 2000). To further decrease the risk of cross-reactions with insertion elements nearly identical to IS*900* and hypothetically residing in yet unknown mycobacteria, a real-time PCR system was designed for F57. However, because it is a single copy gene, a PCR system based on F57 is expected to be less sensitive than the IS*900* systems (albeit Möbius *et al.* (2008) reported the same sensitivity of PCR systems based on IS*900* and F57). Other considered MAP specific genes were *hspX* and IS*Mav2* (Strommenger *et al.*, 2001; Ellingson *et al.*, 1998), but because of promising reports on the specificity of F57 (mentioned above) and previous experience of F57 in this laboratory, it was chosen as target gene.

Multiplex PCR, mentioned in the previous section, can also be used to detect a pathogen by multiple gene targets simultaneously, as an alternative to subsequent confirmation of positive samples. It has been used for MAP detection in conventional PCR setups by Cousins *et al.* (1995) and Tasara *et al.* (2005) and has also been combined with real-time PCR (Schönenbrücher *et al.*, 2008; Slana *et al.*, 2008a). However, as multiplex PCR makes optimization complex and may cause lower sensitivity due to competition of reagents and increased primer dimer formation (Rachlin *et al.*, 2005), we chose to use one single target for MAP in each PCR system. Competition due to the duplex of IS*900* and the IAC is kept to a minimum by adding only low amounts of IAC copies.

3.3 Enumeration and sensitivity

When assessing and stating the sensitivity of agent detection methods, the conventionally used unit for bacterial quantification is CFU per weight or volume, often used in MAP studies (Bögli-Stuber *et al.*, 2005; Stabel & Bannantine, 2005; O'Mahony & Hill, 2004; Mason *et al.*, 2001; Odumeru *et al.*, 2001). However, for many reasons, this is not appropriate for stating the sensitivity of molecular detection methods. First of all, the number of CFU in a sample is virtually irrelevant, since PCR can detect bacterial DNA regardless of the state of viability of the bacteria. This should be considered

one of the strengths of molecular methods, since detection of dead bacteria is also suggestive of infection; especially since samples may be frozen without significant reduction of the sensitivity. Secondly, the colony count, when used to quantify the bacterial suspension for spiking, will be dependent on growth medium, state of viability of the organisms in the primary culture, proportion of aggregated cells and laboratory practice. Therefore, estimations of the number of CFU in suspensions of MAP will vary between laboratories, and more importantly, they are likely to be underestimated, leading to overestimation of the analytical sensitivity when validating molecular detection methods. As mentioned in Paper I, when MAP suspensions with microscopically determined concentrations were cultured for colony counting, the number of CFU would vary between 0.1% and 5% of the number of actual organisms. This shows how quantification of MAP by culture will give inconsistent, false and misleading results.

For a fair and accurate estimation of the analytical sensitivity of developed methods, the organisms should be separated from aggregates and counted visually or by some automatic cell counting device. Any other factors that may cause higher sensitivity on spiked samples than on clinical samples should be investigated and minimized. One such factor proved to be the presence of free DNA residing in cultured MAP colonies.

4 Results and discussion

In this section, results from various studies that contributed to reaching the declared aims are presented and discussed. When covered subjects were originally discussed in a paper, it is indicated in the heading by the relevant paper numbers.

In this thesis, the measure for amounts and concentrations of MAP refers to visually counted bacteria, viable or not, rather than CFU. The reason for this is discussed in the previous section "Enumeration and sensitivity".

4.1 Extraction

4.1.1 Boiling and free DNA

When confirming the identity of suspected growth of MAP on solid medium by PCR, boiling or heating (95˚C – 99˚C) of the suspended colony for 10 – 15 min is the only preparation necessary to obtain enough DNA for detection. Therefore, it was presumed that boiling caused a sufficient proportion of the bacteria to lyse, thereby allowing detection. In an attempt to optimise conditions for lysis, real-time PCR demonstrated little or no quantitative difference in DNA yield caused by difference in temperature (data not shown), leading to an increasing suspicion of the presence of free DNA in MAP colonies. To investigate this possibility, an experiment was performed to compare washed and untreated bacterial suspensions by incubation at both 99˚C and room temperature. Real-time PCR examination of the supernatants showed that washing drastically decreased the signal. Untreated suspensions, however, yielded strong signals, but incubation of the colonies at 99˚C for 15 min caused a four-fold decrease of detectable DNA (Fig. 5). The strong signal of untreated bacteria and the evident reduction of DNA after washing means that high amounts of detectable DNA were already present outside the bacterial cells before

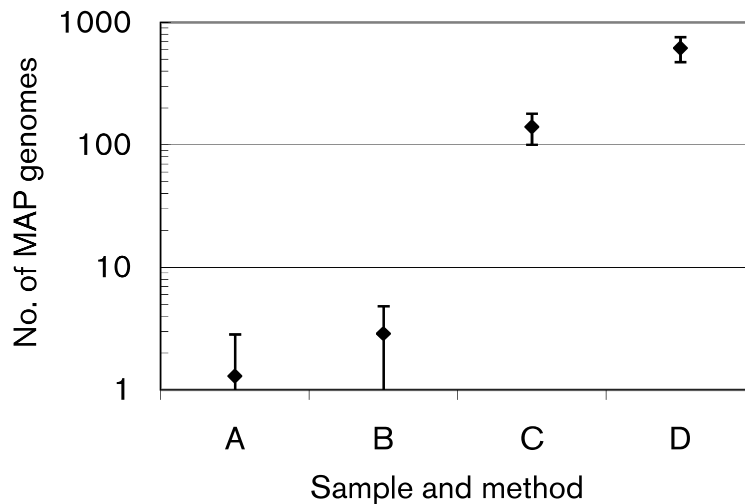

Figure 5. Quantification of detectable MAP genomes by IS*900* real-time PCR: Indication of the presence of free DNA in suspended MAP colonies and demonstration of the detrimental effect heat treatment has on DNA. A) Washed MAP incubated at 99°C, B) Washed MAP at room temperature, C) Untreated MAP incubated at 99°C, D) Untreated MAP at room temperature.

incubation at 99°C, thus referred to as free DNA. Heat treatment of the bacteria destroyed detectable DNA more than it increased the amount by lysis.

This observation suggests that boiling of a suspension of cultured MAP should merely be thought of as a laboratory safety precaution. With reports of unsatisfactory pasteurisation procedures in mind (Grant *et al.*, 2005; Grant *et al.*, 2002b; Chiodini & Hermon-Taylor, 1993), culture of MAP, previously incubated for 5 min at 80°C and 99°C, was performed and showed that 80°C incubation was sufficient to kill all bacteria.

The discovery of free DNA present in MAP colonies explained why boiling previously appeared to be a sufficient lysis method for MAP suspensions, but not for clinical samples. During its slow colony formation, dying MAP appears to spontaneously lyse and release its nucleic acids into the colony. Hypothetically, this could be a natural part of the process of all proliferating MAP, perhaps as a means of releasing substances necessary to stifle the immune response of the host, while it also contributes to the slow development of disease and the slow growth of the bacteria. Alternatively, this could be a phenomena appearing only in culture tubes, perhaps because of the suboptimal milieu that the media constitutes. Either way, once

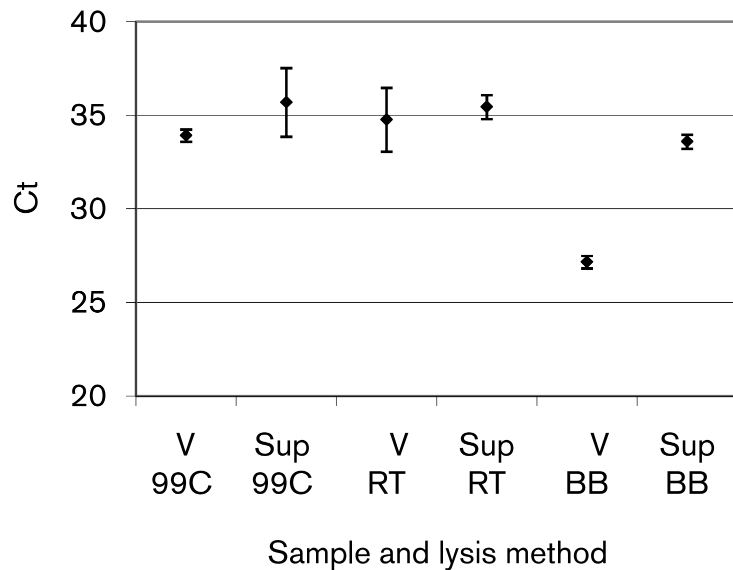

Figure 6. Comparison of bead beating and heat treatment of washed MAP. V indicates that a vortexed homogeneous bacterial suspension was used, while Sup designates cell-free supernatant. RT, 99°C and BB designate incubation at room temperature, 99°C, and bead beating, respectively.

released, exposed DNA is subject to degradation and cannot be expected to remain in high amounts in clinical samples, such as faeces, milk or semen. This finding affects the way MAP should be prepared for spiking experiments, discussed in the section "Assessment of sensitivity", but more importantly, it stresses that MAP must be successfully lysed for detection of its DNA. Probably, free DNA is also present to some extent in liquid cultures, suggested by the fact that Sweeney *et al.* (2006) detected MAP in liquid cultures by direct transfer of culture medium to the PCR tube.

4.1.2 The importance of bead beating

Further experiments with MAP suspensions, washed to reduce the amount of free DNA in suspension, clearly showed that bead beating was necessary to achieve efficient cell lysis (Fig. 6). Without bead beating, vortexed homogeneous suspensions yielded only slightly lower Ct values than did the cell-free supernatant (lower Ct indicates a higher amount of target DNA; up to a two-fold difference per ΔCt). This indicates that the bulk of the nucleic acids in the washed colony suspensions did not come from the cells, but was still present as free DNA (as shown in Paper I, free DNA can only be

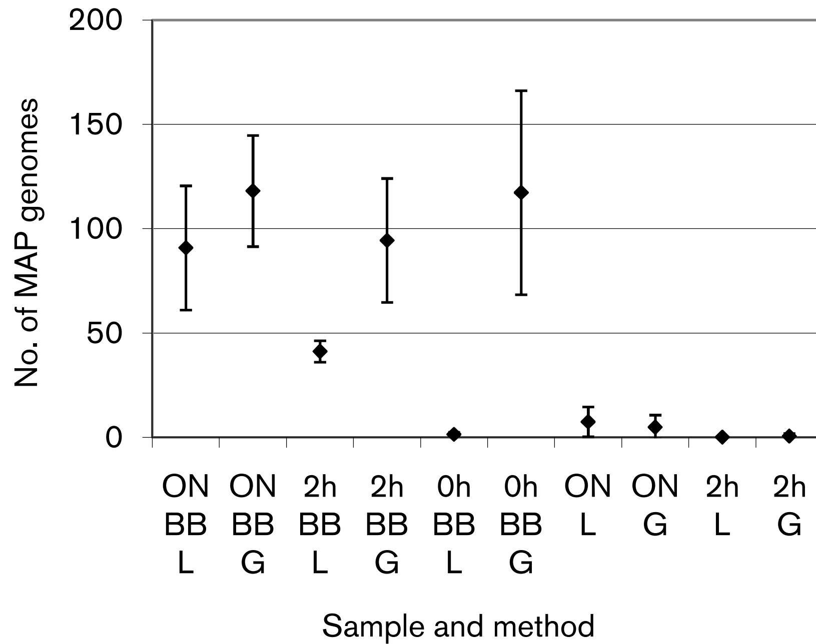

Figure 7. Investigation of factors critical for lysis. The incubation time is indicated by ON (overnight), 2h or 0h. BB indicates that bead beating was performed. L and G indicate default lysis buffer and buffer containing guanidinium thiocyanate, respectively.

reduced by the current method, not completely removed). Incubation of the suspension at 99°C caused an insignificantly lower Ct (P=0.3), which confirms that heat is inefficient for lysis. Would ever a significantly lower Ct be observed by using heat treatment, it could be due to resuspension of free DNA that adhered to the cell surface. When a suspension was treated by bead beating and phenol/chloroform extraction, the Ct was clearly lower than for any other sample, indicating a hundred-fold increase in DNA yield, when compared to the samples treated with high temperature incubation.

The results of several other experiments (data not shown) supported the importance of bead beating for lysis, while others, sometimes when investigating other parameters, inconsistently indicated that bead beating did not cause clear differences in DNA yield. Therefore, an experiment to roughly evaluate the importance of lysis buffer formula, buffer incubation time and bead beating was performed. From the result, bead beating proved to be the most important factor for successful extraction (Fig. 7). Inconsistencies in other experiments may depend on the age of cultures, freezing of samples or something else that can make the bacteria easier to

lyse, or maybe even release free DNA prior to the experiment. Generally, however, MAP should be considered difficult to disrupt and protocols with bead beating should therefore be used. As evident from the above experiment, the lysis buffer containing guanidinium thiocyanate was the most efficient and required a minimal incubation time. It was however not used in subsequent experiments, as it is highly toxic.

4.1.3 Phenol/chloroform extraction on disrupted semen (Paper I)

After bead beating has been performed, it is necessary to remove proteins, lipids and PCR inhibiting substances from the sample. As shown in paper I, a two-step phenol/chloroform extraction with isopropanol precipitation of DNA proved sufficient for removing inhibitors in bovine semen samples while retaining a high sensitivity; 10 organisms per 100 μl sample. The efficiency of the purification was shown to be nearly independent of the degree of extension (dilution) of the semen; only raw semen caused notably higher Ct values.

It should be considered, that it is the analytical sensitivity that was assessed, not the diagnostic sensitivity. For determination of the latter, several semen samples from bulls known to shed MAP in their semen are needed, and such samples are very difficult to obtain. The prevalence and amount of MAP in bovine semen is unknown, as is the natural distribution of the organisms in the semen. Depending on whether the bacteria were shed from within the genital organs, or whether it was faecally contaminated, the organisms may be distributed more or less heterogeneously, and may reside within macrophages or freely, in the seminal fluid. Although it can be assumed that bead beating will release any MAP DNA present in a sample, regardless of whether MAP was intracellular or not, it is still unknown whether a 100 μl sample from an infected bull will contain any MAP. Therefore, this test is not intended for diagnosis of the disease, but for reduction of the risk of MAP transmission, by ascertaining a sample free from MAP (i.e. containing less than 10 MAP), thus deeming it likely that the rest of the batch will also be free. The diagnostic sensitivity will also depend on the degree of extension of the raw semen. The less it is extended, the more sensitive the test will be, as long as the semen is extended at least to 1:2, as shown in paper I.

In contrast to these results, when bead beating and phenol/chloroform extraction were performed on faeces, PCR inhibitors were usually still present in the samples.

4.1.4 Immunomagnetic separation (Paper I)

Initially, it was assumed that bovine semen would be a very troublesome material, because of the high protein content in semen and the extender used to dilute semen for breeding purposes. A protocol including IMS was tried on spiked semen samples. It has previously been shown to successfully concentrate MAP and separate it from milk with antibody-coated magnetic beads (Khare *et al.*, 2004; Grant *et al.*, 1998). This did however yield an unexpectedly low sensitivity of 10^6 organisms per 100 µl sample, and when it was demonstrated that immunomagnetic beads *not* coated with a MAP-specific secondary antibody yielded similar results as the coated beads, the laborious IMS-step was removed from the protocol. Donaghy *et al.* (2008) observed the same, but yet chose to use magnetic bead separation, employing the unspecific binding capacity of the beads. Considering the fact that MAP usually resides intracellularly, problems with antibody-assisted capture of MAP ought not to be surprising.

4.1.5 PCR on short-term faecal cultures

Our first attempts to develop a diagnostic method for faeces that was faster than standard culture was based on the idea that growth of MAP on solid media will be detectable by PCR long before visible colonies appear. An in-house method was adopted and modified to rinse faecal culture slopes, wash the resuspended material from inhibitors and then boil it to effect lysis of the cells. This was performed before it was demonstrated that boiling has an insignificant lysing effect on MAP, but due to the spontaneous release of nucleic acids that appears to occur in cultures, some interesting results were obtained. To determine an appropriate culture period before harvesting the bacteria, growth was monitored by culture of faecal samples spiked with ten-fold dilutions of MAP and rinsing of the tubes at certain time points. The results of one such experiment is shown in Fig. 8. The quantification of MAP carried out used only a limited set of positive controls as reference in the PCR and should therefore not be considered absolute; rather as a unit for relative comparison of the respective MAP yields. Evident growth can be observed in the faeces with the highest spiking level (D1 and D2) while MAP in the lower levels appear to linger in a long latency phase.

Some attempts with bead beating and phenol/chloroform-extraction were later made on the resuspended culture material and as it was soon discovered that this yielded positive results even on rinsed material from time point zero, the protocol eventually evolved into the direct PCR method that is described in the next section.

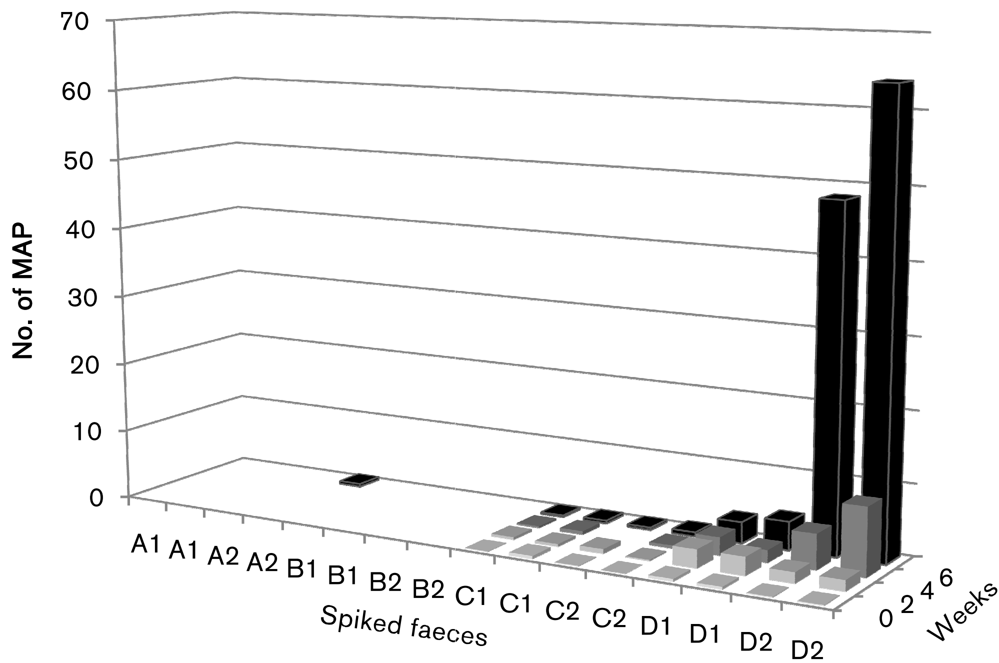

Figure 8. IS*900* real-time PCR on rinsed cultures of spiked faeces of different spiking levels (A-D) and harvested at different time points (0-6 weeks)

4.1.6 Direct PCR on faeces (Paper II, V)

Analytical sensitivity

For extraction of MAP DNA from faecal samples, separation of the bacteria from solid material was followed by bead beating and purification by the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with a slightly modified protocol to enhance MAP lysis and to reduce the risk of cross contamination. Limited tests indicated that the method also appeared to work for ileum tissue samples. An analytical sensitivity of approximately 10^4 organisms (corresponding to less than 500 CFU) per gram faeces was obtained, and inhibitory substances could be removed from most samples without needing to dilute the DNA. Weak signals (i.e. with Ct values above the cut-off; ~ 40 cycles) were yielded from samples spiked with 10^3 organisms per gram.

The method appeared to be at least as sensitive as culture when a limited set of spiked faecal samples were used. From 10^4 MAP, only about 10^2 organisms are viable and recoverable and as previously reported, faeces must contain at least 10^2 CFU for detection by culture (Jørgensen, 1982; Merkal, 1970), due to the decontamination steps. The sensitivity of culture was therefore not expected to be higher than that of direct PCR. Culture yielded colonies from faeces spiked with 10^6 MAP/g or more, either suggesting that the direct PCR method is highly superior to culture or that there were experimental errors in this comparison. Possible explanations are discussed in the section "Assessment of sensitivity". Either way, it would be both interesting and necessary to repeat the experiment before any conclusions can be drawn.

Clinical samples

Parallel testing on both culture and direct PCR was mainly performed on previously frozen samples. Based on 202 samples from several proficiency tests, the direct PCR method appeared to be more sensitive than culture. Of 117 valid and positive proficiency test samples, 97% were positive with this method, while all of the 64 negative samples were negative. Of the 21 low abundance samples that were omitted from the test because less than 70% of the participants could detect MAP in them, 15 were positive with our method. By culture, 86% of the valid samples and four of the omitted samples were positive. The observed lower sensitivity of culture may at least partly depend on the freezing of the proficiency test samples, known to be detrimental to the viability of MAP (Khare *et al.*, 2008; Richards & Thoen, 1977). In addition to proficiency test samples, 33 Swedish fresh faecal and ileum tissue samples were tested. Two tissue samples and one faecal sample were positive by both PCR and culture, while the rest were negative by both methods. For a better comparison of the methods, further investigation by parallel testing on fresh samples should be performed.

Streamlining

Since Qiagen's stool kit was not directly used on faeces with this method, but rather on the liquid lysate resulting from bead beating, it was not clear whether the InhibitEX tablet contained in the kit was necessary for removal of inhibitors. An attempt was made to replace the laborious manual processing of the kit with automated processing by the Biorobot EZ1 (Qiagen), based on an approach with magnetic beads. It was clearly demonstrated that PCR inhibitors remained in the sample, the effect of which diminished only after a 10-fold dilution of the template, resulting in a

decreased sensitivity. A larger elution volume would probably decrease the inhibition to some extent. Fortunately, the promising alternative of Qiagen's QIAcube, automating the applied stool kit, offers a possible solution for future needs.

Some minor experiments, briefly described in paper IV, highlighted the following details about the method. MAP was shown to be evenly distributed in the supernatant after the initial sedimentation, which makes the method independent of where the volume is taken out for further processing and allows for parallel testing of more of the supernatant for increased sensitivity. The 95°C step after the addition of sample to the ASL-buffer was neither necessary for proper lysis nor measurably harmful for the DNA. For the time being, the step was therefore kept, but if these results can be confirmed on clinical samples, it could probably be completely removed.

4.1.7 Extraction of MAP DNA from milk (Paper III)

Initially, the protocol used by Corti and Stephan (2002), including a three-step phenol/chloroform extraction, was tested. However, PCR-inhibitors were often still present in the DNA extracted with the method. Probably, Ca^{2+} ions, reported to inhibit PCR by competition with Mg^{2+} ions (Bickley *et al.*, 1996), stayed in the water phase throughout the process. To remedy this, the milk pellet resulting from initial centrifugation was washed to reduce Ca^{2+} content. However, the complete protocol was quite laborious at this point. Since the addition of a pooling step of cream and pellet was to be investigated, a simpler purification approach suitable for high fat content was needed. Attempts with the Biorobot EZ1 proved successful.

Analytical sensitivity

As both pellet and cream of centrifuged raw tank milk may contain MAP (Gao *et al.*, 2005; Millar *et al.*, 1996), those fractions were pooled and processed together after discarding the whey fraction. It was experimentally confirmed that this approach yielded more MAP DNA; approximately four times more from spiked milk (two ΔCt). An analytical sensitivity of 100 organisms (corresponding to less than 1-10 CFU) per ml was obtained for samples of 10 ml milk, although MAP could also be detected in three of four samples spiked with 10 MAP/ml.

Clinical samples

This direct PCR method was applied to 143 Danish tank milk samples from 56 herds. A simultaneous screening of the herds by culture of faeces from the stable environment was performed, and 38 herds proved positive for

MAP in at least one of 6-18 cultures. MAP was found by direct PCR in the milk of 16 of those herds (42%), and also in one culture negative herd. The amount of MAP in all individual positive samples (n=19) was quantified by reference to spiked samples, and it was found that the concentrations of MAP were usually less than 100 MAP/ml and often below 50 MAP/ml. Considering that this is equal to or below the assessed sensitivity of the method, it is obvious that there is a high risk of false negative results if applied on similar milk. Because of the low concentration of MAP, there were great problems with confirming the positive results by testing of backup samples and by our alternative PCR systems, described in paper II. Only 6 of 19 positive samples could be confirmed by both alternative PCR and testing of a backup sample. The routine for confirmation is discussed in the section "Validation of specificity"

We expected that the tank milk would contain more MAP, either due to heavier contamination or shedding of MAP, but apparently, the milk from most participating farms with paratuberculosis was too diluted by MAP-free milk for MAP to be readily detected. Alternatively, good hygienic measures were effective in avoiding most contaminations and/or the cows did not shed MAP in milk to a high extent.

Optimization experiments not discussed in Paper III

At the end of the validation testing period and for the assessment of analytical sensitivity, 5 µl template DNA was used in the real-time PCR instead of the otherwise used 2 µl. Prior to the change of template volumes, a multifactorial experiment was performed to study the effect of elution volume and template volume on two levels of spiked milk. It was hypothesized that a greater elution volume would yield a greater total amount of DNA, due to a more thorough elution (which in retrospect, while being true for spin columns, might not necessarily be true for magnetic beads), although the dilution would decrease the DNA concentration. Increasing both the elution volume and the template volume could therefore be a way to slightly increase the sensitivity without increasing the level of PCR inhibition. The results showed that increased template volume yielded more DNA (-1.2 ΔCt for both the elution volumes 50 µl and 100 µl [P=0.13 and 0.03, respectively] when milk with 100 MAP/ml was used, while the percentage of positives increased on milk with 10 MAP/ml) and that a maintained elution volume of 50 µl did not cause problems with inhibition.

An attempt to get rid of the bulk of the bovine DNA present in the white blood cells of milk was made. The fact that separation of nucleic acids was done by magnetic particles suggested that there may be a limited surface on the particles, a level of saturation, which could hamper sensitivity due to competition between MAP DNA and bovine DNA for that surface. As a

first step to reduce the amount of bovine DNA, the lysis efficacy of a nucleic lysis buffer on white blood cells was tested and approved by relative quantification using primers for cattle DNA and SYBR Green real-time PCR. MAP yields were compared after different treatments of the milk pellet. As shown in Table 1, although the bovine DNA was washed away (to different degrees) before lysing MAP in the processing of the remaining sample, the Ct-values yielded by the treated samples did not differ significantly to the untreated samples. In all cases, the average Ct was higher for the washed samples, indicating a loss of MAP in the process. It was concluded that the surface of the magnetic particle used in the kit was not a limiting factor.

Before choosing to pool the pellet and cream fractions, another method was considered for harvesting the MAP potentially partitioned into the cream. By heating the milk to 44°C and centrifuging at 40°C, the cream remained liquid and to a higher extent, emulsified with the whey, which would theoretically allow for more of the bacteria to pellet. Results from limited tests were promising, but the method was deemed too laborious and unpractical and therefore rejected in favour of pooling. Reposing on a similar theory as the above, other authors heated milk to make MAP leave the cream fraction, but extracted the DNA from cooled samples (Gao *et al.*, 2007).

Table 1. *The effect of reduction of bovine DNA in milk on MAP yields, analysed by IS900 real-time PCR and represented by Ct-values and statistical significance.*

Wash/Lysis	Average Ct	σ	P*
A (control)	32.2	0.55	-
B (water)	33.4	0.77	0.14
C (lysis buffer 1:10)	32.9	0.88	0.34
D (lysis buffer)	33.0	0.59	0.14

* T-test of four replicates of each group with four replicates of control group A.

4.2 Assessment of sensitivity (Paper I)

When comparing measured cell densities in MAP suspensions, obtained by manual counting in Bürker chamber and by counting of CFU on cultures, the microscopically determined number of organisms per volume was approximately 100 times greater than the number of CFU per volume. As previously mentioned, this may have several causes; low viability, sub-optimal culture methods and aggregation of the bacteria. When CFU is used to express the sensitivity of diagnostic methods, the actual number of

bacterial cells in spiked samples is underestimated, and thus, the sensitivity is overestimated. In addition, the presence of free DNA in suspensions of MAP colonies (and probably also in liquid cultures) results in further overestimation of the sensitivity by allowing detection of DNA in spiked samples without proper lysis of the cells. Thus, a completely inefficient lysis method may be deemed efficient and put in use, if evaluated on samples containing high amounts of free DNA. Three measures were taken to overcome these identified problems with sensitivity assessment:

- A suspension virtually free of aggregated bacteria was obtained by weak and short centrifugation of a rich suspension of MAP colonies followed by transfer of most of the supernatant, now containing almost only dispersed bacteria, to a new tube. This method was fast, easy and gentle to the bacteria.
- The suspension was then washed three times in sterile water to reduce the amount of free DNA to approximately 0.3 ‰ of the original amount, as estimated by relative quantification during optimisation (paper I).
- The organisms were visually quantified by microscopic counting. Although this included all MAP in the enumeration, independent of their viability, it also involved some sources of errors. Deformation of the thin cover glass, caused by capillary forces, sample drought and the resulting migration of bacteria could all lead to errors in estimations of cell concentration. Presumably, however, not greater than approximately 10 to 20%.

4.2.1 Is this the ultimate solution?

Although the above concept appears to work well for making correct and reproducible assessments of the analytical sensitivity of molecular diagnostic methods, some concerns were raised regarding the possible effect separation and washing of the bacteria could have on the culturability of MAP, which needs to be normal and typical when the sensitivity is compared to that of culture. As previously mentioned, this method of cell separation should be very gentle on the cells, but some questions linger. Rather than separating aggregated bacteria from each other, centrifugation separates those that are aggregated from those that are not. Do the latter bacteria have some properties that distinguish them from the other, and in particular, in regard of culturability? MAP is notorious for its tendency to aggregate due to the hydrophobicity of its cell wall. It has been suggested that this is to become more resistant to stress (Grant *et al.*, 1996), but theoretically it is also to

facilitate growth. It is possible that the bacteria that were not aggregated, chosen by separation, lack an extracellular milieu that allows them to be cultured. Aggregation might also make MAP more resistant to chemical stress (Rowe & Grant, 2006), such as decontaminators. There were some indications of this kind of specific decrease of culturability when comparing the analytical sensitivities of the direct PCR on faeces with culture, since culture was less sensitive than expected.

The possibility that the dispersed state of MAP in a prepared suspension is the reason for the observed low rate of culturability creates a disappointing image of incomparability of molecular and bacteriological detection methods for MAP. Quantification of MAP by culture is a bad and uninformative standard for reasons stated in the previous section, giving unreliable data about the actual number of organisms, while separation of MAP for direct visual quantification could instead cause culture to perform worse than normal. Maybe, this is part of the problem, but certainly not the whole truth, since it is a previously established fact that MAP is fastidious and hard to culture (Lilenbaum *et al.*, 2007). One laborious way to get around this potential problem and thereby investigating its validity would be to use extracted DNA from a MAP suspension prepared as described above as a reference in an absolute real-time PCR quantification of extracted DNA from unseparated (but washed) MAP. This suspension would contain aggregates, and may therefore be easier to culture, and yet contain a well defined number of cells.

4.2.2 What is a high sensitivity?

Needless to say, a method with a high sensitivity needs only a low number of targets in the sample to be analyzed. But what is a low number? In this thesis, that was determined by consideration of different factors for each of the sample matrixes. The analytical sensitivity of our direct PCR on faeces (10^4 MAP/g) was theoretically similar to culture, the gold standard. This follows from that approximately one CFU per culture is expected when 10^4 MAP from one gram faeces is multiplied with the factors for viability rate and survival rate after decontamination. The method performed well in proficiency tests, even on the samples with the lowest level of infection. During the extraction process of a highly PCR inhibitory material, some loss of material is expected. The theoretical loss in our method was estimated and considered acceptable.

With semen and milk, it was a little harder to deem the methods sensitive or not. Very little is known about the abundance of MAP in semen of an infected bull, and as for tank milk, there is no lower limit for how

diluted MAP can be. In addition, several stochastic factors determine to which degree milk is contaminated by MAP. So, the question remains: are the methods sensitive enough for their purposes? For semen, it was established that, of the DNA from 10 MAP in a sample, a maximum of 0.4 genomes would be transferred to a PCR tube (10 MAP ÷ 50 μl × 2 μl). That corresponds to less than seven copies of IS*900*; hence, the method cannot be expected to be more sensitive. The sample would have to contain extremely minute amounts of MAP for the method to yield false negatives.

The milk from the Danish herds proved to contain MAP in very low concentrations, and for herd screening, the diagnostic sensitivity would be quite low. However, if the primary purpose of the method is defined as the control of consumers' milk and to avoid human exposure to MAP via milk products, it should be sensitive enough. Viability of MAP in milk will be reduced at least 10^4-fold (Rademaker *et al.*, 2007) by pasteurization which would leave no likely survivors in milk that yields negative results for MAP with the present method.

4.3 Validation of specificity (Paper II)

4.3.1 Testing on different strains and isolates

The three newly designed real-time PCR systems, described in paper II, were tested on various bacterial strains, most of them mycobacterial and often of unknown species, isolated during routine diagnostic testing. MAP strains were of different origin countries and source animals – primarily from cattle, but also from goat, sheep, deer and human. All tested strains, 112 strains of MAP and 155 other strains, are listed in Table 1 and 2 in paper II.

Complete specificity regarding MAP for the F57 system DH3 was demonstrated. The systems DH1 and DH2 targeting IS*900*, however, yielded weak false positives on a MAA isolate and a *Mycobacterium kubicae*-related isolate (Floyd *et al.*, 2000), respectively. Because the signals were very weak for being produced by a resuspended colony, they were probably caused by unspecific reactions rather than reactions on sites identical to the target in MAP. Therefore, if the F57 system is always used as the main confirmatory PCR for IS*900* positives and the DH1 and DH2 only as secondary alternatives when better sensitivity is needed, these false positives are highly unlikely to be manifested. However, as discussed in paper II, mycobacterial strains causing false positives in any of the new systems may appear at any time in the future, since most naturally occurring bacterial strains are not genetically investigated. As positive clinical cases of

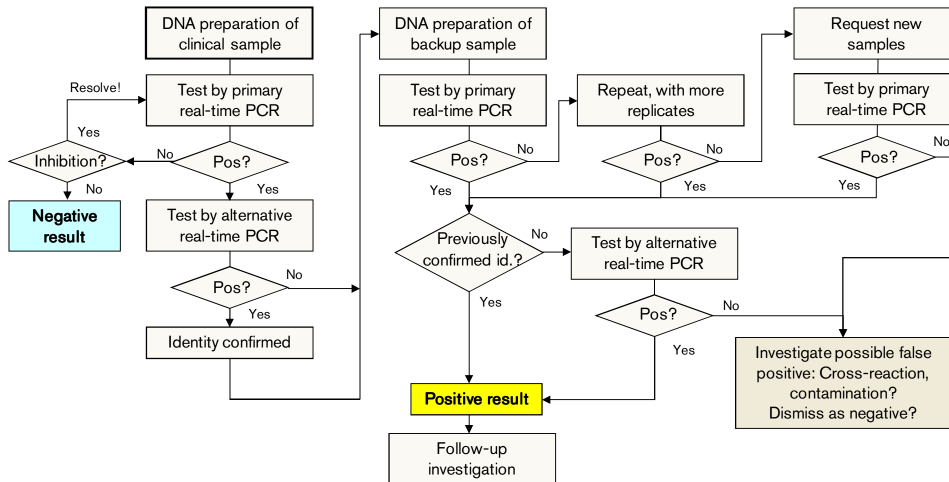

Figure 9. Scheme over the suggested test routine for confirmation of clinical samples that are tested for MAP by real-time PCR. Samples negative by PCR, which are not PCR inhibitory, are considered negative. Samples positive by PCR and confirmed both by identity and on a backup sample are considered true positives.

paratuberculosis are rare in Sweden, all available PCR-systems will probably be used for confirmation in the case of a positive PCR result.

Mycobacteria other than MAP were chosen from both pathogenic and non-pathogenic strains, from slow growers (colonies require more than seven days to be visible) as well as rapid growers. While they make up a relevant assortment of mycobacteria, the quality of the validation would probably have gained from testing of additional strains. Thus, the validation may yet be enhanced by future occasional testing of other mycobacteria. The few tested non-mycobacterial species were chosen because of their natural occurrence in clinical samples from cattle.

4.3.2 Routine for confirmation

A clear outline for the steps to be taken in the routine confirmation of positive clinical samples was established, and schematically described in Fig. 9. It was deemed important that both the true identity of MAP was confirmed, by testing with an alternative PCR system (preferably DH3 on F57), and that the true source of the detected MAP was proven to be the sample and not a laboratory contamination, by testing an identical backup sample. If problems occurred with the identity confirmation due to a low concentration of MAP, confirmation by alternative PCR systems could also be performed later on the backup sample, which may be an important

possibility when the DNA yield is low but the template volume large, as in the case of milk.

4.3.3 Testing on clinical samples (Paper II, III, V)

The systems were validated by direct PCR on clinical bovine faeces with concealed status from USA, Denmark and the Czech Republic, allowing for concurrent comparison of the sensitivity of the systems on clinical material. DNA from the 58 faecal samples that were previously positive by the primary PCR system MP was analysed by the new systems. The alternative IS*900* systems confirmed all positive samples, while the F57 system DH3 required additional replicates for 11 of the samples for successful confirmation, 5 of which were culture negative. One sample, also culture negative, could not be confirmed by DH3, but was judged positive by the IS*900* systems alone. Such cases have to be treated separately and in routine testing, other aspects have to be taken into account (such as animal status) when judging the sample positive or negative. In this case, the sample was obtained from an inter-laboratory proficiency test, and was announced a low-level positive.

The routine for confirmation described above was applied to Danish milk samples and the results are given in paper III and in the section "Extraction of MAP from milk". Because of the low concentration of MAP in milk, it proved difficult to confirm both the identity and the source of the positive samples.

4.3.4 Sensitivity test of the PCR systems

The anticipated lesser sensitivity of the F57 system was confirmed by testing on MAP DNA, serially diluted to half-multiples of ten genomes per ml in the most relevant range of concentrations. The primary IS*900* system MP, the alternative IS*900* systems DH1 and DH2, and the F57 system DH3, could detect 0.1, 0.1, 0.3 and 1 MAP genomes/µl, respectively. The former numbers are less than one, due to the multiple copies of IS*900* in the MAP genome.

4.4 Isolation and typing of MAP in guanacos (Paper IV)

Culture of faecal samples from 501 wild guanacos, inhabiting the Tierra del Fuego Island in Chile, yielded MAP colonies from 21 guanacos (4.2%). This was the first isolation of MAP from free-ranging wild animals in the Chilean Patagonia and the first isolation of MAP from guanaco. Hence, guanaco is proposed to be added to the list of animals that can be infected by

paratuberculosis. No histopathological examination could be performed, but although isolation alone does not prove infection, it is suggestive and should be suspected. The identity of all isolates was confirmed; the three first by real-time PCR on F57 and IS*900* and the rest by conventional IS*900* PCR. Typing was performed by IS*1311* PCR-REA. All strains proved to be of C type, although the wild guanacos often shared grazing land with domestic sheep. There were a few so called hot spots in forested areas, where 85% of the guanacos shed MAP and where thousands of sheep gathered annually. However, MAP infection in domestic livestock has not been reported in the surveyed region.

Transmission from sheep, infected with MAP of C type, would still be one possible explanation for the prevalence of MAP among the wild guanacos that may now be acting as a reservoir of the pathogen. To a lesser extent than sheep, domestic cattle also share grazing land with the wild guanacos, perhaps being the original source of the bovine MAP in the area. The absence of MAP of S type was possibly due to the used culture medium HEYM, which appears to be most suitable for bovine MAP strains, while Löwenstein-Jensen medium is a more favourable medium for ovine strains (Juste *et al.*, 1991). If both media would have been used in parallel and MAP of S strain indeed exists in the guanaco population, a higher incidence of MAP would probably have been found.

5 Materials and Methods

In this section, experiments that are not described in the papers but discussed in the previous sections are described. Refer to the corresponding sections in paper I-V for details about other performed experiments.

5.1 Experiments investigating lysis and free DNA

5.1.1 Indication of free DNA in suspended MAP colonies

A suspension of 10^4 MAP per ml was washed twice by centrifugation at 10 000 *g* for 5 min, discarding of the supernatant and resuspension in the previous volume of sterile water. Two samples of 100 µl washed suspension and two samples of 100 µl untreated bacterial suspension were subjected to incubation at 99°C for 15 min. For comparison, two samples of 100 µl from both washed and untreated suspensions were incubated at room temperature. All tubes were centrifuged at 10 000 *g* for 1 min and duplicates of 2 µl supernatant from each sample (in total, four replicates per set of parameters) were analysed by real-time PCR using the IS*900* MP system.

5.1.2 Comparison of bead beating and heat treatment

A suspension of 10^5 MAP per ml was washed as described above. To obtain a cell-free supernatant, two centrifuge tubes of 1 ml washed MAP suspension were centrifuged at 13 000 *g* for 10 min and 500 µl supernatant from each tube was pooled in a new centrifuge tube. From both the bacterial suspension of washed MAP and from the supernatant, three 100 µl samples were taken for incubation at 99°C for 15 min, three 100 µl samples as untreated controls and three 50 µl samples for treatment by bead beating. To the latter samples was added 50 µl of bovine extended semen as carrier DNA, stabilising the DNA pellet in the subsequent precipitation step. Incubation in lysis buffer, bead beating and extraction were performed as

described in paper I. Duplicates of 2 μl template DNA from all samples, resulting in six replicates per set of parameters, were used in real-time PCR, as described in paper I.

5.1.3 Multi-parameter investigation of factors critical for lysis

Samples of bovine extended semen were spiked with 100 washed MAP, as described in paper I, and frozen before use. One group of samples was subjected to bead beating, while one group, only to the requisite phenol/chloroform extraction. In each group there were two different lysis buffers used. Half of the samples were incubated in the lysis buffer described in paper I, and the other half, in a lysis buffer containing guanidinium thiocyanate (Odumeru *et al.*, 2001). In each subgroup, two samples were incubated in the lysis buffer for 2 h at 37°C and two samples overnight at 37°C. In the bead beating subgroups, two additional samples were incubated for only a few minutes at room temperature, while being transported to the bead beating machine. Duplicates of 2 μl template (in total, four replicates per set of parameters) were used in real-time PCR, as described in paper I.

5.2 Faecal culture

The standard method for faecal culture in this laboratory is based on decontamination according to Beerwerth (1967) and the use of Löwenstein-Jensen medium, as described by Jørgensen (1982). However, some modifications of this method have been made. The volume of NaOH used was decreased from 50 ml to 8 ml. The centrifugal force was specified to 2200 *g*. Two Löwnstein-Jensen tubes were replaced by two HEYM tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Incubation of the cultures was done for one to four months and the first one to two months in 5% CO_2.

5.3 Fast culture-PCR on faeces

A bacterial suspension of MAP was prepared as described in Paper I and used to spike pooled faeces from eight paratuberculosis-free cows to four levels of MAP concentration. In one of the experiments, these levels were calculated so that each culture tube would grow approximately 0.1 – 100 CFU (designated A – D). Since the average viability of MAP cells harvested from a colony is approximately 1% and the survival rate after decontamination is also around 1%, the tubes would have to contain 10^4 times more counted MAP than the number of expected CFU, i.e. $10^3 – 10^6$

organisms. The spiked faeces was decontaminated and cultured as described above, except that only Löwenstein-Jensen tubes (in-house) were used. After one day, two, four and six weeks, MAP was harvested from the culture slopes by rinsing the surface of the culture medium with phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+}. In each tube, a 10 µl plastic loop was used to rub the surface and to suspend all solid faecal material with the liquid. The bulk of the solid material was allowed to sediment for 3 – 5 min at room temperature. From the supernatant of each tube, 500 µl was transferred to a centrifuge tube and centrifuged for 10 min at 3000 g. The new supernatant was discarded and the pellet resuspended in 500 µl water (Sigma-Aldrich, St. Louis, MO, USA). One part (300 µl) was frozen and stored, while 200 µl was boiled for 15 min. The tubes were vortexed and centrifuged for 1 min at 10 000 g. From this supernatant, 2 µl was used as template in a real-time PCR, in the same way as described in Paper I.

5.4 Direct PCR on faeces

The protocol for DNA extraction from faeces is fully described in Paper V, but some details about related experiments are described here. When the analytical sensitivity was compared to that of culture, spiked faeces of each spiking level was cultured as described above, but with one extra HEYM slope.

5.4.1 Comparison of Qiagen's Stool kit and EZ1 kit

Four samples from each of two levels of spiked faeces (10^4 and 10^6 MAP/g) were processed according to the protocol described in Paper V, until the step after the mechanical disruption when the samples were put on ice. Two samples from each level were then processed by the Biorobot EZ1 robot (Qiagen, Hilden, Germany) with the belonging EZ1 DNA Tissue Kit (Qiagen), while the rest followed the original protocol. The EZ1 was programmed to process 200 µl sample and elute 50 µl DNA. The DNA preparations from both protocols were compared by real-time PCR with the system MP, described in Paper I. When inhibition was noted in the EZ1 eluate, a tenfold and a hundredfold dilution of the sample were analysed in the same way.

5.5 Direct PCR on milk

The method for pooling pellet and cream, lysing the bacteria and purifying the DNA by automated magnetic bead separation is fully described in paper

III. In the following experiments, the sample loading volume to the Biorobot EZ1 had not yet been increased from 200 µl to 400 µl.

5.5.1 Investigation of the effect of different elution and template volumes

Tubes with 10 ml spiked milk, four of each of two different concentrations (10 and 100 MAP/ml) were processed according to the described protocol until the samples were loaded into the Biorobot EZ1. Two tubes of each spiking level were processed and eluted in 50 µl while the two others were eluted in 100 µl. From each of the eight tubes with DNA, duplicates of 2 µl and 5 µl template were analysed by real-time PCR using the system MP, described in paper I and II, but without glycerol in the PCR mixture.

5.5.2 Bovine DNA reduction

The effect of either washing the milk pellet in H_2O (Sigma-Aldrich) or lysing the white blood cells in the pellet with undiluted or 10-fold diluted Nucleic Lysis Buffer (Promega Corporation, Madison, WI, USA) before centrifuging, decanting and proceeding with the ordinary protocol (paper III), was examined. The reduction of bovine DNA in the milk was studied by analysing duplicates of 2 µl of the total DNA from each duplicate preparation by real-time PCR with SYBR Green chemistry (14.63 µl of H_2O [Sigma-Aldrich], 2.5 µl of 10x SYBR Green buffer II [Applied Biosystems, Foster City, CA, USA], 2 µl of $MgCl_2$ [25 mM, Applied Biosystems], 2µl of GeneAmp dNTP with UTP [2.5 mM dA,C, GTP, 5mM dUTP, Applied Biosystems], 0.13 µl of AmpliTaq Gold DNA Polymerase [5 U/µl, Applied Biosystems] and 0.25 µl of AmpErase [UNG, 1 U/µl, Applied Biosystems]) and 0.75 µl of each of the newly designed bovine-specific primers B.taurus2F (5'-ATG GGA ACC TTG GAT ACC TG-3', 10 µM) and B.taurus2R (5'-CAG GAG CAT AGC ATG GGT TA-3', 10 µM). DNA from milk extracted without pellet wash or lysis was included as a control. In this and the following experiment, the cream fractions were discarded.

The method of reduction of bovine DNA was then applied to see whether it could increase the yield of MAP DNA. Eight spiked milk samples (10^4 MAP/ml, spiked as described in paper III, but less stringent, as the bacterial suspensions had been stored at 8°C for more than one month) were divided into four pairs and given different treatments (A-D). A: control, B: washing with H_2O (Sigma-Aldrich), C: eukaryote cell lysis with diluted Nucleic Lysis Buffer 1:10, D: eukaryote cell lysis with pure Nucleic Lysis Buffer. The duplicate samples were then processed according to the

ordinary protocol (paper III), and the effect of the treatments was analysed in duplicate by using the IS*900* MP system, described in paper I.

5.6 PCR-REA

The PCR system IS*1311*-2, including the primers M56 and M94 and described by Marsh *et al.* (1999), was chosen for amplification of an IS*1311* sequence. The patterns obtained by subsequent digestion by H*inf*I and gel electrophoresis (shown in Fig. 4) were sufficiently informative for the desired basic typing, and discrimination between MAP and MAA, possible with other suggested systems, was not considered necessary as confirmation of MAP was already performed.

6 Concluding remarks

Sensitive and robust direct PCR methods for MAP detection in three different kinds of clinical matrixes; bovine semen, milk and faeces, were developed and evaluated. Critical for efficient lysis of MAP in all methods was the application of mechanical disruption with ceramic beads. However, other pretreatments and DNA purification methods were chosen to suit the properties of the respective matrixes. All protocols were successful in removing PCR inhibiting substances and the assessed analytical sensitivities were high, as shown in Table 2.

Table 2. *Analytical sensitivities and purification principle of the different methods*

Material	Sensitivity	Sample amount	Final purification method
Semen	10^2 MAP/ml	100 µl	Phenol/Chloroform extraction
Faeces	10^4 MAP/g	1 – 1.2 g	QIAamp DNA Stool Mini Kit*
Milk	10^2 MAP/ml	10 ml	EZ1 DNA Tissue Kit*

* Qiagen

The diagnostic PCR method for faeces was evaluated on 202 proficiency test samples. Of 117 positive and valid samples, MAP was detected in 113 samples by PCR and 101 samples by culture, indicating that the evaluated method was more sensitive, at least on previously frozen faeces. Future parallel testing by culture and direct PCR on more fresh faecal samples would be valuable for the comparison of the methods.

The direct PCR method for milk was applied to 143 Danish bulk tank milk samples from 56 herds and yielded positive results in milk from 42% of the herds that had been shown positive for paratuberculosis by environmental faecal culture. The abundance of MAP in the tank milk was generally very low (less than 100 MAP/ml) which resulted in a low diagnostic sensitivity for the milk PCR. However, the analytical sensitivity

of the method would make it useful for control of MAP presence in consumers' milk.

Detection of DNA from dead or non-viable MAP in clinical samples is also suggestive of disease in a herd, and the ability of a molecular method to do that is one of its advantages. Stating the analytical sensitivity of a molecular diagnostic method in CFU per weight or volume underestimates the number of detectable units, thereby overestimating the sensitivity of the test. Overestimation also occurs when the presence of free DNA from cultured MAP colonies is not recognized. Consequently; when evaluating the analytical sensitivity of molecular diagnostic methods by performing spiking experiments, quantification of MAP used for spiking should be performed by visual counting in a microscope, or by some other method suitable for quantifying both viable and non-viable bacteria. Prior to that, they should be separated – as suggested in this thesis, by light centrifugation – and washed, to reduce the amount of free DNA.

Three novel real-time PCR systems were designed, all of which yielded positive results on all tested MAP strains. After testing on several different strains and validation on clinical samples, the system DH3 on F57 was shown to be the most specific, thus suitable as the primary system for confirmation of previously IS*900* positive samples. However, since it is slightly less sensitive than system DH1 and DH2 on IS*900*, these may be used as alternative confirmatory systems for low-grade infected clinical samples.

MAP was isolated from 21 of 501 tested wild guanacos in Patagonia in Chile and represents the first isolation of MAP from this species. All strains were of C type and the definitive source of this infection remains unclear, although one theory involves faecal spread from domestic cattle to guanacos, alternatively via the more numerous sheep which share the same grazing land. Future studies may include screening of these sheep for MAP, followed by typing.

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