Detection and Confirmation of *Mycobacterium avium* subsp. *paratuberculosis* in Clinical Samples

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Abstract

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Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of the serious disease paratuberculosis in ruminants. It is a chronic enteric disease that causes considerable economic losses worldwide. Paratuberculosis leads to reduced milk production and eventually, diarrhoea, weight loss and death. Its slow development, the inappropriate immune response of the host and the fastidiousness of the bacteria all contribute to the difficulty of early diagnosis, necessary to restrict spread of the disease.

Thanks to rigorous control measures, paratuberculosis is rare or absent in Sweden. However, occasional import-related outbreaks have occurred, during which all animals in the infected herds were culled. Freedom from paratuberculosis is mainly monitored by slow culture methods. In some situations, fast and reliable alternative methods are needed, for instance, when semen is imported for breeding purposes. Donor bulls may be asymptomatic carriers of MAP and the risk for venereal transmission of the disease is insufficiently investigated. In this thesis, the development and sensitivity assessment of a protocol for detection of MAP in bovine semen by real-time PCR is described. Beadbeating with zirconia/silica beads and phenol/chloroform extraction was used to purify the bacterial DNA and it was shown to successfully remove PCR inhibiting substances. A method for accurately evaluating the analytical sensitivity was also developed. By analysis of artificially infected samples, a sensitivity of 10 organisms per 100 µl sample was assessed.

The target gene, for the method described here and for many other PCR methods for detection of MAP, is the insertion element IS900. Although specific for MAP, it has been shown to share similarities with genes in other mycobacteria. Positive PCR results must, therefore, be confirmed by an alternative method. Three novel real-time PCR systems were designed and validated on 267 strains and 58 positive clinical faecal and tissue samples. Two systems were based on IS900 and one system on the MAP-specific gene F57. The latter was the most specific for MAP and is therefore the recommended system for confirmation, but as it was slightly less sensitive when tested on pure DNA, the other systems may be applied when necessary.

Keywords: bacterial infection, bovine semen, confirmation, detection, diagnosis, F57, faeces, IS900, Johne's disease, microbiology, *Mycobacterium avium* subsp. *paratuberculosis*, real-time PCR.

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Svensk sammanfattning

Mycobacterium avium subspecies *paratuberculosis* (MAP) orsakar den allvarliga sjukdomen paratuberkulos hos idisslare. Det är en kronisk tunntarmsinflammation, som förorsakar stora ekonomiska förluster världen över. Sjukdomen kan ha en inkubationstid på flera år, och när den bryter ut, leder den till minskad mjölkproduktion, diarré, uttorkning och kraftig viktminskning innan djuret slutligen dör. Infektionens långsamma utveckling, det ofullständiga immunsvaret, samt svårigheterna att odla bakterien bidrar till svårigheterna med en tidig diagnos, vilket är nödvändigt för att stoppa spridningen av sjukdomen.

Tack vare rigorösa kontrollprogram så är paratuberkulos, om inte helt obefintligt, mycket ovanligt i Sverige. Enstaka importrelaterade utbrott har dock ägt rum hos nötkreatur, vilket har lett till att alla djur i de infekterade besättningarna har slaktats ut. Prevalensen av paratuberkulos övervakas vanligen med långsamma odlingsmetoder på träck. I vissa situationer är det dock önskvärt med snabba och pålitliga alternativa metoder, till exempel när sperma importeras för att användas i avel. Tjurar kan vara symptomfria bärare av MAP och risken för venerisk smitta av sjukdomen är inte tillräckligt undersökt. I den här avhandlingen beskrivs utvecklingen och valideringen av ett protokoll för detektion av MAP i nötsperma med realtids-PCR. Homogenisering med kulor av zirkonium/kisel och extraktion med fenol och kloroform användes för att separera och rena bakteriellt DNA. I samband med detta utvecklades en metod för att göra en rättvisande bedömning av den analytiska känsligheten för molekylärbiologiska detektionsmetoder. PCR-inhiberande ämnen kunde framgångsrikt avlägsnas, och genom att testa metoden på artificiellt infekterade prover uppskattades känsligheten till 10 organismer per 100 µl prov.

Målgenen för denna och många andra PCR-metoder för detektion av MAP, är insertionselementet IS900. Trots att den är specifik för MAP, så har den visat sig ha flera likheter med gener i andra mykobakterier. Positiva PCR-resultat måste därför konfirmeras med en alternativ metod. Tre nya system för realtids-PCR konstruerades och validerades på 267 stammar och 58 positiva kliniska träck- och vävnadsprover. Två system baserades på IS900 och ett på den MAP-specifika genen F57. Den sistnämnda visade sig vara den mest specifika för MAP och är därför det huvudsakligt rekommenderade systemet för konfirmering, men eftersom det var något mindre känsligt än de andra systemen när den testades på rent DNA, så kan även de andra systemen användas vid behov.

Contents

Appendix

Papers I-II

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

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*. (Submitted)

Things should be made as simple as possible, but not any simpler.

Albert Einstein (1879-1955)

Abbreviations

AGID	agar gel immunodiffusion
bp	base pair
CF	complement fixation
CFU	colony forming units
CMI	cell-mediated immunity
Ct	cycle threshold
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
IFN-γ	interferon gamma
IS900	insertion sequence 900
IL2	interleukin 2
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
TH1	T (thymus) helper cell 1
TNF-β	tumour necrosis factor beta
UNG	uracil N-glycosylase

Background

Ever since 1895, when Johne and Frothingham described the slowly progressing enteric disease of ruminants 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. 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.

MAP is a gram-positive, acid-fast, fastidious and extremely slow growing bacillus. Culture of visible colonies may take more than 16 weeks (Collins, 1996) and practically all strains require supplement of mycobactin, an iron-chelating agent that most other mycobacteria produce self-sufficiently (Cocito *et al.*, 1994). 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.*, 2002; Grant *et al.*, 2005), low pH, salt and chemicals, such as chlorine (Donaghy, Totton & Rowe, 2004; Rowe & Grant, 2006). MAP has been shown to be able to survive for long periods of time in the environment, complicating eradication of the disease (Whittington, Marsh & Reddacliff, 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. Another characteristic of MAP is the presence of 15-20 copies of the 1451 base pair long insertion element IS900 (Green *et al.*, 1989; Moss *et al.*, 1991). Although there are sequentially similar insertion elements in related mycobacteria, the sequence as a whole is considered specific for MAP and has been widely used in molecular diagnosis of paratuberculosis.

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 (Shinnick & Good, 1994). At the time of writing, there were 117 recognized and proposed mycobacterial species, and several subspecies, (Euzéby, 2006; http://www.bacterio.cict.fr/m/mycobacterium.html; 2-Jul-2006), taxonomically grouped mainly according to speed of growth and temperature requirements, pigmentation and resistance to antibiotics.

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 (Johnson-Ifearulundu & Kaneene, 1997; Kennedy & Benedictus, 2001). In these surveys, estimates of herd prevalence in Europe range from 7% in Austria to 55% in Denmark (Kennedy *et al.*, 2001). 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 however difficult to assess, as methods to diagnose the disease have generally been lacking in sensitivity, 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, Asanome & Ribeiro, 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 1993. Investigations identified a total of 53 infected cattle herds, 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 or import-connected cattle 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 prevail in nature, both in the environment 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 (Harris & Barletta, 2001; Judge *et al.*, 2005). Corn *et al.* isolated MAP from various wild monogastric mammals and birds (Corn *et al.*, 2005). It has also been shown that MAP may survive in protozoa, insects and biofilms in the environment and has been reported in rivers and catchments areas (Pickup *et al.*, 2005; Rowe & Grant, 2006; Whan *et al.*, 2005).

One of the outcomes of several investigations of the potential relationship between MAP and Crohn's disease in humans (further discussed below) was that MAP was found in biopsies and blood of a relatively high percentage of the healthy control patients (Bull *et al.*, 2003; Naser *et al.*, 2004). These findings together with the above 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.

Susceptibility and pathogenesis

Fecal-oral transmission of MAP is the most common way for the disease to spread among cattle (Chiodini, van Kruiningen & Merkal, 1984; Stabel, 1998). The animals are most susceptible when younger than 30 days and usually ingest the bacteria when suckling from an infected dam with fecally contaminated teats or, probably less likely, from one that sheds MAP directly into her milk and colostrum

(Streeter *et al.*, 1995; Sweeney, Whitlock & Rosenberger, 1992). Heavily infected dams may also convey the organisms to fetuses in-utero (Lawrence, 1956; Sweeney, 1996). 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 and recover from the infection while others become asymptomatic carriers, shedding the bacteria without showing any signs of infection.

MAP has been isolated from the reproductive organs and semen of infected bulls (Ayele *et al.*, 2004; Buergelt, Donovan & Williams, 2004; Eppleston & Whittington, 2001; Glawischnig *et al.*, 2004; Larsen & Kopecky, 1970; 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 ordinary 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 (Chiodini, van Kruiningen & Merkal, 1984; Corn*, et al.*, 2005). The possible relevance of MAP in Crohn's disease, a human inflammatory bowel disease, is discussed below.

When MAP is ingested, it penetrates the intestinal mucosa and is soon phagocytosed by macrophages, its target cells (Cocito*, et al.*, 1994; Sigurethardottir, Valheim & Press, 2004). 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 the 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, Valheim & Press, 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.

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, 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, van Kruiningen & Merkal, 1984). Tissues in the terminal small intestine have become distorted, swollen and leaks plasma, causing continuous or intermittent diarrhoea, protein-loss and malabsorption (Chiodini, 1996; Chiodini, van Kruiningen & Merkal, 1984; Stabel, 1998). Shedding of bacteria in stage III can exceed 10^8 CFU per gram faeces (Chiodini, van Kruiningen & Merkal, 1984), and diagnosis by detection of MAP is usually not a problem. Despite remaining a good appetite, the clinically ill animal emaciates and produces less milk. Its hair coat becomes rough, sometimes with fading colour. Death is often preceded by bloody diarrhoea, debilitation and loss of appetite.

Zoonotic aspects

An association between MAP and the human enteritis Crohn's disease was first suggested by Dalziel in 1913, as he noted its clinical and histopathologic similarities with Johne's disease. Crohn's disease 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. Assays by culture and PCR from blood and intestinal tissue biopsies from Crohn's disease 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 Crohn's disease (Bull*, et al.*, 2003; Naser*, et al.*, 2004; Schwartz *et al.*, 2000; Sechi *et al.*, 2005). However, some authors find more or roughly equal occurrence of MAP in patients with ulcerative colitis (Collins *et al.*, 2000) and yet some other authors failed to find any MAP in humans at all (Ellingson *et al.*, 2003). Most importantly, and independent 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 Crohn's disease. There is also support for theories combining the potential role of MAP with other theories concerning immune dysregulation or immune deficiency (Chamberlin & Naser, 2006). 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 patients without any inflammatory bowel disease contained MAP (Bull*, et al.*, 2003), and in other surveys, the equivalent values were at least remarkably high (Naser*, et al.*, 2004; Sechi*, et al.*, 2005; Stabel, 2000a). This raises a question. How and from where did these patients ingest MAP? As mentioned above, MAP has been found in the environment and may be more widespread than we know, also in food. Dairy cattle

with Johne's disease do to some extent shed MAP in milk (Sweeney, Whitlock & Rosenberger, 1992) and it has been shown that small amounts of MAP can remain viable after pasteurisation (Ayele *et al.*, 2005; Grant*, et al.*, 2005). MAP also survived the ripening process of Cheddar cheese, experimentally manufactured from artificially contaminated milk (Donaghy, Totton & Rowe, 2004).

Diagnostic methods

Several methods has been suggested and used to diagnose Johne's disease, but many of them suffer from inferior specificity and sensitivity (Chiodini, van Kruiningen & Merkal, 1984; 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. During the early cell-mediated immunity (CMI), detection of cytokines, such as IFN-γ, a product of T lymphocytes, can be done in vitro by enzyme-linked immunosorbent assay (ELISA) (Collins, 1996; Harris & Barletta, 2001). 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 *Mycobacterium avium* subsp. *avium* 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, still suffers from insufficient specificity (Collins, 1996; Stabel, 1998).

Greater specificity is achieved when the presence of the aetiological agent, MAP, can be demonstrated and confirmed with bacteriological or molecular methods, if 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 acid-fast bacilli, is suggestive of paratuberculosis, but for specific diagnosis, culture and/or PCR is needed. Likewise, characteristic acid-fast bacilli in fecal samples are suggestive of paratuberculosis, but culture and/or PCR is necessary for a reliable diagnosis.

Bacteriology

MAP is a fastidious organism and has a generation time of 1.5 to 4 days when cultured in liquid media (Lambrecht, Carriere & Collins, 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 supplemented with mycobactin. Optimal sensitivity is difficult to achieve, as chemicals used for decontamination of the faster growing sample microflora also kill some of the MAP, or decrease their viability (Whittington, 2002). If the animal is an intermittent or low shedder, false negatives may 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

with molecular methods or with the classical methods to judge growth and morphological characteristics. Colonies should be small, raised and white or pale yellow. Lack of growth in a control tube, without mycobactin, guarantees that the organism is mycobactin-dependent.

Because of the specificity and the high sensitivity, compared to other methods, culture is since long considered the "gold standard" for diagnosis of paratuberculosis. However, for subclinical infection, 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 from clinical cases that nearly always are shedding MAP. 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).

PCR

The polymerase chain reaction (PCR) has been gaining ground over the last two decades as a sensitive, specific and fast method to diagnose paratuberculosis by detection of MAP. The basis of this molecular method is the enzymatic, temperature-controlled exponential amplification of a target DNA-sequence, the choice of which is crucial for specificity. Two target-specific DNA fragments, called primers, confine the range of the sequence elongation, by acting as extension points for the polymerase. 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, which therefore has to be removed. In theory, 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 visualized by gel electrophoresis and the position of the gel bands then reveal if the amplicons were of expected size.

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 probe sequence hybridises to the target sequence near one of the primers (Fig. 1). To the probe, a reporter (a fluorescent dye), and a quencher dye are attached. The presence of the quencher prevents the reporter to emit light, but as the PCR reaction proceeds, the Taq polymerase digests the probe. The reporter is then released and can emit light (from now on, this technique is referred to as only real-time PCR, as other real-time PCR techniques, such as Sybr Green, are not described here). The amplification reaction takes place in a machine that cycles the temperature, emits light that excites the fluorophore, and most importantly, measures the light emitted from the freed fluorophore, thus monitoring the amplification. Depending on the amount of starting target DNA, it will take a certain number of cycles before the fluorescent signal reaches a certain 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

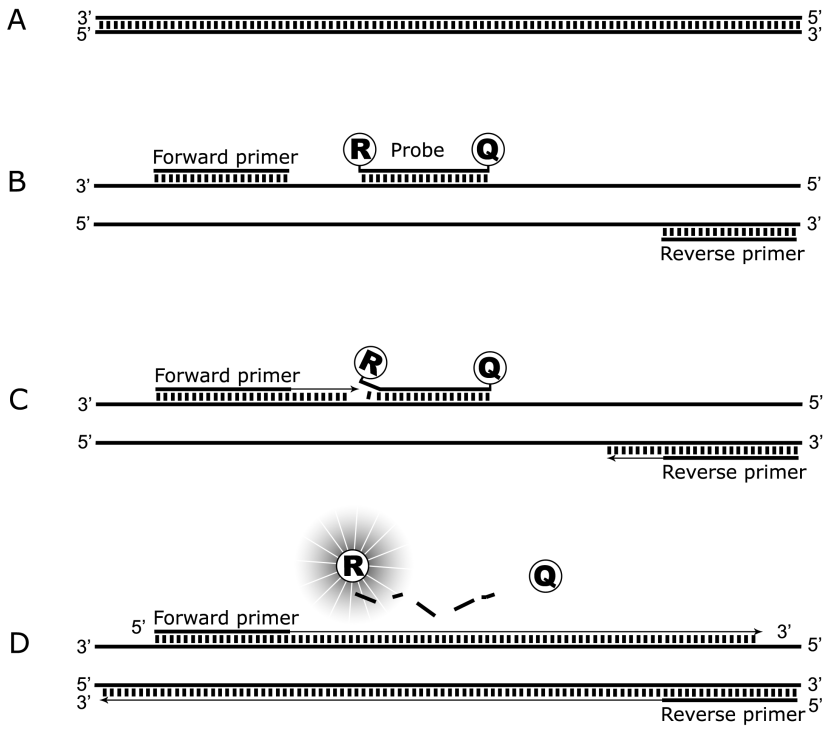

Fig. 1. The principle of TaqMan real-time PCR. Double-stranded template DNA (A) is denatured by high temperature. At low temperature, primers and probe anneal to the template (B). When elongation of the primer reaches the probe, labelled with a fluorescent reporter and a quencher, it is digested (C). In the absence of the quencher, the reporter dye emits light, detected by the instrument, and indicative of amplification (D).

perhaps would not be visible on a gel, the method is very sensitive. The probe can be chosen to make the method more species-specific than conventional PCR.

The most widely used target gene for detection of MAP is IS900, first described in 1989 (Green*, 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 IS900 is not necessarily definitive for identification of MAP, as previously presumed. It has many similarities with genes 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 (Harris & Barletta, 2001; Tasara, Hoelzle & Stephan, 2005). This is especially true with the equivalent gene in strain 2333, with 94% identity to

IS900 (Englund, Bölske & Johansson, 2002). Suggested measures to increase PCR specificity for IS900 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 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 bad viability, molecular methods should be used.

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. Many methods, such as chemical or enzymatic treatment and boiling, have been suggested (Grant & Rowe, 2001), but in this thesis, the method of so-called bead-beating is used, and further discussed below.

Aims of the present study

- To establish an accurate and reproducible method to evaluate the analytical sensitivity of molecular diagnostic methods.
- To develop a fast and sensitive protocol for detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bovine semen and to make a critical evaluation of the analytical sensitivity.
- To design and validate alternative real-time PCR systems for confirmation of a positive PCR test for MAP.
- To develop a robust protocol for detection of MAP in bovine faeces.

Methodological considerations

Real-time PCR

Conventional PCR with visualization of the product on an agarose gel is probably the cheapest 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 electrophoreses should be conducted in a separate lab. 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 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 base uracil, which sometimes 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 products containing uracil are digested. When the temperature program starts, only real template, without uracil, remains.

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

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

Specificity

Although PCR, with certain primers and probes for IS900 (Green*, et al.*, 1989), 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 IS900, 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, IS900 was aligned with five long and two short sequences, with high degrees of similarity, from other bacteria. The long sequences were IS1613, IS901, IS902 and ORF 1179 and 940, present among some *Mycobacterium avium* but not in MAP, and the equivalent to IS900 in the mycobacterial strain 2333. The latter was virtually identical to IS900 among the first 450 bases. The short ones were from *Streptomyces avermitilis* and *Rhodococcus erythropolis*. By studying this alignment, oligos could be chosen so that the new PCR systems would contain at least a few bases that differ in respect to other known similar genes.

To further decrease the risk of cross-reactions with genes, nearly identical to IS900 and hypothetically residing in yet unknown mycobacteria, a real-time PCR system was designed for the gene F57 (Poupart *et al.*, 1993). F57 is hitherto considered specific for MAP and has no known similarities in genes of other bacteria. However, because it only appears once in the MAP genome, a PCR system based on F57 is expected to be less sensitive than the IS900 systems. Other considered MAP specific genes were hspX and ISmav2 (Ellingson, Bolin & Stabel, 1998; Strommenger, Stevenson & Gerlach, 2001), but because of promising results (Coetsier *et al.*, 2000) and previous experience of F57 in this laboratory, F57 was the chosen target gene.

The newly designed systems were tested on various bacterial strains, most of them mycobacterial and often of unknown species, isolated during routine diagnostic testing. All tested strains are listed in Table 1 and 2 in paper II. MAP strains were of different origin countries and source animals – primarily cattle, but also goat, sheep, deer and human. The systems were validated by direct PCR on clinical bovine faeces with concealed status, from USA, Denmark and the Czech Republic, also allowing for concurrent comparison of the sensitivity of the systems on clinical material.

Counting and sensitivity

When assessing and stating the sensitivity of agent detection methods, the conventionally used unit for bacterial quantification is CFU (Colony Forming Units) per weight or volume, often used in MAP studies (Mason, Marsh & Whittington, 2001; Odumeru *et al.*, 2001; O'Mahony & Hill, 2004; Stabel &

Bannantine, 2005). However, for many reasons, this is not appropriate for stating the sensitivity of molecular detection methods. First of all, the number of CFU is 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. Also, samples may be frozen or old 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 labs, and more importantly, they are likely to be underestimated, leading to overestimation of the analytical sensitivity when validating detection methods.

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.

Results and discussion

Extraction

Boiling and free DNA

When confirming the identity of suspected growth of MAP with PCR, boiling or heating (95 ˚- 99 ˚C) of the suspended colony for 10 – 15 minutes is the only preparation necessary to obtain enough DNA for detection. Therefore, it was presumed that boiling lysed a sufficient proportion of the bacteria, thereby allowing detection. When trying to optimise lysis conditions, real-time PCR demonstrated little or no quantitative difference caused by difference in temperature (data not shown). To investigate the suspected presence of free DNA, an experiment was performed on untreated and washed 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 suspension yielded a strong signal (i.e. low Ct-values), but incubation of the colonies at 99 ˚C for 15 min caused a four-fold decrease of detectable DNA (Fig. 2). 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 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.

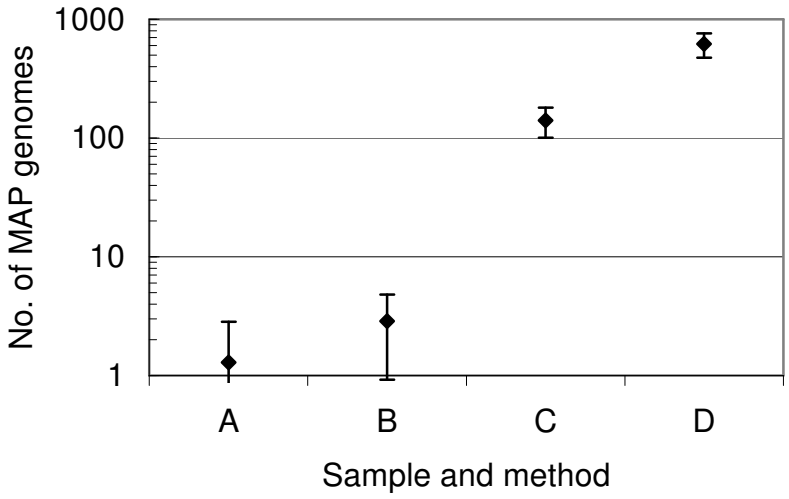

Fig. 2. Indication of the presence of free DNA in suspended MAP colony. A) Washed MAP incubated at 99 ˚C, B) Washed MAP at room temperature, C) Untreated MAP incubated at 99 ˚C, D) Untreated MAP at room temperature.

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

The importance of beadbeating

Beadbeating is a general term for using small beads mixed with the sample, usually in the presence of a proteolytic enzyme and lysis buffer, to disrupt tissues or tough cell walls and spores, by forceful shaking in a machine. It is one of several suggested methods to lyse MAP. Others include combinations of enzymatic treatment, freeze-thaw/boiling and kits for plant DNA purification (Chui *et al.*, 2004; Odumeru, *et al.*, 2001; Zecconi *et al.*, 2002).

The discovery of free DNA present in MAP colonies explained why boiling previously appeared to be a sufficient lysis method for suspensions of MAP, but not for clinical samples. During its slow colony formation, dying MAP appears to spontaneously lyse and release its DNA 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 sub-optimal milieu that the media constitutes. Either way, once 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 under the section “Assessment of sensitivity”, but more importantly, it stresses that MAP must by successfully lysed for detection of its DNA.

Further experiments with MAP suspensions, washed to reduce the amount of free DNA in suspension, clearly showed that beadbeating was necessary to effectively lyse the cells (Fig. 3). Without beadbeating, vortexed homogeneous suspensions yielded only slightly lower Ct than did the cell-free supernatant (lower Ct indicates a higher amount of target DNA; up to a two-fold difference per ΔCt). Incubation of the suspension at 99 ˚C could cause a very slight decrease of Ct, either because high temperature indeed has some lysing effect, notable in the absence of excessive amounts of free DNA, or because it causes free DNA, stuck to the cell surface, to re-suspend. When treated with beadbeating 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 sample treated with high temperature incubation.

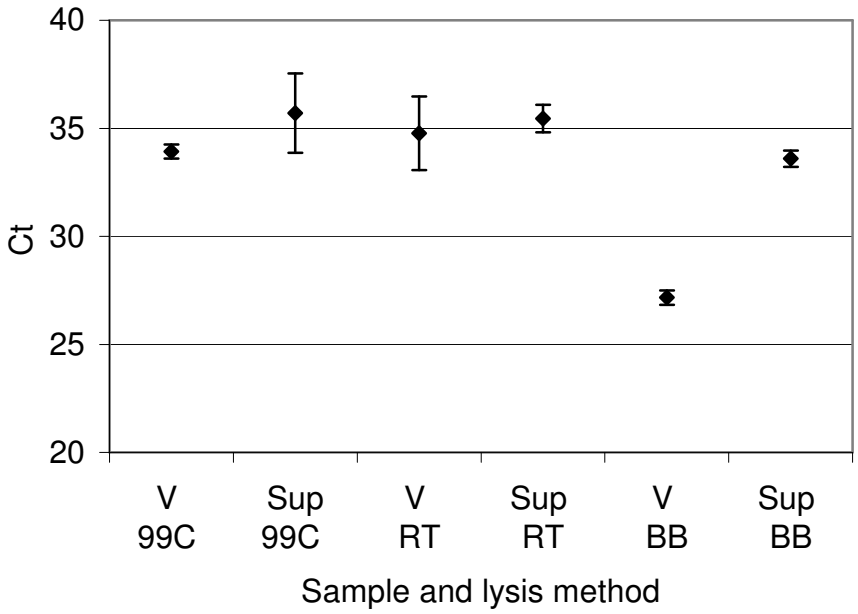

Fig. 3. Comparison of beadbeating and heat-treatment of washed MAP. V indicates that a vortexed homogeneous bacterial suspension was used, while Sup designates cell-free supernatant. RT, 99C and BB designate incubation at room temperature, 99 ˚C, and beadbeating, respectively.

The results of several other experiments (data not shown) supported the importance of beadbeating for lysis, while others, sometimes when investigating other parameters, inconsistently indicated that beadbeating did not cause clear differences in DNA yield. Therefore, an experiment to roughly evaluate the importance of lysis buffer formula, buffer incubation time and beadbeating was performed. From the result, beadbeating proved to be the most important factor for successful extraction (Fig. 4). 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 beadbeating 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.

Phenol/Chloroform extraction on disrupted semen

After beadbeating has been performed, it is necessary to remove proteins and inhibiting substances from the sample. As shown in paper I, a two-step

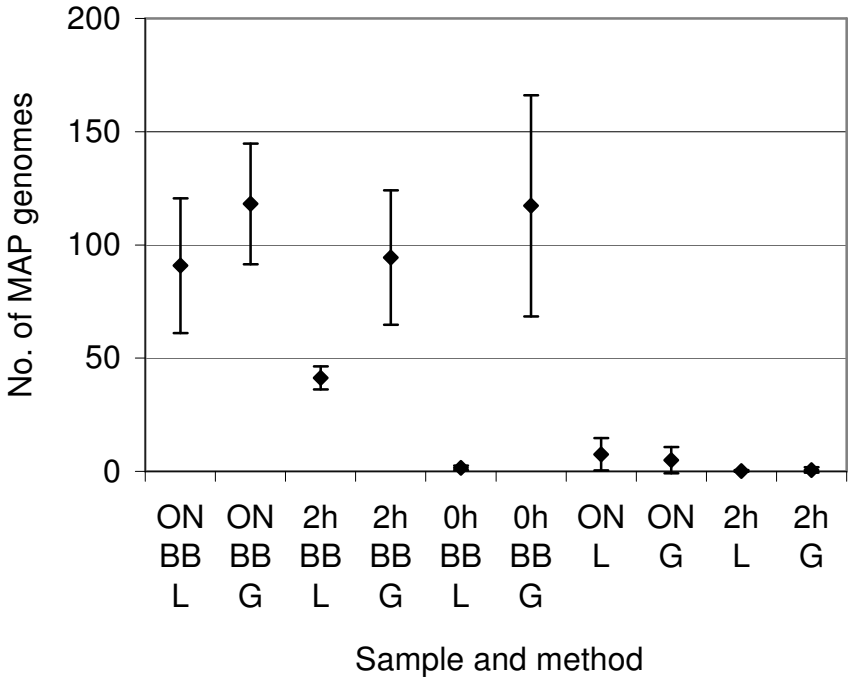

Fig. 4. Investigation of factors critical for lysis. The incubation time is indicated by ON (overnight), 2h or 0h. BB indicates that beadbeating was performed. L and G indicate default lysis buffer and buffer containing guanidinium thiocyanate, respectively.

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 all but independent of the degree of extension (dilution) of the semen; only raw semen caused notably higher Ct values.

It should be considered, that the assessed sensitivity was the analytical sensitivity, 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 their natural distribution 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 beadbeating 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, 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 beadbeating and phenol/chloroform extraction were performed on faeces, PCR inhibitors were usually still present in the samples.

Immunomagnetic separation

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 immunomagnetic separation (IMS), previously shown to concentrate and separate MAP from milk (Grant, Ball & Rowe, 1998; Khare *et al.*, 2004), was tried on spiked semen samples. 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.

Modification of QIAamp DNA Stool Mini Kit

For extraction of MAP DNA from faecal samples, beadbeating was combined with a modification of QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). A sensitivity of approximately 10^4 organisms per gram faeces was obtained, and inhibitory substances could be removed from most samples without the need for dilution of the DNA. Weak signals (i.e. with unacceptably high Ct) were yielded from samples spiked with 10^3 organisms per gram. This 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 recoverable, and considering that the decontamination steps lowers the viability even further, the sensitivity of culture was not expected to be higher than that of our method. According to Merkal, faeces must contain at least 10^2 organisms for detection by culture (Merkal, 1970).

As for clinical samples, parallel testing was only performed on previously frozen samples, and then appeared to have a sensitivity comparable to that of culture. For a correct comparison of the methods, however, further investigation on fresh samples is required.

Assessment of sensitivity

When comparing cell densities in MAP suspensions, obtained by either manual counting in Bürker chamber or by counting of CFU on cultures, the visually determined number of organisms was approximately 100 times greater than the number of CFU. 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 the above problems with sensitivity assessment.

1) A suspension virtually free of aggregated bacteria was obtained by weak and short centrifugation of a rich MAP suspension 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.

2) The suspension was then washed three times in sterile water to reduce the amount of free DNA to approximately 0.1 ‰ of the original amount, as estimated during optimisation. One possible drawback with the centrifugation in the washing procedure is the potential, but unlikely, decrease in viability of MAP, which would give culture a disadvantage when comparing detection methods.

3) The organisms were visually quantified by microscopic counting. Although this allowed all MAP to be counted, 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%.

Validation of specificity

Application of the three newly developed real-time PCR systems, described in paper II, on 112 strains of MAP and 155 other strains, demonstrated complete specificity regarding MAP for the F57 system DH3. The systems DH1 and DH2 targeting IS900, however, yielded weak false positives on a *Mycobacterium avium* subsp. *avium* isolate and a *Mycobacterium cubicae*-related isolate, respectively. Because the signals were very weak for being produced by a resuspended colony, they are 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 IS900 positives and the DH1 and DH2 only as secondary alternatives, 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 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 requires 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.

The systems were validated by application on 58 clinical faecal samples, previously shown positive by direct PCR using the primary PCR system. The alternative IS900 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 IS900 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 came from an inter-laboratory ring test, and was announced a low-level positive.

The suspected 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 IS900 system in this laboratory, called MP, the alternative IS900 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, thanks to the multiple copies of IS900 on the MAP genome.

Materials and Methods

Here, some methods discussed in the previous sections are described. Refer to the corresponding section in paper I-II for details about other performed experiments.

Extracting DNA from faecal samples

Bovine faeces, 1.0 to 1.2 g, was transferred to 10 ml screw cap tubes, containing 5 ml 1 M NaOH. If possible, the faeces were stuck to the inner wall of the tube, until all samples had been transferred. The tubes were shaken by hand to mix the NaOH with the faeces, thereafter incubated for 5 min. The tubes were vortexed for 10 s and let to sediment at room temperature for 30 min. From the middle of the supernatant, 1 ml was transferred to labelled 1.5 ml centrifuge tubes with a disposable pipette, and then centrifuged at 5000 *g* for 5 min. The supernatant was discarded and the opening of the tube was briefly touched against a soft paper tissue to remove the remaining liquid. The pellet was resuspended, or at least dissolved to fragments, with a mixture of 500 µl lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.6% SDS) and 2 µl proteinase K (10 µg/µl), then transferred to beadbeating tubes (BioSpec Products, Inc., Bartlesville, OK, USA), containing 200 µl of beads (0.1 mm Zirconia/silica beads, BioSpec Products, Inc.). Depending on the time of the day, the tubes were either incubated at 37 ˚C overnight or at 52 ˚C for 2 h, in both cases with shaking at 600 rpm.

The tubes were shaken in a beadbeating-machine (BioSpec Products, Inc.) for 60 s and put on ice to settle for 10 min. Short vortexing removed foam and beads from the inner walls of the tubes. From this point on, the kit QIAamp DNA Stool Mini Kit (Qiagen), modified to suit the characteristics of MAP and reduce the risk of cross-contamination, was used. The modifications were as follows, in the steps indicated by the numbers:
1-2) Buffer ASL was added before the sample, and only 1.1 ml was used. Instead of using solid stool, all of the beadbeated lysate from above was transferred to the tubes.
3) Incubation at 95 ˚C instead of 70 ˚C.
5-6) The InhibitEX tablet was added to the tube before the lysate.
9-11) Proteinase K and buffer AL were added before the supernatant from step 8.

While incubating at high temperatures, screw cap tubes or safe-lock tubes were used. All suggested optional centrifugations (step 12-13 and 16), to decrease the risk of cross-contamination or to remove rests of washing buffer, were performed.

Experiments investigating lysis and free DNA

Indication of free DNA in suspended MAP colony

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 suspensions and two samples of 100 µl untreated bacterial suspensions 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 used as template in real-time PCR reactions using the IS900 MP-system, described in paper I.

Comparison of beadbeating and heat-treatment

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

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 were subjected to beadbeating, 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 above and in paper I, and the other half, in a lysis buffer containing guanidinium thiocyanate (Odumeru*, et al.*, 2001). In each sub group, two samples were incubated in the lysis buffer for 2 h at 37 ˚C and two samples overnight at 37 ˚C. In the beadbeating sub groups, two additional samples were incubated for only a few minutes at room temperature, while being transported to the beadbeating 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.

Concluding remarks

Reliable methods for detecting MAP in bovine semen and faeces by real-time PCR were developed and evaluated. The analytical sensitivity in semen was 10 organisms per sample of 100 µl, intended for making certain that the semen does not contain high amounts of MAP, although not to declare the donor bulls free from paratuberculosis.

The here developed method of combining sedimentation, beadbeating and a commercial kit proved able to efficiently lyse MAP and remove inhibiting substances from most of the tested clinical faecal samples, yielding a sensitivity of 10^4 organisms per gram. This is a satisfactory sensitivity, comparable to that of culture methods.

Detection of DNA from dead or non-viable MAP in clinical samples is also suggestive of disease, and the ability of molecular methods to detect it is one of its advantages. Therefore, stating sensitivity in CFU per weight or volume underestimates the number of detectable units, thus overestimates the sensitivity of the test. When evaluating the sensitivity of molecular diagnostic methods by performing spiking experiments, quantification of MAP in the bacterial suspensions used for spiking should be performed by visual counting in a microscope, or with some other method suitable for quantifying both viable and non-viable bacteria. Before that, they should be separated – as suggested in this thesis, by light centrifugation – and washed, to reduce the amount of free DNA, present in cultured MAP colonies.

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 validated 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 IS900 positive samples. However, since it is slightly less sensitive than system DH1 and DH2 on IS900, these may be used as alternative confirmatory systems for low-grade infected clinical samples.

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The friendly cow all red and white,
I love with all my heart:
She gives me cream with all her might;
to eat with apple tart.

Robert Louis Stevenson (1850 - 1894)

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ORIGINAL ARTICLE

Sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine semen by real-time PCR

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Abstract

Aims: To develop a fast and sensitive protocol for detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bovine semen and to make a critical evaluation of the analytical sensitivity.
Methods and Results: Processed semen was spiked with known amounts of MAP. Semen from different bulls as well as semen of different dilutions was tested. The samples were treated with lysing agents and beadbeating and the DNA was extracted with phenol and chloroform. Real-time PCR with a fluorescent probe targeting the insertion element IS900 detected as few as 10 organisms per sample of 100 μl semen. PCR-inhibition was monitored by inclusion of an internal control. Pre-treatment with immunomagnetic separation was also evaluated, but was not shown to improve the overall sensitivity.
Conclusions: Real-time PCR is a sensitive method for detection of MAP in bovine semen. Lysis by mechanical disruption followed by phenol and chloroform extraction efficiently isolated DNA and removed PCR-inhibitors.
Significance and Impact of the Study: The high sensitivity of the applied method allows reliable testing of bovine semen used for artificial insemination to prevent the spread of Johne's disease, caused by MAP.

Introduction

Paratuberculosis, a chronic enteric disease in ruminants also known as Johne's disease, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Chiodini *et al.* 1984). At the later stage of its slow development, it causes weight loss, diarrhoea and death. Considerable economic losses for farmers throughout the world are caused by loss of milk production and by the necessary actions taken when the disease is detected (Kennedy and Benedictus 2001). MAP has also been associated with Crohn's disease by detection of the bacteria in human intestinal biopsy specimens (Bull *et al.* 2003), though not proven to be the causative agent of the disease (Hermon-Taylor *et al.* 2000; Harris and Lammerding 2001). The risk that MAP may infect humans makes control of Johne's disease of yet greater importance.

The main route for introduction of MAP infection into a herd is animal trade. Due to the long incubation period and shortcomings in the available test methods there is a great risk of introducing the disease with purchased animals despite serological and/or 'agent testing' before introduction.

There is an opinion that a way to reduce the risk to introduce MAP in the herd is to import bovine semen instead of bulls for breeding purposes. However, MAP has been isolated from the semen and the reproductive organs of infected bulls (Larsen and Kopecky 1970; Larsen *et al.* 1981; Ayele *et al.* 2004; Buergelt *et al.* 2004; Glawischnig *et al.* 2004), but whether semen can transmit the disease *via* uterus is not fully investigated (Eppleston and Whittington 2001). Although semen should be taken only from healthy bulls, these may carry MAP without showing any clinical symptoms and there is thus a risk of the semen being infected.

As a precaution, semen-donor bulls should be tested regarding MAP. According to the SCAHAW opinion (Anon 2004), it is recommended to perform repeated herd

tests for the bull's herd of origin or to test the bull himself after the lay-off period of approximately 4 years. However, a fast, sensitive and specific method to test semen for MAP would be useful to further decrease the risk of certified herds becoming infected. This is of special interest in those cases when the donor bull is no longer alive for further tests and when no other material is available for testing.

Extraction of genomic DNA and detection of species-specific sequences by PCR has proven to be a fast and specific method for identification of pathogens. Since primary culture of MAP may take up to 12–16 weeks (Whipple *et al.* 1991), the rapidity of detection by PCR is of particular importance. In our study, the use of real-time PCR to detect MAP in semen was evaluated and the analytical sensitivity was determined. The target gene was the insertion sequence IS900, regarded as unique to MAP (Green *et al.* 1989; Moss *et al.* 1991). Different pre-treatment methods were investigated and compared, one of which was Immunomagnetic Separation (IMS), previously shown to work well with milk samples (Grant *et al.* 1998; Khare *et al.* 2004). The potential presence of PCR-inhibitors, previously reported to be a problem when analysing semen (van Engelenburg *et al.* 1993; Guerin *et al.* 1995), was monitored in all experiments.

Materials and methods

Bacterial strains and growth conditions

Mycobacterium avium subsp. *paratuberculosis* (ATCC 19698, American Type Culture Collection, Rockville, MD, USA) was cultured on slopes of Löwenstein-Jensen-medium supplemented with mycobactin (4 mg l^{-1}, Allied Monitor, Fayette, MO, USA) in CO_2-incubator at 37°C for 8–12 weeks. Several large colonies of MAP were resuspended in 1 ml sterile water in a microtube. The tube was centrifuged for 20 s at 3000 ***g*** to pellet aggregated material. Care was taken not to disturb the loose pellet while transferring the bulk of the still cloudy supernatant to a new tube. This suspension was pelleted by centrifugation for 5 min at 8000 ***g*** and then resuspended in 1 ml sterile water. This washing procedure was repeated twice to ensure a low level of free DNA in the suspension. The suspension was diluted 10-fold and the bacterial density was determined by manual counting in a Bürker-chamber (0·01 mm, Assistent, Sondheim/Rhön, Germany) with a phase-contrast microscope (Olympus, Tokyo, Japan). The suspension was diluted to 10^8 organisms ml^{-1} and serially diluted to 10 or 100 organisms ml^{-1}. To determine the viability of the bacteria, 100 μl from dilutions with 10^2–10^6 organisms ml^{-1} were cultured on slopes of Löwenstein–Jensen-medium supplemented with mycobactin in air at 37°C for up to 4 months. CFU was counted and compared with the number of organisms counted in Bürker-chamber. Dilutions with 10^4–10^6 organisms ml^{-1} were also spread on slopes without mycobactin for control of identity.

Evaluation of the bacterial washing

MAP was cultured and harvested as described above and aggregated material was discarded. The bacterial suspension was washed 4 times by centrifugation for 5 min at 5000 ***g*** and resuspension of the pellet in 1 ml of sterile water. The supernatant was sampled for measurement of free MAP-DNA after each centrifugation.

Preparation of spiked semen

Bovine semen was prepared in three ways:
i Processed frozen semen from six bulls (Svensk Avel, Skara, Sweden), previously extended 10- to 20-fold in Triladyl (Minitüb Abfüll-und Labortechnik, Tiefenbach, Germany), was blended to even out individual differences in semen constitution. Ten microlitres of diluted bacterial suspension was mixed with 90 μl semen to obtain spiked samples of 100 μl containing serial 10-fold dilutions of 1–10^6 organisms and stored at −20°C.
ii Processed semen from five bulls was spiked separately with 100 organisms per sample of 100 μl.
iii Raw semen (provided by Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Uppsala, Sweden) was diluted two-fold in Triladyl to 1 : 2, 1 : 4, 1 : 8 and 1 : 16 semen. Raw and diluted semen was spiked with 100 organisms per sample of 100 μl.

Immunomagnetic separation

The coating procedure of the magnetic Dynabeads (Dynal, Smestad, Norway) was performed as previously described (Grant *et al.* 1998) with polyclonal rabbit anti-MAP IgG (provided by I.R. Grant, diluted 1 : 10). To obtain uncoated Dynabeads suitable for comparison with the coated ones, beads were treated as above but with buffer free of antibodies. The IMS step was performed as described by Grant (Grant *et al.* 2000), but instead of milk, 100 μl of spiked processed semen was used.

Extraction of DNA

Eight hundred microlitres of lysis buffer (2 mmol l^{-1} EDTA, 400 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris–HCl pH 8·0, 0·6% SDS) containing 20 μg proteinase K (Sigma–Aldrich, St. Louis, MO, USA) was added to the immunomagnetic beads used in the IMS-step or to a sample of 100 μl spiked semen and incubated overnight at 37°C with shaking at 600 rev min^{-1} in a Thermomixer

(Eppendorf, Hamburg, Germany). The samples were transferred to beadbeating-tubes containing 200 μl of beads (0·1 mm Zirconia/silica beads, BioSpec Products Inc., Bartlesville, OK, USA) and run in a beadbeating-machine (BioSpec Products) for 60 s and put on ice to settle for 10 min. Five hundred microlitres of phenol (Sigma–Aldrich) was added to each tube, vortexed 20 s and centrifuged for 10 min at 10 000 ***g***. The upper phase was transferred to a new screw-cap tube. An equal volume of chloroform : isoamylalcohol (24 : 1) was added. Vortexing and centrifugation was performed as in the previous step. The upper phase was transferred to a new tube and 0·6 volumes of isopropanol were added. The DNA was precipitated at −20°C for 30 min. The tubes were centrifuged for 10 min at 14 000 ***g***. The supernatants were discarded and the pellets washed once with ice-cold 70% ethanol. The tubes were centrifuged for 5 min at 14 000 ***g*** and the remaining liquid was removed with a pipette. The DNA pellets were left to air dry for 15 min and then resuspended in 50 μl sterile Dnase/Rnase free water (Sigma–Aldrich).

Real-time PCR

The primer pair MPF/MPR and IS900-specific probe were designed using the Primer Express software (PE Applied Biosystems) (Willemsen *et al.* 1999).

In order to monitor the inhibition of the PCR reaction, a mimic IS900 template was developed and used as an internal control. The construction is illustrated in Fig. 1. In an IS900 region, defined by the primers P90A (5′-dTTCTTGAAGGGTGTTCGGGGCCGTCG-3′) and P11 (5′-dGCTGCGCGTCGTCGTTAATAACCATGCAG-3′) (Moss *et al.* 1992), the two fragments flanking the target site of the IS900-specific probe were amplified using the primers (i) P90A and R (5′-dAGTGTATACCCAGCAGACGACCACGCCGACGT-3′) and (ii) P11 and F (5′-dGCTAAGCTTGTGGCACAACCTGTCTGGGCGGG-3′). The fragments, containing the primer-binding sites for MPF and MPR, were ligated to a synthetic ds-oligo, obtained by annealing ss-oligos 5′-dATACGTCGTCTAAGTCCGATTCA-3′ and 5′-dAGCTTGAATCGGACTTAGACGACGT-3′, and inserted into the vector pGEM-T (Promega, Madison, WI, USA) resulting in the plasmid pWIC9.

The 21 μl PCR-mixture comprised 6·625 μl H_2O (Sigma–Aldrich), 2·0 μl glycerol (Sigma–Aldrich), 2·5 μl 10× PCR-buffer II (Applied Biosystems, Foster City, CA, USA), 5·0 μl $MgCl_2$ (25 mmol l^{-1}, Applied Biosystems), 2·0 μl GeneAmp® dNTP with UTP (2·5 mmol l^{-1} dA, C, GTP, 5 mmol l^{-1} dUTP, Applied Biosystems), 0·75 μl primer MPF (10 pmol μl^{-1}, 5′-dCCGCTAATTGAGAGATGCGATT-3′), 0·75 μl primer MPR (10 pmol μl^{-1}, 5′-dCCAGACAGGTTGTGCCACAA-3′), 0·5 μl IS900-specific probe (10 pmol μl^{-1}, 5′-6-FAM-dACCTCCGTAACCGTCATTGTCCAGATCA-BHQ-1-3′), 0·5 μl mimic-specific probe (10 pmol μl^{-1}, 5′-ROX-dGAATCGGACTTAGACGACGTATACCCAGCAG-BHQ-2-3′), 0.125 μl AmpliTaq Gold® (5 U μl^{-1}, Applied Biosystems) and 0·25 μl AmpErase® (Uracil *N*-glycosylase, 1 U μl^{-1}, Applied Biosystems). The addition of glycerol allowed freezing of the PCR-mixture. Two microlitres of template DNA and 2 μl of mimic molecule pWIC9 (150 fg μl^{-1}) were added to each reaction tube, except for the PCR-negative controls. DNA extracted from ATCC 19698 was included in triples of 10, 100 and 1000 MAP-genomes as standard controls.

The real-time PCR reaction was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) with the following program: 50°C 2 min, 95°C 10 min, repeat 95°C 15 s and 60°C 1 min 45 times. The results were analysed with the Rotor-Gene software Version 5·0 and the built in analytical tools Dynamic Tube Normalization and Slope Correction. Real-time PCR curves of normalized fluorescence for FAM crossing a threshold value (T) of 0·01 at less than 40 cycles were considered positive, as long as the curves had a normal and expected shape. FAM-negative curves with a positive corresponding ROX-curve (i.e. a positive mimic-signal) were considered as true negatives, otherwise inhibition was suspected.

Results

Sensitivity

Beadbeating and phenol/chloroform extraction performed directly on extended semen samples yielded a high sensitivity. As few as 10 organisms per 100 μl sample were detected with real-time PCR (Fig. 2). Lower levels of spiked semen could not be detected. From the number of CFU on cultures of the washed MAP, used for spiking the semen (data not shown), it follows that the Bürker-chamber counted stock solution of 10^9 organisms ml^{-1} contained $2{\cdot}6 \times 10^7$ CFU ml^{-1}. Similar relations between CFU and manual count (viability) were observed when preparing MAP for other experiments. However, viability could occasionally be as low as 0·1% and as high as 5%.

Semen from separate bulls

No major difference in PCR-signal could be found between samples of spiked extended semen from the five individual bulls. They all yielded similar Ct-values, as shown in Table 1.

Semen of different dilutions

All samples of raw semen diluted in Triladyl 1 : 2 and more, spiked with 100 organisms per 100 μl sample, were

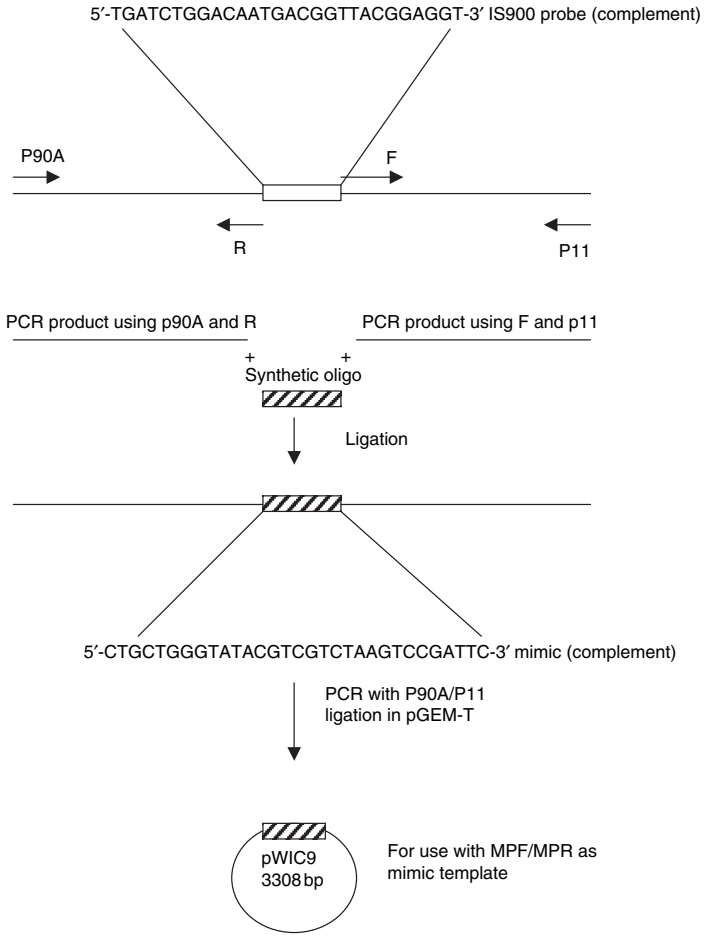

Figure 1 Construction of IS900-PCR internal control (mimic template) for real-time PCR. Two fragments flanking the target site of the IS900-specific probe were amplified by separately using primers P90 and R for one fragment and primers P11 and F for the other, thereby introducing terminal restriction enzyme sites AccI and HindIII, respectively. After enzymatic digestion, a synthetic ds-oligo was ligated to the two fragments and subsequently inserted into the vector pGEM-T, resulting in the internal control plasmid pWIC9.

found positive and yielded similar Ct-values in the real-time PCR reaction. As shown in Fig. 3, only the spiked undiluted semen yielded higher Ct-values or fell out of the range of detection, suggesting that the semen should be diluted at least 1 : 2 to yield reliable results with this method.

Immunomagnetic separation

The application of IMS prior to beadbeating did not yield a sufficient sensitivity. The minimum number of organisms that could be detected was 10^6 per 100 μl sample (data not shown). Immunomagnetic beads coated with secondary antibodies did not yield a considerably stronger PCR-signal than uncoated beads. When coated and uncoated beads were used on 100 μl samples spiked with 10^6 organisms, the uncoated beads caught bacteria that after extraction yielded the Ct-values 37·2, 36·5 and 37·5 in the real-time PCR, while coated beads resulted in the Ct-values 35·9 and 36·7 and one negative.

Spiking procedure

Manual counting of the bacteria was more reproducible and reliable than counting CFU when spiking semen for measuring sensitivity as shown by the low and variable viability rates. Moreover, washing of the bacterial suspension prior to the spiking experiments was shown to be necessary to avoid overestimation of the extraction efficiency caused by free DNA. Table 2 shows how washing of the bacteria could drastically reduce the amount of free DNA in suspension. Two or three washes were found to

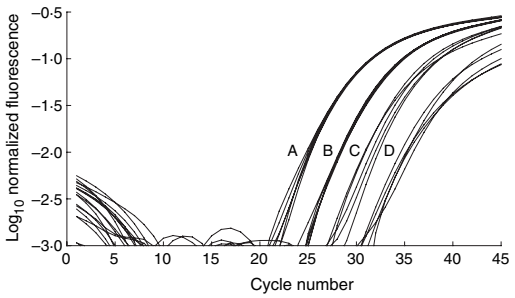

Figure 2 Real-time PCR analysis for MAP isolated from spiked processed bovine semen. The clusters of normalized fluorescence curves, labelled A–D, correspond to template from samples of 100 μl spiked with 10^4, 10^3, 10^2 and 10^1 organisms, respectively. The Ct-values are defined as the cycle numbers where the curves intersect a threshold set at −2.

Table 1 Real-time PCR analysis of MAP isolated from spiked processed semen from five individual bulls

Bull	Ct range*
1	33·6–35·2
2	33·4–34·8
3	32·5–34·6
4	33·3–35·0
5	33·2–34·2

*Threshold $T = 0{\cdot}01$; $n = 4$.

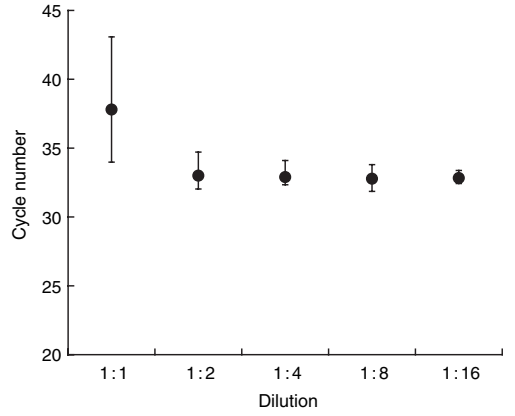

Figure 3 Average Ct values from real-time PCR analysis of MAP isolated from raw and diluted bovine semen. The dilutions made prior to spiking with 100 organisms per 100 μl were 1 : 16, 1 : 8, 1 : 4, 1 : 2 and raw, undiluted. All six replicates (triplicates of samples and duplicates of PCR-reactions) from each dilution yielded approximately the same Ct. Only four out of six replicates from the raw spiked semen were detected. The error bars represent the Ct range.

Table 2 Real-time PCR analysis of the supernatant of centrifuged suspensions of MAP after 0–4 washing steps with sterile water

No. of washes	Ct values*
0	17·9, 18·1
1	22·6, 22·8
2	27·1, 27·2
3	27·6, 27·6
4	28·4, 28·4

*Threshold $T = 0{\cdot}01$.

be sufficient. Overall, the applied method of separating, washing and counting MAP for spiking was shown to be reliable.

Discussion

A fast and sensitive PCR-test would help to monitor MAP in semen prior to artificial insemination. Although the application of PCR-tests for MAP in semen has been reported (Ayele *et al.* 2004; Buergelt *et al.* 2004), the analytical sensitivity has not previously been evaluated. We have developed a protocol that includes beadbeating, phenol and chloroform extraction and real-time PCR. Particular care was taken not to overestimate the sensitivity of the tests in the evaluation study. Because of reported presence of PCR inhibitors in the seminal fluid (van Engelenburg *et al.* 1993; Guerin *et al.* 1995), an internal amplification control was developed and used in the PCR-mixture. Real-time PCR was chosen as it has several advantages over traditional PCR. It is more sensitive, allows for quantification and decreases the risk of contamination in the laboratory by making it unnecessary to open tubes with PCR-product. Quantification is useful in the development of the extraction method and for determining the degree of infection in a sample.

The best results were achieved by the direct method, where beadbeating was performed on the material without preceding attempts to separate the bacteria from the semen. This was shown to work surprisingly well, considering that from 10 extracted genomes from the weakest sample, a maximum of 0·4 genomes were actually transferred to the PCR-reaction (2 μl of 50 μl DNA). The high sensitivity was probably possible due to the fact that each MAP genome contains 15–20 copies of the IS900 element (Moss *et al.* 1991). With lower levels of MAP in spiked semen, the probability of at least one IS900 element to occur in the PCR tube is quite low. Even so, we tested samples of 100 μl spiked with one organism in a separate experiment and found that the rate of detection was very low (data not shown).

The sensitivity of IMS-PCR performed on spiked semen was not as good as expected. Others have claimed

to get high sensitivity of detection of MAP or other pathogens by using IMS-PCR on milk and faeces (Zhang and Weintraub 1998; Grant *et al.* 2000; Khare *et al.* 2004). However, judging from our experiments, IMS–PCR did not improve the sensitivity on this material. It may instead be more appropriate to use IMS when rescuing bacteria from highly PCR-inhibiting samples in which the bacteria are more abundant. Furthermore, several parallel comparisons with Dynabeads not coated with a secondary antibody showed that the effect of the antibody was very small, suggesting that the transfer of bacteria was largely due to nonspecific sticking to the surface of the beads.

In many articles about detection of MAP or other bacterial pathogens, CFU ml^{-1} is used as a measure of sensitivity (Grant *et al.* 2000; Odumeru *et al.* 2001; Vansnick *et al.* 2004). However, in this study we have shown that this is not appropriate for molecular detection, as CFU accounts only for viable bacteria. Viability will depend on the age of the cultures and the growth will vary in different media. In addition, one CFU may correspond to many more than one organism, since MAP tends to aggregate. To avoid the problem with uncertainty in the number of detectable genomes when preparing spiked samples, dispersed organisms were separated from clumps by light centrifugation, and then manually counted in Bürker-chamber. Comparison with culture on Löwenstein–Jensen medium shows that the number of CFU was often between 0·1 and 5% of the actual number of inoculated organisms. This should be kept in mind when comparing sensitivities stated in the present paper with sensitivities stated in CFU ml^{-1}.

During the work on the direct approach without the IMS step, a background signal independent of lysing agents and beadbeating was detected by real-time PCR. This signal was present in analysed supernatant even of hard-centrifuged suspensions of organisms and it was concluded that solutions of resuspended colonies contained a considerable amount of free DNA that could cause overly optimistic results in the evaluation of extraction methods. This further supports the theory that CFU ml^{-1} is an inappropriate measure of sensitivity for molecular detection methods. The effect of simple washing of the bacteria by pelleting and resuspension in sterile water was evaluated by measuring the amount of free DNA in the supernatant after each centrifugation for four washes. Real-time PCR showed that the supernatant of the unwashed bacteria contained high levels of free MAP-DNA that were drastically reduced by washing (Table 2). Possibly, continuous release of free DNA from the surface of broken cells etc., made it impossible to completely eliminate the presence of free DNA. Adding three washing steps of the bacteria to the spiking procedure in subsequent experiments was considered sufficient to reduce the amount of free DNA to a minimum. This ensures that successful lysing of the bacteria will be required for detection of MAP DNA in spiked samples.

Although PCR offers many advantages over culture and immunological methods, there are some disadvantages of which a serious one is the problem with PCR-inhibition, also reported to complicate molecular detection in semen. Several methods to remove PCR-inhibiting components in semen have been suggested (von Beroldingen *et al.* 1991; Santurde *et al.* 1996; Manterola *et al.* 2003). To monitor PCR-inhibition in the present study, an internal amplification control was constructed. A small amount of a mimic molecule, consisting of a plasmid with primer binding sites identical to the ones targeting IS900, was added to all samples. By using two differently labelled probes, we could distinguish between the IS900 amplicons (FAM-labelled) and the mimic amplicons (ROX-labelled). If a sample was a true negative, the mimic alone was amplified. The results showed that PCR-inhibition was not a problem for the application of this method.

Since the exact degree of extension in the processed semen was unknown, an inhibition study was done to find out if inhibition of the real-time PCR would occur in raw and less diluted semen. Both the large amount of sperm DNA and other substances in the semen could potentially inhibit the PCR-reaction (van Engelenburg *et al.* 1993) or somehow disturb the extraction process. The results showed no signs of inhibition or other problems with detection of MAP, as long as the semen was extended at least 1 : 2 with Triladyl. This indicates that it should be possible to increase sensitivity further by testing raw semen instead of processed samples. The large amount of background DNA in the semen may even enhance the sensitivity by acting as a carrier for the target DNA. This has previously been suggested to be the case when extracting DNA from foods (Dickinson *et al.* 1995).

Processed semen from five different bulls was spiked individually and analysed to investigate whether the constitution of semen from separate individuals would vary enough to cause a difference in PCR-signal. No such difference could be found in this limited experiment, indicating that semen could be regarded as a uniform material. However, the Ct of these reactions, shown in Table 1, differ from the average Ct of the samples spiked with the same amount of MAP, shown in Fig. 2, by 3·6 cycles. Apart from being due to slight differences in spiking procedure, extraction and PCR-mixture, a major contributor to this divergence is the general difference in Ct-levels between real-time PCR runs. In the diagnostic application, the Ct-values should be standardised against the positive control to minimise the effect of this variation.

Although several properties of spiked semen were investigated with regard to detection, spiked samples still

might differ in some respect from field samples. The study would therefore benefit from including semen samples from infected bulls. Regrettably, such samples are not easily obtained. As the cattle in Sweden are practically free from paratuberculosis, there has been no infected Swedish bull from which to obtain semen. Even in countries with a high prevalence of paratuberculosis there are great difficulties to get semen samples from infected semen-donor bulls, as reflected by the sparse reports in the literature, where the infected semen samples tested came from very few infected bulls.

Many PCR-systems used to identify MAP target sites not entirely specific for the IS900 (Cousins *et al.* 1999; Englund *et al.* 2002). Therefore, a positive IS900 PCR result should be confirmed by using an additional PCR-system targeting another MAP-specific gene, for instance f57 (Poupart *et al.* 1993). Another approach is to sequence a long IS900-product amplified with nested PCR (Bull *et al.* 2003).

With real-time PCR it was possible to detect 10 accurately counted MAP bacteria per 100 μl semen, which is at least as good as culture. Even though it cannot be ruled out that naturally infected semen in some respect differs from spiked semen, it is most likely that the latter closely resembles field samples.

The prevalence and amount of MAP in semen from infected bulls is not known, and it can therefore not be estimated how effective this PCR on semen could be for diagnosis or screening of paratuberculosis in bulls. The practical use of this test is in first place to reduce the risk of transmission of MAP via semen. Especially when the bulls have not been adequately tested for paratuberculosis, this PCR method can be used on a batch of semen to ascertain that it is free from MAP, i.e. contains less than 10 organisms per dose.

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II

New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Background

Johne's disease, a serious chronic form of enteritis in ruminants, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). As the organism is very slow growing and fastidious, several PCR-based methods for detection have been developed, based mainly on the MAP-specific gene IS900. However, because this gene is similar to genes in other mycobacteria, there is a need for sensitive and reliable methods to confirm the presence of MAP. As described here, two new real-time PCR systems on the IS900 gene and one on the F57 gene were developed and carefully validated on 267 strains and 58 positive clinical faecal and tissue samples.

Results

Our confirmatory PCR systems on IS900 were found sensitive and specific, only yielding weak false positive reactions in two strains. The PCR system on F57 was shown to be entirely specific for MAP and only slightly less sensitive than our primary IS900-system. DNA from both naturally and artificially infected faeces that tested positive with our primary system could be confirmed with all new systems, except one weakly infected sample that proved negative on F57.

Conclusions

We recommend using the newly constructed DH3 PCR system on the F57 gene as the primary confirmatory test for PCR positives, but if it should fail due to its lower sensitivity, then the DH1 and DH2 PCR systems should be used.

Background

Paratuberculosis (Johne's disease) is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is a ubiquitous chronic enteric wasting disease of ruminants, though in Sweden the disease is rare or absent, thanks to successful control measures in the past. It is regarded as an exotic disease and falls under the Swedish Epizootic Act, which means that in an event of an outbreak, measures must be taken promptly to combat the disease and to trace the origin of the outbreak [1, 2]. When a positive case is identified, consequences for the farmer are grave and it is usually deemed necessary to slaughter the whole herd.

In the Swedish Paratuberculosis Control Program and in most of the Swedish surveillances undertaken to monitor freedom from paratuberculosis, detection is based on culture. During the culture procedure, suspected colonies are picked and identified as MAP by PCR. Other characteristics of the colony isolate act to confirm the identification made with PCR, namely acid-fast staining (acid-fast bacilli), growth characteristics (small, slow-growing) and dependence on mycobactin. The PCR methods generally used to identify and detect MAP are based on IS900, an insertion sequence considered specific for MAP [3-6]. IS900 is a 1,451 bp segment that lacks inverted terminal repeats and does not generate direct repeats in target DNA [7]. It belongs to the same family of insertion sequences as IS901, IS902, and IS1110, described in *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *avium*, respectively [8-10].

PCR based on IS900 has been used for direct detection of MAP, without primary culture, from milk, faecal specimens, semen, and human intestinal tissue [11-16]. Apart from being the method of choice when speed is a priority, direct PCR is also preferred when MAP is difficult or impossible to cultivate [17].

However, as IS900-like genes have been found in other unrelated *Mycobacterium* species, it is evident that the PCR systems used for IS900 are not completely specific for MAP [18-20]. In a recent investigation, a nested PCR on IS900 was shown to elicit false positive reactions from several mycobacterial strains [21]. It is therefore desirable to use alternative PCR systems to confirm a positive IS900 PCR for MAP. This is useful when PCR identification cannot be confirmed by conventional culture-based methods, as is the case with direct PCR and PCR identification of growth in liquid cultures [22, 23].

In the present paper, two new real-time PCR systems targeting other parts of IS900 and one real-time PCR targeting the F57 gene, specific for MAP [24], were developed and evaluated as confirming tests on strains and clinical samples, found positive with our standard IS900 PCR.

Results

Specificity

All MAP strains and isolates proved positive in the primary PCR system and were confirmed by all three confirmatory systems, as shown in Table 1. Listed in Table 2 are a variety of other mycobacteria, as well as a few other bacterial strains, that were tested with all four real-time PCR systems. With the primary PCR system, strain 2333 proved positive, as previously reported [20]. An isolate of *Mycobacterium avium* subsp. *avium* from a Swedish horse and a *Mycobacterium cubicae*-related isolate from cat gave weak positive reactions (i.e. high CT values) with the IS900 systems DH1 and DH2, respectively. All the other strains tested negative. The F57 system DH3 gave no false positive reactions.

Direct PCR on faecal samples

The procedure of first detecting MAP (or noting PCR inhibition) with the primary system, then confirming positives with the three other new systems, worked well with clinical samples. When inhibitors were still present, a dilution of the DNA or a new preparation of the original sample would usually solve the problem. When the sample produced a positive signal with the primary system, the same result was obtained with the confirmatory systems. However, as shown in Table 3, for 12 of 58 positive samples, re-runs with additional replicates had to be performed on the F57 in order to pick up the dispersed DNA, and for one weak culture-negative sample, DH3 failed to confirm the presence of MAP.

MAP could be detected in the spiked faeces using the primary system at a spiking level of 10^4 organisms/g and readily confirmed at the same concentration by all three confirmatory systems without requiring re-runs.

Sensitivity on pure DNA

The IS900 systems MP, DH1, DH2 and the F57 system DH3 yielded positive signals from DNA suspensions with the concentrations 0.1, 0.1, 0.3 and 1 MAP genomes/µl respectively.

Discussion

Real-time PCR is a sensitive method for detection that eliminates the need to open the tubes when analysing the product, a stage in other PCR techniques that often constitutes a risk of cross-contamination. In particular, the sensitive method nested PCR involves a great risk of contaminating other samples when the product from its first reaction is being transferred to the second. Another advantage of real-time PCR is that the use of probes enhances the specificity of the reaction, as an additional match with the target DNA will be required for a positive signal.

To confirm PCR positives for MAP, one of the most extensively used methods is sequencing of a part of the IS900 [25-27]. However, with current generally available technology, sequencing may not be the most practical method. To obtain a successful sequencing reaction, a relatively large amount of pure amplicon is needed, which weakly infected samples might not yield. Furthermore, sequencing is a laborious and expensive method [28], not suitable for confirmation of numerous positive samples. The sensitivity of real-time PCR makes it easy to use the original template DNA in reactions targeting other sites on IS900 or on other genes. Only when the template contains minute traces of MAP DNA or is slightly PCR inhibiting, can problems arise, as discussed below.

The two systems DH1 and DH2 were selected on the basis of minimal sequential similarity to related IS elements in other mycobacteria, such as the IS900 equivalent in strain 2333 [20], and minimal self-complementarity. It appears that the sequential similarity to strain 2333 predominates among the first 450 base pairs of IS900, which is also the area in which most of the systems suggested by the Primer3 software are found. Most previously described probe-based real-time PCR systems on the IS900 gene are also located there and thus, they have little or no possibility to discriminate against 2333 [29-32]. Many potential systems could therefore be excluded, after which DH1 and DH2 were selected from the remaining oligos. In contrast to the IS900, the F57 has no known similarities to genes on other related organisms, which made the task of selecting suitable oligos for the F57 less complicated.

It may appear simpler to change the primary system to one of the new systems, as they seem to be more specific than the MP system, but it should be noted that it is not certain that each of the other systems alone is entirely specific either. When using only one of the other systems, there is instead a risk of cross-reactions with other, unknown organisms having similarities in other parts of the MAP genome. The four systems eliminate many potential false positives by complementing each other, covering different parts of the IS900 – or, as for the DH3, a part of the F57 gene. However, the DH3 together with the primary system would probably be sufficiently

specific for the routine application, where DH1 and DH2 only need to be used if DH3 fails, as discussed below.

Previously published works on systems targeting F57 [33-35] have reported specificity for MAP and application of their systems on altogether 95 strains of MAP and 188 other strains. Our system DH3, targeting F57, was also entirely specific when applied to 112 strains of MAP and 155 other strains. It is however less sensitive than the systems on IS900, as there are 15 to 20 copies of the IS900 in the MAP genome [3] – and specifically 17 copies in strain K-10 [36]. This is consistent with the results of our sensitivity tests. A suspension of 0.1 MAP genomes/µl contains about 1.7 IS900 elements/µl. Since real-time PCR has been reported capable of detecting a single copy of the target gene [37-39], the successful detection of MAP in 2 to 2.5 µl of this suspension was expected. As there is only one single copy of F57 in the MAP genome, the template volume had to contain at least 1 genome for successful detection. In fact, at such low concentrations, the probability of detection drops well below 100% if an insufficient volume is tested. Use of Poisson distribution shows a 63% theoretical probability of at least 1 genome observed in 1 µl at a concentration of 1 genome/µl. In 2.5 µl of template, the probability of the same is 92% and when duplicates are run, it increases to 99.3%. Similarly, one can show that the probability of finding at least one IS900 in duplicates of 2 µl of the above-mentioned suspension (0.1 genomes/µl) is 99.9%. In reality however, any detection system is less than optimal and an occasional target copy may be lost in the process, which is why one must expect lower probability of detection at these low concentrations. DNA extracted from clinical samples may be highly complex and impure and have a slightly inhibitory effect on the PCR reaction in weak samples, even when a positive internal control indicates that inhibition should not be a problem.

In theory, the confirmatory systems could be combined and optimized to work as one single multiplex confirmatory PCR. Nevertheless, the systems were kept separate, as competition of reagents can occur in a multiplex system, thus lowering its sensitivity [40, 41].

If a clinical sample proves positive with the primary system, yet any of the three confirmatory systems shows negative, an investigation into the cause of this divergence must be undertaken. If the primary system elicited a strong signal and all laboratory errors can be excluded, the likelihood is that the result was false positive, produced by some other mycobacterial strain. In that case, it would probably be of interest to further investigate this strain! The other possibility is of course that the negative system is *not* sensitive to all MAP strains, in spite of our extensive testing.

However, when the positive signal is very weak, there may be several reasons why some of the confirmatory systems show negative. Because the genomes are sparse and attenuated in weak samples, a positive signal may be the outcome of low probability, thus impairing reproducibility. The confirmatory systems must then be employed again, this time using more replicates. In particular, the DH3 system on F57 will have a very low probability of detection at such concentrations. In rare cases, when even repeated analyses with additional replicates yield negative results, one will have to do without confirmation with F57 and instead take other aspects into account when judging the sample as true or false positive. In fact, it was notable that the F57 system could confirm the weakest detected level of spiked faeces (10^4 organisms/g) at the

first attempt. This was probably because the weaker concentration (10^3 organisms/g) was very close to the detection level. It yielded a few weak signals, but with unacceptably high CT values. Another considered explanation for occasional failure of any of the systems DH1-3 to confirm the primary system was that they might be more sensitive to inhibition than was the primary system, containing the internal control plasmid, especially as the confirmatory systems were developed with the use of a template volume of 2.5 µl instead of 2 µl. However, no such difference in robustness has yet been shown by the authors, and it is therefore assumed that they are equally sensitive to inhibition. Ultimately, when trying to confirm a positive test, one must be certain that a weak signal in one single system is not due to laboratory contamination.

The false reactions given by the *Mycobacterium avium* strain originating from horse and the *Mycobacterium cubicae*-related isolate from cat are most likely not a problem, as they were much weaker than would be expected when using template from a resuspended colony. A high CT value by confirmation of a normal-sized colony should alert the investigator that a cross-reaction or a contamination might have occurred. In contrast, when higher CT values are expected, as when direct-PCR is performed on clinical samples, these strains are unlikely to yield positive signals. But if they still do, the remaining systems will show them to be false.

Conclusions

After validation on several mycobacterial strains and on faecal and tissue samples, our new confirmatory systems were found to be both sensitive and reliable. We recommend using the DH3 PCR on the F57 gene as the primary confirmatory test on PCR positives, but if it fails due to its lower sensitivity, then the DH1 and DH2 PCR systems can be used.

Methods

Laboratory strains, growth conditions and extraction of genomic DNA

MAP strains were cultured for 8 weeks on modified Löwenstein-Jensen medium with mycobactin (4 mg/l, Allied Monitor, Fayette, MO, USA). Other mycobacterial strains were cultured on Löwenstein-Jensen medium at 37° or 30°C for up to 6 weeks.

Laboratory strains and their various origins are shown in Tables 1 and 2. The following MAP strains have defined RFLP subtypes: strain Telford 9.2 (RFLP type *S1*) from R.J. Whittington and strains 5001-1425 (RFLP type *B-C12*), P1850/1/97 *(A-C10*), 17 *(Z-C18),* M211 *(H-C1*), 5TSD *(B-C17*), 93/433 *(B-C19*), P1611-15 *(B-C13),* 4064 (*B-C1*), 1038 (*B-C12*), 6/922 (*B-C2*), 6256 (*D-C12*), 7954 (*B-C16*) 25071 (*B-C13*), 9602 (*E-C1*), 9944 (*E-C1*), K126 (*B-C17*), 6042 (*D-C12*), M212 (*B-C2),* from I. Pavlik. Isolates of MAP (*n*=20) from Sweden have been RFLP typed as *B-C1.*

Purified MAP-DNA was obtained from ATCC 19698 (American Type Culture Collection, Rockville, MD, USA) by beadbeating with zirconia/silica beads (0.1 mm, BioSpec Products, Inc., Bartlesville, OK, USA) and phenol/chloroform extraction. However, less pure DNA for confirmation of identity with real-time PCR was

obtained by centrifugation of resuspended MAP colonies, heat-killed at 99°C for 10 min.

Field isolates

Single colonies, suspected to be MAP, were isolated from faecal cultures on modified Löwenstein-Jensen medium [42] or on Herrolds Egg Yolk medium, both supplemented with mycobactin (Table 1). Other mycobacterial isolates, obtained from the TB laboratory at SVA, had been isolated on Löwenstein-Jensen, Stonebrink, or Middlebrook medium (Table 2).

Direct-PCR on faeces

Template DNA for direct testing with real-time PCR was extracted from bovine faeces. The protocol involved lysing of the bacteria by beadbeating with zirconia/silica beads and purification of the lysate with a modified QIAamp protocol (QIAamp DNA Stool Mini Kit, Qiagen). Samples were obtained from External Quality Assessments from USA, Denmark and the Czech Republic. Some Swedish clinical samples as well as artificially contaminated faeces were also tested. The latter was spiked with different dilutions of dispersed MAP bacteria, washed free of excessive free DNA and carefully quantified in a microscope as previously described [16] to avoid overestimation of the analytical sensitivity. The final concentrations of MAP in the spiked faeces were $10^2 – 10^7$ organisms/g.

Real-time PCR based on IS900 and F57

Our primary real-time PCR system used to detect MAP in the samples was the previously described MP system [16], which includes an internal control for indication of PCR inhibition. When positive, the presence of MAP was confirmed with three newly constructed systems: two based on IS900 and one based on the F57 gene. The free on-line primer design software Primer3 [43] was used to find potential primers and probes. From these, the two systems DH1 and DH2 were selected on IS900 and DH3 on F57. Candidate sequences were compared with other known genes using the BLAST Sequence Analysis Tool to check for incidental similarities. Selected oligos are listed in Table 4.

The PCR mixture comprised 6.625 µl H_2O (Sigma-Aldrich) in the case of the primary MP system and 8.625 µl H_2O in the confirmatory systems, 2.0 µl glycerol (Sigma-Aldrich), 2.5 µl 10x PCR-buffer II (Applied Biosystems, Foster City, CA, USA), 5.0 µl $MgCl_2$ (25 mmol/l, Applied Biosystems), 2.0 µl GeneAmp® dNTP with UTP (2.5 mmol/l dA, C, GTP, 5 mmol/l dUTP, Applied Biosystems), 0.75 µl forward primer (10 pmol/µl), 0.75 µl reverse primer (10 pmol/µl), 0.5 µl MAP-specific probe (10 pmol/µl), 0.5 µl mimic-specific probe (10 pmol/µl) in the case of the primary MP-system, 0.125 µl AmpliTaq Gold® (5 U/µl, Applied Biosystems) and 0.25 µl AmpErase® (Uracil N-glycosylase, 1 U/µl, Applied Biosystems). The addition of glycerol allowed freezing of the PCR mixture. In the case of the MP system, 2 µl of template DNA and 2 µl of mimic molecule pWIC9 (150 fg/µl) [16] were added to each reaction tube, except for the PCR-negative controls. In the case of the confirmatory systems, no mimic molecule but 2.5 µl of template DNA was added.

The real-time PCR reaction was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) with the following program: 50ºC 2 min, 95ºC 10 min, repeat 95ºC 15 s and 60ºC 1 min, 45 times. The results were analysed with the Rotor-Gene software versions 5 and 6 and the built-in analytical tools Dynamic Tube Normalisation and Slope Correction. Real-time PCR curves of normalized fluorescence for FAM crossing a threshold value (T) of 0.01 at less than 40 cycles were considered positive, as long as the curves had a normal and expected shape. FAM-negative curves with a positive corresponding ROX curve (i.e. a positive mimic signal) were considered as true negatives; otherwise, inhibition was suspected. DNA extracted directly from faeces was run in duplicates.

If a slightly infected clinical sample yielded positive results with all systems except the DH3 on the F57, additional runs were performed; first with five replicates, then if still negative, with 15 replicates.

Sensitivity test on pure DNA

The concentration of purified MAP-DNA was determined with NanoDrop (Wilmington , DE, USA) and low, specific concentrations were obtained by serial dilution. At the lowest concentrations (less than 100 genomes per µl) the DNA was diluted in half-multiples of ten ($10^{0.5}$) for better resolution of the sensitivity measurement. Real-time PCR was run in duplicates on DNA suspension ranging from $10^{-1.5}$ (~0.03) to 10^{3} MAP genomes per µl with all four PCR systems described in Table 4.

Authors' contributions

DH participated in the design of the study, designed the PCR systems, carried out the laboratory work and drafted the manuscript. GB conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Strains of *Mycobacterium avium* subsp. *paratuberculosis*, tested with the primary and confirmatory PCR systems.

No. of samples	Source	Origin	IS900 MP	IS900 DH1	IS900 DH2	F57 DH3
77	Cattle	Various[a]	+	+	+	+
19	Goat	Various[b]	+	+	+	+
4	Sheep	Various[c]	+	+	+	+
1	Deer	Czech Rep.	+	+	+	+
1	Human	ATCC 49164	+	+	+	+
1	Human	ATCC 43015	+	+	+	+
1	Cattle	NCTC 8578	+	+	+	+
1	Human	Czech Rep.	+	+	+	+
1	Cattle	CIP 107488	+	+	+	+
1	Cattle	ATCC 19698[T]	+	+	+	+
5	Unknown	Various[d]	+	+	+	+

a. Sweden (13), USA (28), Denmark (12), Czech Rep. (16), Switzerland (1), Norway (2), Slovak Rep. (2), Argentina (2), Great Britain (1)

b. Norway(16), UK (1), Greece (1), New Zeeland (1)

c. Australia (1), USA (1), Faroe Islands (1), Czech Rep. (1)

d. Denmark (4), Czech Rep.(1)

T. Type strain

Table 2: Non-target strains, tested with the primary and confirmatory PCR systems.

No. of Samples	Species	Source	Origin	IS900 MP	IS900 DH1	IS900 DH2	F57 DH3
1	MAA[a]	Horse	Sweden	-	+	-	-
1	MAA	Human	ATCC 35718	-	-	-	-
1	MAA	Domestic fowl	ATCC 25291T	-	-	-	-
6	MAA	Various[b]	Various[c]	-	-	-	-
1	*Mycobacterium* species (strain 2333)	Cattle	Sweden	+	-	-	-
1	*Mycobacterium* species[d]	Cat	Sweden	-	-	+	-
1	*Mycobacterium kansasii*	Human	ATCC 12478^T	-	-	-	-
1	*Mycobacterium smegmatis*	Cattle	GB	-	-	-	-
1	*Mycobacterium chelonei*	Cattle	Sweden	-	-	-	-
1	*Mycobacterium fortuitum*	Cattle	Sweden	-	-	-	-
1	*Mycobacterium bovis*	Cattle	Sweden	-	-	-	-
1	*Mycobacterium bovis*	Cattle	ATCC 19210^T	-	-	-	-
1	*Mycobacterium silvaticum*	Dove	Sweden	-	-	-	-
1	*Mycobacterium intracellulare*	Sparrow	ATCC 35767	-	-	-	-
1	*Mycobacterium intracellulare*		ATCC 13950^T	-	-	-	-
1	*Mycobacterium intracellulare*	Cattle	ATCC 35771	-	-	-	-
1	*Mycobacterium scrofulaceum*	Human	ATCC 19981^T	-	-	-	-
1	*Mycobacterium gordonae*	Human	ATCC 14470^T	-	-	-	-
1	*Mycobacterium marinum*	Guppy	Sweden	-	-	-	-
2	*Mycobacterium marinum*	Dolphin	Sweden	-	-	-	-
1	*Mycobacterium malmoense*	Human	CIP 105775^T	-	-	-	-
1	*Mycobacterium celatum*	Human	CIP 106109	-	-	-	-
1	*Mycobacterium flavescens*	Guinea pig	CIP 104533	-	-	-	-
1	*Mycobacterium komossense*	Sphagnum moss	CIP 105293^T	-	-	-	-
1	*Mycobacterium marinum*	Saltwater fish	CIP 104528^T	-	-	-	-
1	*Mycobacterium terrae*	Human	CIP 104321^T	-	-	-	-
1	*Mycobacterium vaccae*	Cattle	CIP 105934^T	-	-	-	-
1	*Mycobacterium xenopi*	Toad	CIP 104035^T	-	-	-	-
1	*Mycobacterium* species[e]	Turtle	Sweden	-	-	-	-
1	*Mycobacterium porcinum*	Swine	CIP 105392	-	-	-	-
1	*Mycobacterium porcinum*	Cattle	Italy	-	-	-	-
1	*Mycobacterium hiberniae*	Soil	CIP 104537	-	-	-	-
1	*Mycobacterium cookii*	Sphagnum moss	ATCC 49103	-	-	-	-
110	*Mycobacterium* species	Various[f]	Various[g]	-	-	-	-
1	*Staphylococcus aureus*		ATCC 25923	-	-	-	-
1	*Streptococcus dysgalactiae*	Cattle	CCUG 27436	-	-	-	-
1	*Streptococcus uberis*	Cattle	CCUG 27444	-	-	-	-
1	*Escherichia coli*		ATCC 25922	-	-	-	-
1	*Arcanobacter pyogenes*	Cattle	CCUG 39326	-	-	-	-
1	*Pseudomonas aeruginosa*	Human	CCUG 17619	-	-	-	-

a. *Mycobacterium avium* subspecies *avium*

b. Cat (1), Cattle (1), Deer (1), Sheep (3)

c. Sweden (3), Faroe Islands (1), Iceland (2)

d. *Mycobacterium kubicae*-related strain

e. *Mycobacterium nonchromogenicum*-related strain

f. Cattle (59), Sheep (23), Monkey (1), Deer (2), Buffalo (1), Horse (1), Elephant (3), Ciclide (1), Antelope (1), Alpaca (2), Environmental (1), Cell culture (1), Peat (1)

g. Sweden (82), Czech Republic (3), USA (11), Australia (2), Denmark (11), Great Britain (1)

T. Type strain

Table 3: Confirmation of samples of bovine feces, positive for MAP with the primary PCR.

Samples	*n*	Confirmed*	Additional 5 replicates†	Additional 20 replicates‡	F57 failure§
Culture-confirmed	49	43	4	2	0
Not culture-confirmed	9	3	5	0	1

* Confirmed by all three alternative systems, using two PCR replicates

† Confirmed by F57, only after running 5 additional PCR replicates

‡ Confirmed by F57, only after running 20 additional PCR replicates

§ Confirmation with the F57 system failed after a total of 22 PCR replicates

Table 4: Primers and probes in the primary and confirmatory real-time PCR systems.

Oligo	Nucleotide sequence	5' annealing position*	Product length
MPF	5'-CCG CTA ATT GAG AGA TGC GAT T-3'	139	115
MPR	5'-CCA GAC AGG TTG TGC CAC AA-3'	253	
MP probe	5'-6-FAM-ACC TCC GTA ACC GTC ATT GTC CAG ATC A-BHQ-1-3'	231	
DH1F	5'-AAG ACC GAC GCC AAA GAC-3'	536	69
DH1R	5'-CAG AGG CTG CAA GTC GTG-3'	604	
DH1 probe	5'-6-FAM-GAT CAT CGC CGA TCA GGC CC-BHQ-1-3'	559	
DH2F	5'-GCC TTC GAC TAC AAC AAG AGC-3'	737	65
DH2R	5'-GCG TCG GGA GTT TGG TAG-3'	801	
DH2 probe	5'-6-FAM-GCC GCG CTG ATC CTG CTT ACT-BHQ-1-3'	761	
F57F	5'-AAC TAA GCG GAT CGA CAA TTC-3'	493	80
F57R	5'-TGG TGT ACC GAA TGT TGT TG-3'	572	
F57 probe	5'-6-FAM-TGC AAC TCG AAC ACA CCT GGG A-BHQ-1-3'	519	

* According to deposited sequences X16293 and X70277 in GenBank