Abstract

Englund, S. 2002. Molecular Biology Techniques as a Tool for Detection and Characterisation of *Mycobacterium avium* subsp. *paratuberculosis*. Doctoral dissertation. ISSN 1401-6257, ISBN 91-576-6366-1.

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative agent of paratuberculosis, also known as Johne's disease, a chronic intestinal infection in cattle and other ruminants. Paratuberculosis is characterised by diarrhea and weight loss that occurs after a period of a few months up to several years without any clinical signs. The considerable economic losses to dairy and beef cattle producers are caused by reduced milk production and poor reproduction performance in subclinically infected animals. Early diagnosis of infected cattle is essential to prevent the spread of the disease. Efforts have been made to eradicate paratuberculosis by using a detection and cull strategy, but eradication is hampered by the lack of suitable and sensitive diagnostic methods.

This thesis, based on five scientific investigations, describes the development of different DNA amplification strategies for detection and characterisation of *M. paratuberculosis*. Various ways to pre-treat bacterial cultures, tissue specimens and fecal samples prior to PCR analysis were investigated. Internal positive PCR control molecules were developed and used in PCR analyses to improve the reliability and facilitate the interpretation of the results. The sensitivity of the ultimate methods was found to be approximate that of culture and allowed detection of low numbers of *M. paratuberculosis* expected to be found in subclinically infected animals.

Genomic DNA of a Swedish mycobacterial isolate, incorrectly identified by PCR as *M. paratuberculosis* was characterised. The isolate was closely related to *M. cookii* and harboured one copy of a DNA segment with 94% similarity to IS*900*, the target sequence used in diagnostic PCR for detection of *M. paratuberculosis*. This finding highlighted the urgency of developing or evaluating PCR systems based on genes other than IS*900*.

A PCR-based fingerprinting method using primers targeting the enterobacterial intergenic consensus sequence (ERIC) and the IS*900* sequence was developed and successfully used to distinguish *M. paratuberculosis* from closely related mycobacteria, including the above mentioned mycobacterial isolate.

In conclusion, the molecular biology techniques developed in these studies have proved useful for accelerating the diagnostic detection and characterisation of *M. paratuberculosis*.

Keywords: Animal; bacterial infection; diagnosis; fastidious bacteria; microbiology; sensitivity and specificity

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Contents

Appendix

This thesis is based on the following five papers, which will be referred to by their roman numerals.

I. Englund, S., Ballagi-Pordány, A., Bölske, G. and Johansson, K.-E. 1999. Single PCR and nested PCR with a mimic molecule for detection of *Mycobacterium avium* subsp. *paratuberculosis.* Diagnostic Microbiology and Infectious Disease 33: 163-171.

II. Englund, S., Bölske, G., Ballagi-Pordány, A. and Johansson, K.-E. 2001. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples by single, fluorescent and nested PCR based on the IS*900* gene. Veterinary Microbiology 81: 257-271.

III. Halldorsdottir, S., Englund, S., Fredsvold Nilsen, S. and Olsaker, I. 2002. Detection of *Mycobacterium avium* subsp. *paratuberculosis* by buoyant density centrifugation, sequence capture PCR and dot-blot hybridisation. (Submitted for publication).

IV. Englund, S., Bölske, G. and Johansson, K.-E. 2002. An IS*900*-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. FEMS Microbiology Letters 209: 261-265.

V. Englund, S. IS*900*/ERIC-PCR as a tool for distinguishing *M. avium* subsp. *paratuberculosis* from closely related mycobacteria. (Manuscript).

Abbreviations

AFLP	amplified fragment length polymorphism
AGID	agar gel immunodiffusion
AP	alkaline phosphatase
ATCC	American type culture collection
bp	base pair
BSA	bovine serum albumin
CF	complement fixation
CFU	colony forming units
DR	direct repeats
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
ERIC	enterobacterial repetitive intergenic consensus sequence
IFN-γ	interferon gamma
IS	insertion sequence
M. paratuberculosis	*M. avium* subsp. *paratuberculosis*
nt	nucleotide
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PGRS	polymorphic GC-rich repetitive DNA elements
PPD	purified protein derivate
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal DNA
REA	restriction enzyme analysis
rep-PCR	repetitive element PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
rrn	ribosomal RNA operon
tRNA	transfer RNA

Introduction

In 1895 Jöhne and Frothingham described the first case of paratuberculosis in cattle, which also became known as Johne's disease (Jöhne & Frothingham, 1895). They assumed that the acid-fast bacilli found in the intestine of the diseased animal were an atypical variant of the mycobacterium causing disease in birds, later known as *Mycobacterium avium.* Ever since then their assumption has been extensively investigated and debated in the scientific community. The causative agent was isolated in 1910 by Twort and Ingram who named it *Mycobacterium enteritidis chronicae pseudotuberculosis bovis,* Jöhne, with the intention of characterising the disease it caused in cattle (Twort & Ingram, 1912). The name was later altered to *Mycobacterium paratuberculosis* and so used until 1990, when studies based on numerical taxonomy showed *M. paratuberculosis* to be closely related to *M. avium* and hence the name reverted to *Mycobacterium avium* subsp. *paratuberculosis* (Saxegaard & Baess, 1988; Thorel et al., 1990). For the reader's convenience, and that of myself, the appellation *M. paratuberculosis* is used in this thesis.

Fig. 1.
Guernsey cow with clinical signs of paratuberculosis observed as diarrhea and weight loss. Affected animals remain bright and alert, without fever, and eating well. *This photo was republished with kind permission from the University of Wisconsin-Madison, School of Veterinary Medicine, USA*.

Paratuberculosis

M. paratuberculosis causes slowly developing granulomatous enteritis, primarily in cattle, goats and sheep, but the infection also occurs in other ruminants and wildlife such as deer, rabbits, and foxes (Beard et al., 1999; Beard et al., 2001; Chiodini et al., 1984; Greig et al., 1999). The clinical symptoms are observed as intermittent or chronic diarrhea and weight loss. The disease is characterised by a long period free from any clinical signs in infected animals. This period can range from a few months up to several years, but usually the disease appears within 3 to 5 years after infection. It may therefore take several years before a herd is recognised as infected (Chiodini et al., 1984). Not all animals exposed to the bacterium become infected and some may remain as asymptomatic carriers, that is, shedding *M. paratuberculosis* but never developing clinical signs. The existence of asymptomatic carriers and subclinically infected animals makes it difficult to combat and eradicate the disease. The possible transmission of paratuberculosis from wildlife to domestic animals and the long survival time of *M. paratuberculosis* in the environment are also important factors that have to be taken into account in control and eradication programmes (Collins et al., 2001; Greig et al., 1999; Larsen et al., 1956; Lovell et al., 1944). The major hinder to successful eradication of paratuberculosis is the lack of tests, sensitive enough to detect infected animals in the earliest stage of disease (Whitlock & Buergelt, 1996).

The *first stage* is characterised by a silent infection without clinical signs. No or minimal quantities of *M. paratuberculosis* may be shed. In some cases, infection might be detected by tests based on the cell-mediated immune response.

In *stage two*, the subclinical phase, there are no visible signs of the disease. Adult animals may bear detectable antibodies to *M. paratuberculosis* and/or a modified cellular immune response and may shed low to moderate numbers of *M. paratuberculosis*. In this stage a part of infected animals are detectable by available diagnostic techniques.

In *stage three*, clinical signs such as weight loss and diarrhea appear. Most animals test positive in faecal culture, and some serological tests.

Stage four is the final stage of the disease characterised by advanced clinical symptoms such as emaciation, dehydration and profuse shedding of *M. paratuberculosis*. These animals are easily detected by fecal culture, and serological tests and can be culled from the herd immediately on detection.

Transmission

M. paratuberculosis is shed by infected animals into manure, milk and colostrum. Transmission occurs by ingestion of fecally contaminated feed, water, or milk. Young calves, especially newborn, are most susceptible to infection because of their immature immune defence and the risk of ingesting large volumes of infected milk by suckling from an infected dam who excretes *M. paratuberculosis* into the milk, or has faecally contaminated teats (Hagan, 1938; Larsen et al., 1975). Other theoretically possible transmission routes are semen of an infected bull, embryo transfer, or from wildlife ruminant reservoirs (Sweeney, 1996).

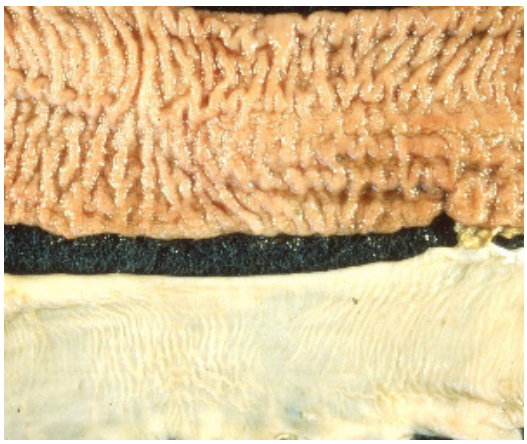

Fig. 2. Pathological changes. The upper part of the photo shows an ileum thickened due to inflammatory response caused by *M. paratuberculosis* infection. Below is a piece of a normal non-infected ileum. *This photo was republished with kind permission from the University of Wisconsin-Madison, School of Veterinary Medicine, USA.*

Pathogenesis

The infection route of *M. paratuberculosis* is not entirely understood. The current hypothesis is that after the organism has been ingested, it crosses from the lumen of the small intestine into the lymphoid system. It is then taken up by macrophages in the wall of the intestine and in regional lymph nodes. Within the macrophages the mycobacteria remain viable and replicate and they are protected against humoral factors. This initial subclinical stage can last for months to years as long as the mycobacteria are contained within the macrophages. During this time, minimal numbers of *M. paratuberculosis* are shed into feces, but as the infection develops the number of organisms gradually increases. Over time the environment becomes heavily contaminated and the infection spreads throughout the herd (Chiodini et al., 1984). It is not clear what makes the disease progress to the clinical and terminal stage, but stress factors such as parturition or heavy milk production might contribute. The clinical phase is characterised by chronic or intermittent diarrhea and sudden weight loss. The malabsorption of proteins is due

to the influx of inflammatory cells into the intestinal wall, which thickens and corrugates until it is no longer functional (Buergelt et al., 1978). The clinical stage can last from 3 to 6 months or longer but the disease invariably leads to the death of the animal if not slaughtered (Manning & Collins, 2001). This stage is also characterised by reduced milk production and infertility and prolific fecal shedding of the microorganism (Chiodini et al., 1984). The animal's age at the time of exposure and the dose of organisms ingested probably determine the duration of each stage of the infection (Larsen et al., 1975).

Immunology

In the early stage of the infection the cell-mediated immune response predominates, but as the disease progresses towards the final clinical stage, humoral immune responses take over (Bendixen, 1978; Chiodini et al., 1984; Chiodini, 1996). Asymptomatically infected animals are therefore difficult to identify by immunological methods such as the complement fixation test (CF), the agar gel immunodiffusion test (AGID), and the cutaneous test with johnin, a *M. paratuberculosis* sensitin produced as PPD tuberculins. In addition, low sensitivity and cross-reactions with other mycobacteria sometimes hamper a correct interpretation of the immunological test results (Merkal et al., 1968; Bendixen, 1977). In some cases animals with obvious clinical symptoms may lack humoral antibodies and cellular reactivity due to anergy (Chiodini, 1996). It is thought that the observed clinical findings are more probably caused by the immunological reactions in the host rather than by the infectivity or metabolic activity of the organism (Bendixen, 1978).

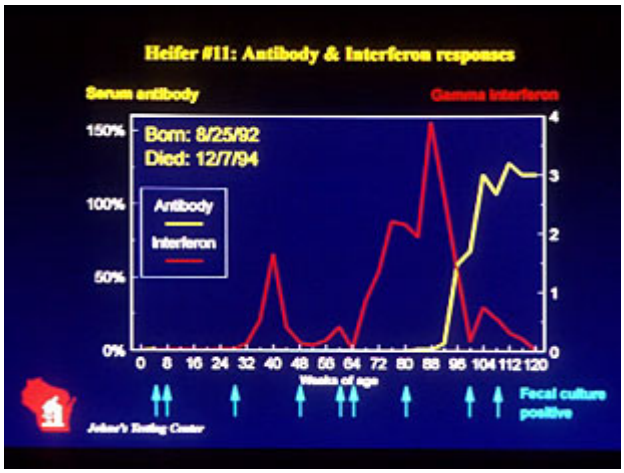

Fig. 3. Heifer experimentally infected with *M. paratuberculosis* and tested every 4th week for paratuberculosis using BACTEC fecal culture, ELISA for serum antibody and the gamma interferon test.

Serum antibody

Gamma interferon

Faecal culture positive

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Treatment

Antimicrobial treatment is not regarded as a viable option as it merely alleviates the clinical signs and the shedding of the organism, but does not eliminate the infection. Moreover, the drugs are expensive and need to be administered over extended periods and when the therapy is discontinued the disease will progress (Stabel, 1998). Vaccination, with live and killed vaccines, has been used since 1926 but has not succeeded in defeating paratuberculosis. The vaccinated animals still shed the organism and are positive in antibody tests, which may interfere with serological testing for paratuberculosis or tuberculosis. Vaccination of calves does not prevent transmission of *M. paratuberculosis* and is therefore used in endemic herds only to reduce the number of animals developing clinical disease and to restrict the shedding of *M. paratuberculosis* (Chiodini, 1996; Kormendy, 1994). Instead, interest is focused on herd management, which is by far the most important factor to hold back the spread of paratuberculosis (Kalis et al., 2001).

Prevalence

Paratuberculosis is universally endemic and causes serious economic losses due to reduced milk production and poor reproduction performance in animals with subclinical disease. Animals showing clinical symptoms are usually culled from the herd and exacerbate the economic loss because of increased cow-replacement costs. The herd prevalence of bovine paratuberculosis in the European countries ranges from 7% up to 55% (Kennedy et al., 2001). In the USA the infection is spread throughout the country and the prevalence in herds of more than 300 animals is close to 40% (Weels & Wagner, 2000). A less even distribution of paratuberculosis has been observed in Australia, where the infection rate in dairy herds is 22% in the state of Victoria, but only 9% in New South Wales (Kennedy & Allworth, 2000). Paratuberculosis is a great problem in the Australian sheep industry.

In Sweden, paratuberculosis has been regulated since 1952 by the law on epizootics, which requires suspected cases to be reported to the veterinary authorities, the herd to be placed under restriction and full economic compensation is consequently offered to the owner. When *M. paratuberculosis* is detected in a single animal the whole herd has to be slaughtered, the premise disinfected, and up to 3 years of quarantine of pastures is enforced (Engvall et al., 1994). During the period 1962-92, no cases of paratuberculosis were reported in Sweden, but in 1993 an imported cow showed clinical signs of paratuberculosis. The source of the infection was traced back to animals imported in the mid- 1980s and resulted in a number of surveys to investigate the prevalence of the infection in Swedish cattle (Bölske et al., 1999; Viske et al., 1996). A voluntary state-funded control program was introduced in 1998 to ascertain the extent of the infection in beef production. So far, in this program, more than 35, 000 fecal samples have been analysed from over 800 herds and only one case of paratuberculosis has been found. This observation confirms previous surveys, where the prevalence of *M. paratuberculosis* in Sweden was demed to be below

1% (Viske et al., 1996). As yet, *M. paratuberculosis* has not been detected in any dairy cattle in Sweden (Holmström et al., 2000).

Zoonotic aspects

M. paratuberculosis is suspected to be the causative agent of Crohn's disease, a chronic inflammatory bowel disease in man (Chamberlin et al., 2001; Chiodini, 1989; Chiodini & Rossiter, 1996; Hermon-Taylor et al., 2000). The symptoms of Crohn's disease do to some extent resemble those of paratuberculosis. A majority of the patients require surgical resection of the damaged intestine and this often has to be repeated. The mortality is about 6% and the lifelong suffering from this painful and incurable syndrome makes it a fearful disease. So far, the etiological relationship between *M. paratuberculosis* and Crohn's disease has not been confirmed and further investigation is needed (Harris & Lammerding, 2001). The finding of *M. paratuberculosis* in milk has raised the questions as to whether or not milk could act as a reservoir for exposure of the general public to *M. paratuberculosis* (Giese & Ahrens, 2000; Millar et al., 1996; Streeter et al., 1995). Moreover, laboratory-scale and commercial-scale studies on pasteurisation of *M. paratuberculosis*-containing milk indicated that the mycobacterium could survive this heat treatment (Grant et al., 2002; Lund et al., 2000; Pearce et al., 2001; Stabel, 2000; Stabel, 2001).

The genus Mycobacterium

The genus *Mycobacterium* comprises over 100 species many of which are obligate parasites, saprophytes, or opportunistic pathogens. The mycobacteria are found in environments such as water and soil but also in warm-blooded and cold-blooded animals. Among the mycobacteria of importance in veterinary medicine are the slow-growing mycobacteria found in two major complexes, *M. tuberculosis* and *M. avium* (Runyon et al., 1974). The *M. tuberculosis* complex includes of *M. tuberculosis* and *M. bovis*. *M. tuberculosis* causes tuberculosis in man, primates, dogs and other animals. *M. bovis*, also known as the bovine tubercle bacillus, causes tuberculosis in cattle, domestic and wild ruminants, man and other primates, swine and other animals. The *M. avium* complex comprises *M. avium* subsp. *avium* (*M. avium*), *M. avium* subsp. *silvaticum* (*M. silvaticum*), *M. paratuberculosis,* and *M. intracellulare*. Organisms in the *M. avium* complex, except for *M. paratuberculosis*, are frequently found and widely distributed in the environment. *M. avium*, especially the virulent serotype 2, cause avian tuberculosis. In humans, patients with HIV infections are frequently encountered with *M. avium-M. intracellulare* infections (Nolte & Metchock, 1995). *M. intracellulare* is not as pathogenic as *M. avium* and it rarely causes disease in birds, but does cause severe pulmonary diseases in man and is sometimes isolated from swine and cattle. *M. silvaticum*, also known as the wood-pigeon mycobacterium, has not been isolated from the environment and it is an obligate pathogen for birds. *M. paratuberculosis* is an obligate parasite, a strict pathogen for ruminants but is not free living in the environment.

M. leprae is of major importance in human medicine as an obligate pathogen and this species cannot be grown *in vitro*. For that reason, it has been practically impossible to compare *M. leprae* to other mycobacteria by conventional numerical methods.

Genome sequencing projects

Little is known about the gene function in mycobacteria, but the number of whole genome sequencing projects will substantially contributed to new knowledge in this area of the fastidious mycobacteria. The complete genome sequence has been determined for *M. tuberculosis* (Cole et al., 1998), *M. leprae* (Cole et al., 2001), and *M. bovis* (http://www.sanger.ac.uk/Projects/M_bovis/: Accessed 31-Mar-2002). Genome projects underway are *M. avium* (TIGR, http://www.tigr.org/: Accessed 31-Mar-2002), *M. paratuberculosis* (University of Minnesota, http://www.cbc.umn.edu/ResearchProjects/Ptb/: Accessed 31-Mar-2002), and *M. smegmatis* (TIGR, http://www.tigr.org/: Accessed 31-Mar-2002).

Phenotypic characteristics

The organisms within the genus *Mycobacterium* are characterised as aerobic, non-motile, acid-alcohol-fast rods, having mycolic acids in their cell walls, and a DNA guanine-plus-cytosine (G+C) content of 61 to 71 mol% (Levy-Frebault & Portaels, 1992; Runyon et al., 1974). Depending on the species, the optimum growth temperature ranges between 30° and 45ºC (Wayne, 1985). Mycobacteria often grow by aggregating into clumps, which for some species give the characteristic dry breadcrumb-like appearance on solid media. Of all known bacteria, the mycobacteria are probably the ones with the highest lipid composition. Mycobacterial mycolic acids are possibly the most characteristic component of the mycobacterial cell wall and are recognised by their long branched carbon atom chains (60 to 90 carbon atoms) and high molecular weight. The sugar constituents of the cell wall are arabinose and galactose. Together with other lipophilic molecules these form an envelope, which effectively encapsulates the rest of the cell. The cell wall composition contributes to the hydrophobicity and the resistance to acids, alkalis and other disinfectants used to kill other bacteria. It also provides the mycobacteria with a highly resistant protection against the bactericidal mechanisms of the macrophages. Cell wall deficient forms of mycobacteria, spheroplasts, have been encountered in cultures of human tissues (Chiodini, 1989).

The mycobacteria can be divided into two major categories, based on their growth rate. Rapid-growing mycobacteria form visible colonies within 7 days under ideal culture conditions from a dilute inoculum. Slow-growing mycobacteria form visible colonies after 7 days or more under comparable conditions (Goodfellow & Wayne, 1982). Slowly growing species are often pathogenic for humans and other animals, whereas rapidly growing species are usually considered non-pathogenic, though there are exceptions (Shinnick & Good, 1994). While the rapid growers are metabolically active and easy to

cultivate, the slow-growing mycobacteria are more difficult to cultivate and analyse.

Taxonomy of the slow-growing mycobacteria

Bacterial taxonomy is defined as classification, nomenclature and identification. According to the new taxonomy of bacteria (http://www.cme.msu.edu/Bergeys/: Accessed 3-Apr-2002), mycobacteria belong to the phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, suborder *Corynebacterineae*, family *Mycobacteriaceae,* and genus *Mycobacterium*.

Analysis of the lipid composition of the mycobacterial cell wall by gas-liquid chromatography has been used to classify mycobacteria and efforts have been made by this method to differentiate *M. paratuberculosis* from other closely related mycobacteria (Chiodini & van Kruiningen, 1985; Levy-Frebault et al., 1986; Levy-Frebault & Portaels, 1992; Saxegaard et al., 1988). However, fatty acid analysis alone is not enough to identify mycobacteria to the species level, but it can be combined with other criteria for classification.

The mycobacteria may be divided into four groups based on pigmentation and growth rate according to the Runyon classification (Levy-Frebault & Portaels, 1992; Sommers & Good, 1985; Wayne & Kubica, 1986). Group 1 consists of the photochromogenic slow growers whose pigmentation is light dependent. Group 2 are the scotochromogenic slow growers whose pigmentation is light independent. Group 3 consists of the non-chromogenic (non-pigmented) slow growers, finally, group 4, consists of the rapid growing mycobacteria.

The *M. avium* complex

The organisms in the *M. avium* complex belong to the non-chromogenic group (Nolte & Metchock, 1995). Because these mycobacteria may be lightly or moderately pigmented, they can be misinterpreted as group 2 scotochromogenic organisms, making the Runyon groups less useful for species classification (Sommers & Good, 1985).

The genetic relationship of different mycobacteria can be investigated by DNA–DNA hybridisation experiments where the thermal stability of DNA–DNA hybrids is measured (Baess, 1983; Yoshimura & Graham, 1988). Strains with DNA-relatedness values exceeding 90% belong to the same species, whereas values below 80% indicate different species (Baess, 1985; Saxegaard & Baess, 1988; Saxegaard et al., 1988). Based on DNA–DNA hybridisation, *M. avium*, *M. paratuberculosis*, and *M. silvaticum* form a genetically homogeneous cluster and they have therefore been placed in the single genomic species *M. avium* (Hurley et al., 1988; Saxegaard & Baess, 1988; Saxegaard et al., 1988; Yoshimura & Graham, 1988). However, the phenotypic properties are rather heterogeneous and taken into account the pathogenicity and host range characteristics *M. avium* has been divided into three subspecies (Thorel et al., 1990). The distinction between

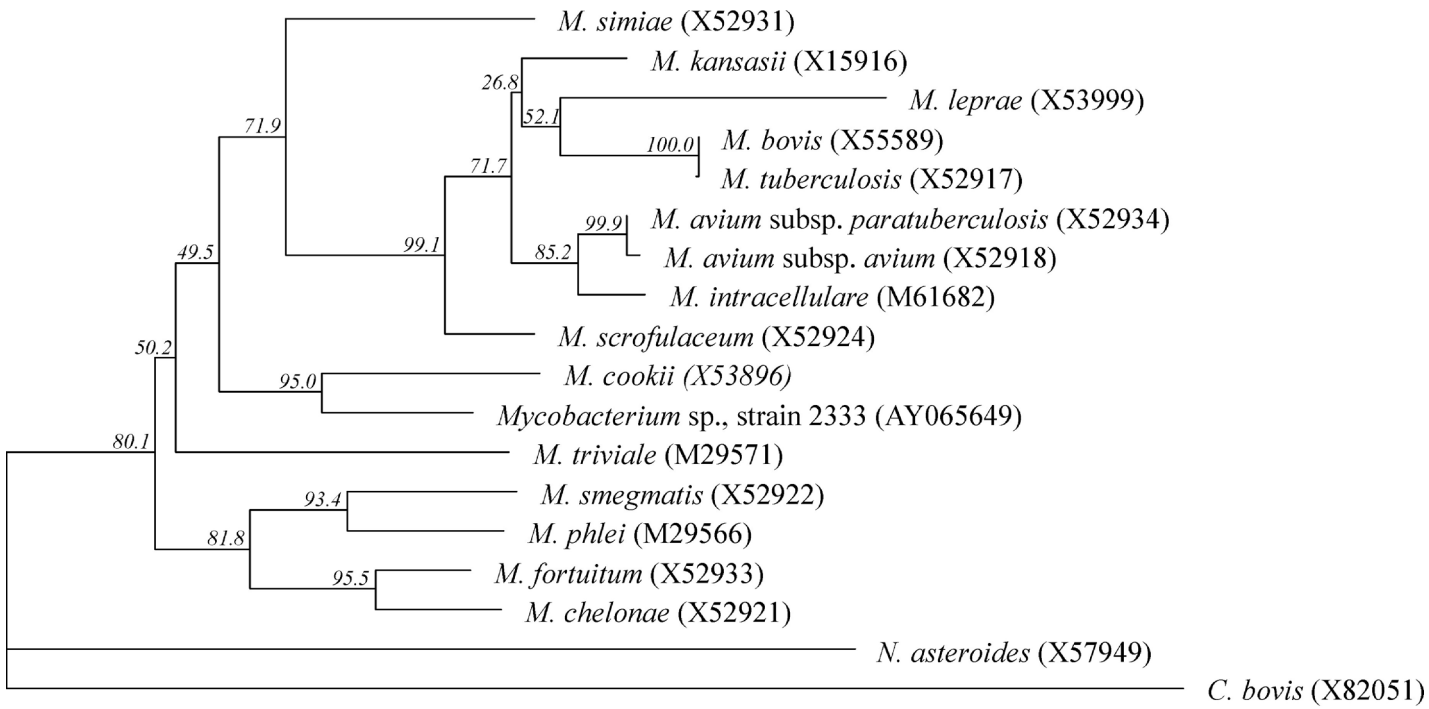
M. avium and *M. intracellulare* is based on agglutination of serovars or hybridisation to specific DNA probes (Baess, 1983; Kirschner & Bottger, 1992; Saito et al., 1989; Thoresen & Saxegaard, 1993; Wayne et al., 1993).

Due to difficulties in separating *M. scrofulaceum* from the *M. avium* group by numerical classification and because of the confusion concerning the identification and classification of *M. avium*, *M. intracellulare*, and *M. scrofulaceum*, the designation MAIS complex has sometimes been used for serovars of the three species. However, this is not a valid nomenclature and should not be used (Baess, 1983; Tsukamura, 1976; Wayne et al., 1993). Phylogenetically, *M. scrofulaceum* is clearly separated from *M. avium* and *M. intracellulare* (Rogall et al., 1990b).

Objections have been raised against the classification of *M. paratuberculosis* as a subspecies to *M. avium* due to phenotypic differences between *M. avium* and *M. paratuberculosis*. (Chiodini, 1990; Chiodini, 2000). Comparison of the complete genomic sequences of *M. avium* and *M. paratuberculosis* may ultimately reveal the true genomic relationship of these two taxa.

In consequence of the difficulties encountered in numerical taxonomic analysis of mycobacteria, the International Working Group on Mycobacterial Taxonomy (IWGMT) was formed in 1967 by a group of researchers working in the field to coordinate the strains and techniques being used (Goodfellow & Wayne, 1982; Wayne, 1981; Wayne et al., 1989; Wayne et al., 1996; Wayne et al., 1993). Studies based on numerical taxonomy, including techniques based on immunology, DNA–DNA hybridisation, and restriction endonuclease analysis have so far failed to provide data for a unified and unambiguous classification of mycobacteria. The use of comparative sequence analysis of 16S rRNA has offered a suitable alternative to numerical taxonomy (Stahl & Urbance, 1990).

Fig. 4 (right). Evolutionary tree based on 16S rRNA sequences showing the phylogenetic relations of a selected group of mycobacteria. *Nocardia asteroids* and *Corynebacterium bovis* were used as outgroups. The tree was computed from a distance matrix corrected for multiple nucleotide substitutions by neighbour joining. The bootstrap percentage values from 1000 re-samplings of the data set are given at each node. The length of the scale bar corresponds to one substitution per 100 nucleotide positions. Strain designation is given for the taxon which has not been assigned species status.


M. simiae (X52931)
M. kansasii (X15916)
M. leprae (X53999)
M. bovis (X55589)
M. tuberculosis (X52917)
M. avium subsp. paratuberculosis (X52934)
M. avium subsp. avium (X52918)
M. intracellulare (M61682)
M. scrofulaceum (X52924)
M. cookii (X53896)
Mycobacterium sp., strain 2333 (AY065649)
M. triviale (M29571)
M. smegmatis (X52922)
M. phlei (M29566)
M. fortuitum (X52933)
M. chelonae (X52921)
N. asteroides (X57949)
C. bovis (X82051)
26.8
52.1
100.0
71.7
99.9
85.2
71.9
99.1
49.5
50.2
95.0
80.1
93.4
81.8
95.5
.01

Phylogeny of slow-growing mycobacteria

Phylogeny is defined as the evolutionary history of a gene from a group of taxa or the evolutionary history of a group of taxa. Phylogeny plays an important role in modern taxonomy, and classification based on phylogeny can be regarded as natural because it reflects natural relations between organisms.

The phylogenetic relationship of organisms may be studied by comparative sequence analysis of the rRNA genes, which are relatively well conserved in all life forms (Fox et al., 1980). Members of the slow-growing mycobacteria have one ribosomal RNA operon (*rrn*) with genes coding for the 16S, 23S, and 5S rRNA, being present in that order, and with no coding regions for tRNA in the intergenic spacer regions (Bercovier et al., 1986; van der Giessen et al., 1994). The fast-growing mycobacteria have two rRNA operons (Bercovier et al., 1986; Ji et al., 1994; Menendez et al., 2002). The finding of one or two rRNA operons in mycobacteria and seven operons in *Escherichia coli*, an organism with a similar genome size, supports the hypothesis that growth rate rather than genome size is correlated to the number of rRNA operons (Bercovier et al., 1986). A phylogenetically valid taxonomy for mycobacteria has been based on data provided by 16S rRNA sequence analysis.

16S rRNA and 18S rRNA molecules, which are fundamental in the translation process in both prokaryotic as well as in eukaryotic cells, are present in all living organisms. Due to its high informative content and conserved nature, the sequence of the 16S rRNA gene has been and still is very useful in studies on the phylogeny of microorganisms (Ludwig & Schleifer, 1999; Woese, 1987). In the 16S rRNA genes, there are regions which are highly conserved and other regions with a considerable amount of variability. The characteristic sequence patterns or structures of the variable regions reflect the natural relationships among members of a common line of descent. The sequence information in these regions is very useful when a new species, not previously described, is characterised. Furthermore, the universal regions of the 16S rRNA genes provide necessary targets for PCR and sequencing primers when analysing organisms not earlier described or organisms that cannot be cultivated. The use of direct sequencing of the amplified DNA fragment eliminates the need for cloning or subcloning of the genes (Edwards et al., 1989; Rogall et al., 1990a). The new taxonomy of bacteria (http://www.cme.msu.edu/Bergeys/: Accessed 3-Apr-2002) is based on phylogenetic relations, but this approach has been criticised and alternative classifications have been suggested (Cavalier-Smith, 2002).

The division of mycobacteria into one group of slow growers and a group of fast growers is supported by sequence analysis of the 16S and the 23S rDNA (Böddinghaus et al., 1990; Stackebrandt & Smida, 1988; Stahl & Urbance, 1990; Stone et al., 1995). It is noteworthy that the phylogenetic relationship does not reflect the Runyon classification within the groups of slow growers. Rogall et al. demonstrated a high degree of 16S rRNA sequence similarity within the

Mycobacterium genus (Rogall et al., 1990b). The slow-growing species had similarity values exceeding 94.8% and formed a disparate group, clearly separated from the tight cluster formed by the fast-growing species. Based on sequence data of the 16S rRNA gene, *M. intracellulare*, *M. avium*, and *M. paratuberculosis* form a distinct cluster. The 16S rRNA similarity between *M. avium*, and *M. paratuberculosis* is 99.9% and confirms the close genetic relationship shown by DNA–DNA hybridisation (Kirschner & Bottger, 1992; Rogall et al., 1990b; Springer et al., 1996).

Length and sequence variations are two examples of structure signatures generally found within a coherent phylogenetic assemblage of bacteria. Such a signature is found at positions 451 to 482 in 16S rRNA (according to the numbering of the 16S rRNA molecule of *E. coli*), where slow growers exhibit a long helix, while the fast growers have a short helix (Stahl & Urbance, 1990). The length of the helix cannot be used as a definitive differential between fast and slow growers as a few slow-growing species with a short helix, for example *M. simiae* and *M. triviale*, have been encountered.

The gene coding for the 23S rRNA in *M. paratuberculosis* and *M. avium* is 3100 bp long and has 9 nucleotides that differ between *M. paratuberculosis* and *M. avium*, giving an overall similarity of 99.7% (van der Giessen et al., 1994). The intergenic spacer region between the 16S and the 23S rRNA genes often shows high sequence variability between closely related organisms, but in *M. paratuberculosis* and *M. avium* only two nucleotides differ (van der Giessen et al., 1994). Restriction endonuclease analysis of the 5S rRNA gene in *M. paratuberculosis* strains isolated from animals and humans from different parts of the world did not show any polymorphisms, thus indicating a close genetic identity of the strains (Chiodini, 1990). The close sequence similarity of *M. paratuberculosis* and *M. avium* has made it difficult to design probes or specific PCR primers targeting the rRNA genes, to be used for diagnostic purpose.

Repeated DNA sequences

A variety of repetitive elements have been found in *M. tuberculosis* and *M. bovis*, which have been successfully used to distinguish between isolates (van Soolingen et al., 1993). The most commonly used elements are the polymorphic GC-rich repetitive DNA element (PGRS) (Ross et al., 1992), the 36-bp direct repeat (DR) (van Embden et al., 2000) and the insertion sequence IS*6110* (Thierry et al., 1990). Double repetitive PCR (DRE-PCR) is a subtyping method for *M. tuberculosis* strains based on amplification of segments located between the two repetitive elements IS*6110* and the polymorphic GC-rich repetitive sequence (PGRS) (Friedman et al., 1995). For further examples, see (Bigi et al., 1995; Caimi et al., 2001; Dale et al., 2001; Glennon et al., 1997; Gordon et al., 1999; Kremer et al., 1999; March et al., 1996). So far, insertion sequences remain the

most useful target in *M. paratuberculosis* for strain identification and strain differentiation.

Insertion sequences

Insertion sequences (IS) are mobile genetic elements, usually 0.8 to 2.5 kb in size, able to insert into new sites on the same or another replicon without leaving their original location. A typical insertion sequence is characterised by terminally perfect inverted repeats (IR) of about 10 to 40 bp and flanking direct repeats (DR), together with a single major open reading frame (ORF) coding for a transposase. In addition, they often carry one smaller ORF within the larger ORF, using the same frame but in the opposite direction from the complementary DNA strand. The insertion can be random, directed, or site-specific. The inverted repeats are thought to provide the recognition and binding sites for the transposase protein, while the direct repeats are probably derived from the staggered cut of the target sequence followed by repair upon transposition (Galas & Chandler, 1989; IIda et al., 1983; Mahillon & Chandler, 1998; Mahillon et al., 1999). The IS elements are grouped into families based on similarities and identities in the DNA sequence of their transposase and in their genetic organisation (Mahillon & Chandler, 1998). Different families of insertion sequences have been detected in mycobacteria and have proved invaluable for diagnostic and epidemiological studies (Dale, 1995; Dvorská et al., 1999; Green et al., 1989a; McAdam et al., 1994). New IS elements will probably be discovered in mycobacteria as a result of the ongoing genome sequencing projects and hopefully provide insight into the mechanism of transposition and function of these elements.

The IS*110* family

The members of the IS*110* family have no or very small inverted repeats and generally do not create direct target repeats. The mechanism of transposition of these elements is not clear (Mahillon & Chandler, 1998). Some of the insertion sequences related to this family are carried on either a phage or a plasmid and phages or plasmids are therefore assumed to be vehicles for these elements, allowing horizontal transfer (Puyang et al., 1999).

IS*900* was discovered in *M. paratuberculosis* independently by two groups (Collins et al., 1989; Green et al., 1989b). This 1451 bp element lacks inverted terminal repeats and does not generate direct repeats in target DNA. IS*900* exists in 15-18 copies in the genome of *M. paratuberculosis*. The insertion sites of different copies of IS*900* are similar and share a common consensus sequence. In *M. paratuberculosis* IS*900* inserts in a target gene in an orientation-specific manner (Bull et al., 2000). The major ORF codes for the *M. paratuberculosis* specific transposase protein p43 (Tizard et al., 1992). A second ORF, found on the opposite strand, encodes a putative transport protein. The transcription and translation of this protein product requires host-encoded sequences adjacent to and outside IS*900* (Doran et al., 1997; Doran et al., 1994). It has been speculated that the insertion of IS*900* might disrupt the expression of genes involved in the

regulation of virulence determinants (Harris & Barletta, 2001; Naser et al., 1998). The discovery of IS*900* made it possible to design a species-specific PCR to distinguish *M. paratuberculosis* from the closely related *M. avium.*

IS*901*, present in *M. avium,* appears to be restricted to strains pathogenic to animals (Kunze et al., 1991; Kunze et al., 1992). An almost identical insertion sequence, IS*902*, is present in some strains of *M. avium* and some strains of *M. silvaticum* and usually in strains pathogenic in animals (Moss et al., 1992a). These elements possess characteristics similar to those of IS*900* but exhibit only 60% sequence identity to IS*900*.

The novel insertion sequence IS*1613* (Accession no. AJ011837) present in *M. avium* and *M. intracellulare* shares 83% sequence identity with IS*900* (Harris & Barletta, 2001). IS*1626*, isolated from a clinical strain of *M. avium*, lacks terminal IRs and does not possess flanking direct repeats and is closely related to IS*900*, showing 82% sequence similarity (Puyang et al., 1999).

Other insertion sequences belonging to the IS*110* family are IS*1110*, a highly mobile element found in *M. avium* (Hernandez Perez et al., 1994), and IS*1547* found in *M. tuberculosis* (Fang et al., 1999).

The IS*256* family
IS*1311* is the second insertion sequence found in *M. paratuberculosis* (Roiz et al., 1995) and this element belongs to IS*256* family. The insertion sequences in this family carry related terminal IRs of 24 to 41 bp and most of them generate 8 bp direct target repeats. Little is known about the way in which the elements in this family are transposed (Mahillon & Chandler, 1998). IS*1311* is not unique to *M. paratuberculosis* as it has also been found in *M. avium* and *M. intracellulare*. Thus, the use of IS*1311* is not as discriminatory as IS*900* for subtyping of *M. paratuberculosis* (Collins et al., 1997).

IS*1245* is a high copy insertion element found in *M. avium* and it has also been reported in one laboratory strain of *M. paratuberculosis* (Guerrero et al., 1995). Collins et al. characterised strains of the *M. avium* complex by using the four insertion sequences IS*900*, IS*901*, IS*1245*, and IS*1311*, but could not confirm the presence of IS*1245* in the 10 *M. paratuberculosis* strains investigated (Collins et al., 1997). However, IS*1245* shares 85% sequence identity with IS*1311* and rigorous hybridisation conditions are essential to obtain reliable results.

ERIC elements

Enterobacterial repetitive intergenic consensus (ERIC) elements and repetitive extragenic palindrome (REP) sequence motif constitute different classes of prokaryotic dispersed repeat elements that have been used to delineate strain relatedness (Lupski & Weinstock, 1992; van Belkum et al., 1998). ERIC elements are 126 bp in size that appear to be restricted to transcribed regions of the genomes of Gram-negative enteric bacteria, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. These elements contain a highly conserved centrally inverted repeat, which could potentially form a stem-loop structure when transcribed into RNA. ERIC sequences can be found in either orientation with respect to the direction of transcription. The precise function of the ERIC element is not known, but it is suggested that the presence of elements like this is important for the structure and evolution of genomes (Hulton et al., 1991; Lupski & Weinstock, 1992; Versalovic et al., 1991).

If repetitive elements cluster sufficiently close to one another, they can be used as PCR targets for outward-directed sequence-specific 18-24mer oligonucleotide primers (Versalovic et al., 1994). In ERIC-PCR a fingerprint is obtained by amplification of genomic DNA located between ERIC elements, or between ERIC elements and other repetitive DNA sequences (Versalovic et al., 1991; Versalovic et al., 1995). ERIC-PCR has been used for fingerprinting analysis of various Gram-negative bacterial strains, such as mixed Gram-negative bacterial strains (Di Giovanni et al., 1999) and Gram-negative soil bacteria (de Bruijn, 1992). ERIC-PCR has also been successfully used for molecular analysis of toxigenic and non-toxigenic strains of *Vibrio cholerae* (Rivera et al., 1995). The results showed ERIC-PCR to be a rapid, reproducible, and highly discriminating fingerprinting method. Even though ERIC sequences were originally detected in Gram-negative bacteria, fingerprinting by ERIC-PCR has been successfully performed for bacteria outside this group. Van Belkum et al. used ERIC-PCR for typing of methicillinresistant *Staphylococcus aureus* (van Belkum et al., 1993). Sechi et al. (Sechi et al., 1998) used ERIC-PCR to demonstrate clonal relationships between different strains of *M. tuberculosis.* However, Gillings et al. (Gillings & Holley, 1997) reported that ERIC primers are not necessarily directed at ERIC elements. Because of the low annealing temperature and the long extension time used in the original protocol, they postulated that ERIC-PCR constitutes a reproducible variation of the randomly amplified polymorphic DNA method (RAPD). Nevertheless, ERIC-PCR may still be a useful tool for differentiation of bacterial strains.

Diagnostic methods

The different stages of paratuberculosis have been extensively described (Whitlock & Buergelt, 1996) and reflect the diagnostic problems often tackled, especially in view of the possibility of detecting infected animals in the earliest stage of disease (Collins, 1996). At present, no single diagnostic method suffices for all purposes.

Histopathology

Acid-fast bacilli can be demonstrated by Ziehl-Neelsen staining, and are sometimes found in enormous numbers especially in the submucosa of the intestine. Lesions essentially remain confined to the intestine, and to mesenteric and ileocecal lymph nodes. In clinical cases the ileum is corrugated and thickened, with enlarged edematous mesenteric lymph nodes, and abundant multinucleated giant cells. Innumerable intracellular acid-fast bacilli may also be observed (Buergelt et al., 1978).

Immunology

Two kinds of cellular immunity test have been employed to identify *M. paratuberculosis* infected animals. One is a skin test, where the sensitin Johnin, a *M. paratuberculosis* antigen prepared as tuberculin PPD, is injected intradermally. A positive reaction is observed as a swelling. This test elicits positive reactions in some animals infected with *M. paratuberculosis*, but cross-reactions are observed with *M. avium* and *M. tuberculosis* (Manning & Collins, 2001). The other test is based on the production of cytokine gamma interferon (IFN-γ) by sensitised T-lymphocytes, which is one of the earliest detectable reactions to *M. paratuberculosis* infection. The test must be performed on freshly heparinised blood within 16 h of collection. However, production of IFN-γ may be more closely related to the pathology than to the immunity of *M. paratuberculosis* (Rook et al., 1987; Rook, 1987; Stabel, 1996). Non-specific reactions and the time-sensitive nature of the protocol limit the diagnostic value of the IFN-γ test (Jungersen et al., 2002).

Diagnostic tests based on detection of serum antibodies are not particularly useful for early detection, as animals do not develop an antibody response until late in the disease. Furthermore, cross-reactivity can occur as a consequence of infection with other mycobacteria (Chiodini et al., 1984; Stabel, 1998). Enzyme linked immunosorbent assay (ELISA) shows low sensitivity in subclinically affected animals and is therefore best applied as a surveillance tool to establish the infection status of entire herds of adult animals (Collins & Socket, 1993; Sweeney et al., 1995; Vanuffel et al., 1994). The agar gel immunodiffusion (AGID) test turns positive in cattle in advanced stages of the disease and is therefore used essentially to confirm paratuberculosis in animals with suspected clinical symptoms (Sherman et al., 1984; Sherman et al., 1990). The complement fixation (CF) test lacks species specificity and is not very sensitive (Colgrove et al., 1989).

Bacteriology

Culture is considered to be the "gold standard" for detection of *M. paratuberculosis*, though the method is slow; it can take up to 4 months to produce visible colonies on solid medium (Whipple et al., 1991). Ovine strains are even more fastidious and difficult to cultivate than those from cattle and can take 12 months or more to show visible growth (Choy et al., 1998; Collins et al., 1993; Juste et al., 1991). Colonies are rough, dull white, raised and circular. After Ziehl-Neelsen staining the bacteria can be observed as 1-2 μm long plump rods. Specimens preferentially tested by culture are feces, which is easy to collect and can be obtained from live animals (Whipple et al., 1991). A heavy shedder can excrete as many as 10^9 *M. paratuberculosis*/ g feces while a low shedder can excrete less than 10^3 *M. paratuberculosis*/g feces. The sensitivity of fecal culture is estimated to 50 to 100 organisms/g (Merkal, 1973; Whipple et al., 1991) and is currently the most sensitive method for detection of *M. paratuberculosis*. Tissue specimens such as intestinal mucosa and the associated mesenteric lymph nodes can be collected at necropsy or, more rarely, as biopsies from live animals and are cultured in the same manner as feces (Benedictus & Haagsma, 1986; Chiodini et al., 1984).

Iron is required for bacterial growth and function. Because this substance is virtually insoluble at neutral pH, microorganisms have developed mechanisms for solubilising iron. By producing and excreting specific iron-binding compounds, known as siderophores, iron can be extracted from iron compounds in the vicinity of the bacterium and help the organisms to survive and proliferate in an iron-deprived environment (Ratledge, 1982). Mycobactin is a siderophore that is essential for transport of iron in mycobacteria. It is produced by all cultivable mycobacteria except *M. paratuberculosis* and some strains of *M. avium* (De Voss et al., 1999; Lambrecht & Collins, 1993; Thorel, 1984). Addition of mycobactin to the medium is therefore needed as a growth factor and is used to distinguish *M. paratuberculosis* from the faster growing *M. avium* and *M. intracellulare* (Merkal & McCullough, 1982).

A radiometric culture method, BACTEC, has been used to reduce the time for diagnosis (Collins et al., 1990b; Cousins et al., 1995; Whittington et al., 1998b). The sample is cultured in a liquid medium in which a ^{14}C-labelled substrate is metabolised by growing microorganisms to radiolabelled carbon dioxide that can be measured in the gas phase above the culture. However, the growth of other mycobacteria and contamination of other microorganisms sometimes limit the possibility of detecting *M. paratuberculosis.*

Biochemical tests

Characterisation of *M. paratuberculosis* based on biochemical properties, is problematic due to its low biochemical activity. Furthermore, the available biochemical tests have been developed for more rapidly growing mycobacteria (Chiodini, 1986).

DNA probes

A number of DNA probes have been developed and compared with culture for detection of *M. paratuberculosis* (Collins, 1996; Hurley et al., 1989; Whipple et al., 1992; Zimmer et al., 1999). The use of specific probes directly on biological samples is hampered by components of the sample, giving the test a low sensitivity, and by the inherent low sensitivity of the technique. Although the use of DNA probes shortens the time needed for detection of fecal excretors of *M. paratuberculosis*, infected animals shedding low numbers of *M. paratuberculosis* cannot be detected. Further limitations are the cost per sample and that the organism cannot be isolated for further characterisation. The commercially available test Accuprobe "*M. avium* complex culture identification test" (Gen Probe, San Diego, CA, USA) is based on a DNA probe complementary to the rRNA sequence. The test can be performed on mycobacterial colonies from primary cultures. (Drake et al., 1987). This probe only confirms the isolate as a member of the *M. avium* complex and phenotypic characteristics need to be supplemented in order to determine if the isolate is *M. paratuberculosis* (Thoresen & Saxegaard, 1991).

PCR

The introduction of molecular biology techniques, especially the polymerase chain reaction, has contributed notably to a major breakthrough in the paratuberculosis research and diagnosis (Stevenson & Sharp, 1997). PCR is a rapid detection method widely employed for the detection and identification of microorganisms (Altwegg & Verhoef, 1995). PCR is based on the enzymatic amplification of a target nucleic acid sequence, using a specific pair of oligonucleotides (primers) and a thermostable DNA polymerase (Mullis & Faloona, 1987; Saiki et al., 1985). As the amplification is exponential, one single copy of the target DNA sequence will in theory suffice to produce millions of copies after repeated cycles of PCR in a thermo cycler.

The insertion sequence IS*900* has been extensively used as a target for PCR-based diagnostics and has been regarded as definitive for the identification of *M. paratuberculosis* (Lisby et al., 1994; Moss et al., 1991; Moss et al., 1992b; Sanderson et al., 1992; Vary et al., 1990). The discovery of new insertion sequences displaying close homology to IS*900* (Harris & Barletta, 2001) and the fact that positive results have been obtained with IS*900* PCR from mycobacteria other than *M. paratuberculosis* (Cousins et al., 1999; Naser et al., 1999) call for alternative, species-specific PCR. A few *M. paratuberculosis* specific sequences have been reported, but are not yet routinely used as the basis for diagnostic PCR.

The *M. paratuberculosis* specific sequence F57 is a single-copy sequence of 620 bp not related to any other known sequence (Poupart et al., 1993). F57 has been used together with a mycobacterial sequence upstream of a gene encoding a 34 kDa protein (Gilot et al., 1992) in order to develop a diagnostic duplex PCR (Coetsier et al., 2000; Poupart et al., 1993). This PCR system distinguishes between *M. bovis*, *M. avium*, and *M. paratuberculosis* and can be used as a supplement to IS*900* based PCR.

The single-copy gene *hspX*, encoding a putative heat-shock-like protein unique to *M. paratuberculosis*, has been used as a PCR target to distinguish *M. paratuberculosis* from other species belonging to the *M. avium* complex (Ellingson et al., 1998; Ellingson et al., 2000).

Amplification of IS*Mav2*, a newly described insertion sequence in *M. paratuberculosis,* may also be a potential alternative to IS*900*-based PCR (Strommenger et al., 2001).

Specimens commonly analysed by PCR are feces, intestinal mucosa and the associated mesenteric lymph nodes, and milk. Various sample preparation methods for mycobacteria have been described for diagnostic PCR (Challans et al., 1994; Choy et al., 1998; Collins et al., 1993; Del Prete et al., 1998; Garrido et al., 2000; Millar et al., 1995; Ros Bascuñana & Belák, 1996; Vary et al., 1990). Treatment of the samples prior to PCR consists of the extraction of the mycobacteria from the specimens, followed by release, purification, and concentration of the mycobacterial DNA. Extraction of the mycobacteria may be accomplished by sedimentation, centrifugation, or extraction using organic solvents (Challans et al., 1994; Garrido et al., 2000; Gwóz´dz´ et al., 1997), or by capturing the mycobacteria by specific antibodies attached to magnetic beads, known as immunomagnetic capture (Grant et al., 2000; Mason et al., 2001). After extraction of the mycobacteria the DNA is released by lysis. The mycobacterial cell wall contains mycolic acids and various carbohydrate polymers, such as arabinogalactan. These structures are very resistant to common enzymatic treatments and are difficult to cleave by conventional methods. *M. paratuberculosis* is most efficiently lysed by physical methods, such as sonication or beadbeating (Hurley et al., 1987; Odumeru et al., 2001) and although this method is likely to shear the DNA into small fragments, the DNA is still functional as a PCR template. The DNA can be purified by phenol and chloroform extraction followed by ethanol precipitation. To separate high molecular weight polysaccharides from the DNA, precipitation of the DNA can be accomplished by means of cetyl trimethyl-ammonium bromide (CTAB) (Hurley et al., 1987). Another way to concentrate the DNA and at the same time remove amplification inhibitors is the use of sequence capture. This method is based on binding of the specific target DNA to a biotinylated DNA probe that is subsequently captured on streptavidin-coated magnetic beads. The beads are washed and the captured DNA is then subjected to amplification (Mangiapan et al., 1996; Marsh et al., 2000; Marsh & Whittington, 2001; Millar et al., 1995).

PCR is a sensitive method which has a theoretical potential for detecting one single gene copy, though in practice a lower degree of sensitivity is achieved, due chiefly to the presence of inhibitors which may be difficult to remove, in combination with the relative sparasity of target organisms in clinical samples. Fecal samples have been extremely difficult to analyse by PCR because of abundant amplification inhibitors of varying composition that differ from one time to another. Traces of compounds used in the DNA extraction methods may also contribute to weaken the sensitivity of the PCR. (Abu Al-Soud & Rådström, 2000; Al-Soud & Rådström, 2001; Monteiro et al., 1997; Wilson, 1997). To check the efficiency and the sensitivity of each individual amplification reaction,

internal control molecules may be used. These are molecule constructs which are co-amplified with the same primers and in the same tube as the sample. The amplicons of the internal control are distinguished from the target amplicons by their difference in size when analysed by agarose gel electrophoresis (Ballagi-Pordány & Belák, 1996; Rosenstraus et al., 1998; Sachadyn & Kur, 1998).

A suboptimal amplification reaction, caused by inhibitors present in the sample, can to some extent be circumvented by performing nested PCR, in which an aliquot of the first-round amplification product is amplified with a new set of primers specific to the internal sequence amplified by the first primer pair (Pierre et al., 1991). The transfer of PCR products from one tube to another increases the risk of aerosol cross-contamination and is therefore not recommended for routine clinical laboratory use. Precautions are imperative to avoid problems of false-positive results due to carryover of PCR products from previous amplification reactions. For example, the use of dedicated equipment and separate rooms for sample treatments, PCR set-up, and analysis of PCR products are standard procedures (Belák & Ballagi-Pordány, 1993; Kox et al., 1994). The application of uracil N-glycosylase (UNG) is an additional precaution to prevent amplicon carryover contamination (Longo et al., 1990). This method is based on the incorporation of dUTP instead of dTTP in the PCR products. Treatment of pre-assembled starting reactions with uracil DNA glycosylase (UDG) will destroy all dUTP-containing DNA and block the replication by DNA polymerases. This method is not practicable for nested PCR and the use of dUTP instead of dTTP sometimes reduces the overall sensitivity of the PCR (Ritzler et al., 1999).

Analysis of the PCR products is usually done by agarose gel electrophoresis, followed by ethidium bromide staining. The size of the obtained amplicons is compared with an external size marker and a positive control. Fluorescently labelled primers can be used in the PCR to enhance sensitivity. The fluorescent amplification products are detected during electrophoresis by laser scanning in a automated DNA sequencer and the size of the products is determined (Versalovic et al., 1995). Another approach is to use real-time PCR, an automated PCR with fluorescent probes in which the PCR products are continuously detected during the amplification (Fang et al., 2002; Heid et al., 1996).

The specificity of the amplification can be determined by dot-blot hybridisation or Southern blot hybridisation of the PCR products (Moss et al., 1991; Vary et al., 1990). Both techniques are based on hybridisation with an IS*900*-specific probe. In dot-blot hybridisation the PCR product is blotted directly on a membrane and a positive sample can be observed as a dot. In Southern blotting the amplicons are separated in an agarose gel that is subsequently blotted on the membrane. A positive sample is observed as a band and the size of the product can be determined. Hybridisation methods promote sensitivity and specificity in the analysis of the PCR products.

Strain differentiation

Subtyping of *M. paratuberculosis* isolates is important in order to determine the source of infection and to trace the spread of the disease. Discrimination power and reproducibility are important properties in any typing system used, as also are ease of use, and ease of interpretation. Other factors to consider when choosing a subtyping method are technical difficulty, cost, and time required to obtain a result. Numerous methods based on molecular biology techniques have been used to distinguish between isolates of *M. paratuberculosis*. Because the slow-growing mycobacteria possess only a single rRNA operon, ribotyping is of limited use. The discovery and study of repeated DNA elements in mycobacterial genomes has provided new and alternative pathways for diagnostic and epidemiological investigations (Poulet & Cole, 1995).

PFGE

Analysis of whole chromosomal DNA by pulsed field gel electrophoresis (PFGE) was one of the first molecular techniques applied to *M. paratuberculosis* strains. The intact mycobacteria are embedded in agarose gel blocks and lysed, and the DNA released is subsequently digested by a rare cutting restriction endonuclease. The plugs with the digests are inserted into an agarose gel and subjected to electrophoresis with the polarity of the current changed at predetermined intervals to yield a unique pattern of discrete bands. By using the enzyme *Xba*I, *M. paratuberculosis* strains could be divided into two subgroups differing in only one band. These two subgroups were found in both bovine and ovine strains. PFGE analysis have confirmed the genetic homology of strains isolated from cattle, sheep, goats, and Crohn's disease patients (Feizabadi et al., 1996; Feizabadi et al., 1997; Tenover et al., 1995).

RFLP

Before the discovery of IS*900*, restriction endonuclease analysis (REA) of genomic DNA was successfully used to distinguish *M. paratuberculosis* from other slow-growing mycobacteria (Collins & de Lisle Geoffrey, 1986; Whipple et al., 1987; Whipple et al., 1989). By using *Bst*EII, *M. paratuberculosis* strains could be divided into two groups, one with strains isolated from cattle and other animals, including some strains from sheep and goats, and a second group with strains from sheep and a goats only (Collins et al., 1990a).

In restriction fragment length polymorphism (RFLP) whole chromosomal DNA is digested with a restriction endonuclease and the fragments are then separated by electrophoresis in an agarose gel. The DNA fragments are transferred from the gel to a nitro-cellulose membrane by Southern blotting and bound to the membrane by UV-illumination or by heating at 80°C for 2 h. The DNA fragments are then hybridised with a labelled IS*900* probe, yielding a banding pattern corresponding to the multiple copies of IS*900* at different sites in the chromosomal DNA. In contrast to PFGE, only the DNA fragments that hybridise to the probe are visible, which simplifies the analysis.

RFLP is robust and reproducible, and the results obtained for individual isolates are stable over time, permitting intra-laboratory comparison of data. Efforts have been made to standardise RFLP and to create a common basis for interpretation of the banding patterns (Pavlik et al., 1999b). Thirteen RFLP types have so far been detected with *Pst*I (15 to 19 bands) and 20 RFLP types with *Bst*EII (24 to 27 bands). The differences in banding pattern between subtypes are minor, however, often only 1 to 2 bands (Pavlik et al., 1999b). The predominant RFLP type found in cattle in western Europe is B-C1.

RFLP has been used worldwide to investigate *M. paratuberculosis* strain variation in different animal species, eg. cattle, sheep, goats, alpaca, and deer. Investigations of a variety of *M. paratuberculosis* isolates by RFLP have confirmed the finding of two major groups: cattle strains and sheep strains, obtained by PFGE. However, cattle strains have been found in sheep and goats too and species-specific strains have not been confirmed (Bauerfeind et al., 1996; Collins et al., 1990a; Cousins et al., 2000; Moreira et al., 1999; Thoresen & Olsaker, 1994; Pavlík et al., 1995; Pavlik et al., 1999b; Whipple et al., 1990; Whittington et al., 2000). IS*900* is firmly integrated in the genome, making it a suitable target for strain differentiation, but the variations found are not sufficient for use in epidemiological studies (Cousins et al., 2000; Pavlik et al., 1999b).

PCR-REA

One approach for subtyping strains is to study by PCR the difference in copy number and the distance between IS elements, yielding a unique banding pattern. In addition the PCR products obtained can be further characterised by restriction endonuclease analysis. PCR amplification of IS*1311* followed by restriction endonuclease analysis (PCR-REA) has been used to distinguish between cattle or sheep strains of *M. paratuberculosis* (Collins et al., 1997). The method is based on a point mutation that has created an extra *Hin*fI site, which is found in some copies of IS*1311* in cattle strains but not in sheep strains of *M. paratuberculosis*. IS*1311* PCR-REA can be performed on primary culture. It is rapid and provides information similar to IS*900* RFLP (Marsh et al., 1999; Whittington et al., 1998a; Whittington et al., 2000; Whittington et al., 2001).

RAPD

Randomly amplified polymorphic DNA (RAPD) is based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence, yielding a DNA fingerprint pattern (Welsh & McClelland, 1990; Williams et al., 1990). This method is not used routinely due to reproducibility difficulties. In the studies on *M. paratuberculosis* by Scheibel et al. (Scheibel & Gerlach, 1997), and Pillai et al. (Pillai et al., 2001) amplifications were performed with low stringency at an annealing temperature of 35° to 36°C with random 10-mer oligonucleotides from commercial kits. Sheibel et al. needed seven different primers to distinguish between strains whereas Pillai et al. needed only one primer. The use of "Ready to go" PCR beads (Amersham Bioscience) probably contributed to the reproducibility reported by Pillai. Nevertheless, a limited number of polymorphisms were observed between strains of *M. paratuberculosis* and even

though more time consuming, RFLP remains more suitable for strain differentiation.

Aims of the present investigations

The objective of these studies was to improve the diagnostic tools used to detect and characterise *M. paratuberculosis*. By developing methods based on molecular biology techniques, the diagnosis of paratuberculosis could be accelerated. Sensitive methods suitable for routine diagnostic use for detection of low-grade infection of *M. paratuberculosis* in cattle were highlighted.

Comments on materials and methods used

Further details on the materials and methods used are given in papers I-V.

Bacterial strains (papers I-V)

M. paratuberculosis ATCC 19698 is the neotype strain possessing the defined characteristics for this species (Merkal, 1979). It was used as the reference strain in these studies. The *M. avium* strains ATCC 25291, ATCC 35714, and ATCC 35718, and the *M. silvaticum* field strain TB228/95 possessed the IS*901* element.

Growth conditions

M. paratuberculosis and *M. silvaticum* were grown on slopes of modified Löwenstein-Jensen medium supplemented with mycobactin (4 mg/l), and cultured at 37°C for 6 weeks to 4 months and 2 to 3 weeks, respectively. *M. avium* and *M. intracellulare* were grown on slopes of Löwenstein-Jensen medium supplemented with glycerol (7.5 ml/l) and sodium pyruvate (2 g/l), at 37°C for 2 to 3 weeks. *M. bovis* and other mycobacteria studied were grown on slopes of Löwenstein-Jensen medium supplemented with sodium pyruvate (2 g/l), at 37°C for 2 to 3 weeks.

Isolation of M. paratuberculosis field isolates

The field strains of *M. paratuberculosis,* isolated from various organs and bovine feces, were collected at the National Veterinary Institute, Uppsala, Sweden, during the period 1993-1998. The isolates were characterised by growth rate, mycobactin dependence, colony morphology, acid fastness, the Accuprobe test for the *M. avium* complex (GenProbe, San Diego, CA, USA), IS*900* PCR, and RFLP analysis according to the protocol of Pavlik et al. (Pavlík et al., 1995). Following subculture on Lövenstein-Jensen medium supplemented with mycobactin the *M. paratuberculosis* strains were harvested and resuspended in 0.9% NaCl and kept at −70°C. Subculturing was performed and genomic DNA was extracted by using the beadbeating protocol (paper II). Extracted DNA was dissolved in TE (10 mM Tris-HCl buffer [pH 7.5], 1 mM EDTA), and the concentration was determined with Gene Quant (Amersham Biosciences). The DNA was aliquoted and kept at −20°C.

Clinical specimens (papers I-III)

In this thesis, ileum tissue specimens, mesenteric lymph nodes, and fecal samples were chosen for analysis by PCR. The specimens were collected from animals in a slaughter survey conducted between 1995 and 1996, and from animals in the surveillance and infection tracing during 1994 to 1999. The presence or absence of *M. paratuberculosis* was determined by culture. The amount of bacteria in positive samples was classified in four categories based on the number of colonies found per tube and the number of tubes showing visible growth (paper II). The clinical specimens were stored individually at –20°C.

Spiked specimens (papers I-III)

In the natural environment of *M. paratuberculosis*, the mycobacteria often aggregate into clumps of cells and can reside inside cells, such as macrophages. When spiking samples, these factors are difficult to mimic and it is therefore important to evaluate the final methods on naturally infected specimens in order to determine the true sensitivity of the methods.

The number of bacteria in one colony-forming unit (CFU) varies between different bacterial species. For mycobacteria, 1 CFU can represent considerably more than one organism. The actual content of *M. paratuberculosis,* determined as CFU, may also be higher than calculated due to non-viable bacteria. Furthermore, *M. paratuberculosis* can aggregate in its natural environment, whereas *M. paratuberculosis* in the broth culture used in the spiking experiments may be more evenly distributed. All these factors have to be considered when comparing the sensitivity of the PCR tested on pure genomic DNA, cultures of *M. paratuberculosis* determined as CFU, or clinical samples.

To obtain a single colony of *M. paratuberculosis* in fecal culture, the minimum amount of *M. paratuberculosis* has to be at least 50–100 organisms/g feces. An animal in the subclinical phase of the infection may excrete less than 3000 CFU/g feces and is therefore considered a light shedder (Sweeney et al., 1992), whereas heavy shedders in the clinical phase can excrete more than 10^8 CFU/g (Cocito et al., 1994).

As the viability of *M. paratuberculosis* is affected by freezing (Richards & Thoen, 1977), spiked fecal samples were cultured directly, whereas PCR analysis was performed on spiked samples stored at –20°C (paper III).

Pre-treatment of samples (papers I-III)

Amount of sample

To increase the likelihood of detection and to reduce the amount of inhibitory substances, the mycobacteria need to be withdrawn and concentrated from the clinical specimens prior to DNA purification. As the infection status is usually not known, a larger amount of sample (1-2 g) is preferably analysed, but the limited amount of positive specimens available obliged us to restrict the studies on ileum specimens and lymph nodes to 0.1 to 0.3 g (papers I and II).

In paper III, *M. paratuberculosis* was isolated from fecal samples by buoyant density centrifugation in Percoll. Efforts were made to analyse 1 g of feces, but this necessitated the use of 38-ml round-bottomed thin-wall centrifuge tubes. It was difficult to sediment the mycobacteria to a defined spot in the tube and the large volume of Percoll needed made the method impractical. To handle 1.5 ml microcentrifuge tubes is preferable as standard laboratory equipment can be used and it also makes it possible to process many samples in a short time. Therefore, a small-scale preparation protocol comprising 0.3 g of feces was developed.

Extraction of M. paratuberculosis from the samples

In papers I and II, an extraction protocol based on xylene was used and evaluated. The mycobacteria were concentrated into the organic phase due to their hydrophobic nature and then collected by centrifugation. Because of difficulties in withdrawing the organic phase layer with the mycobacteria in a standardised way and because of the toxicity of xylene, other approaches were investigated. Percoll® (Amersham Bioscience) is a non-toxic gradient medium, which is easily removed from purified materials and which has been used previously to separate cells, organelles, viruses, and other sub-cellular particles (Anonymous, 1995).

A method based on buoyant density centrifugation in Percoll® was developed and successfully used for fecal samples (paper III). The mycobacteria were separated from the fecal components in the samples due to the differences in their density during centrifugation in the gradient medium. To determine the buoyant density of the fecal components and the mycobacteria, a calibration curve was drawn on the basis of the results obtained with coloured Density Marker Beads in Percoll®. The condition for optimal separation was determined by using coloured Marker Beads with densities representing the mycobacteria and the fecal constituents together with different Percoll concentrations, ranging from 40% to 90%. The different centrifuge tubes were then subjected to various centrifugal forces and centrifugation times (Lindqvist, 2000; Lindqvist, 1997). It was not possible to achieve a complete separation with only one concentration of Percoll. Instead, two concentrations of Percoll were used with the denser Percoll in the bottom and the lighter Percoll on top, forming two cushions. By this procedure the mycobacteria were collected at the bottom of the tube while the fecal debris was trapped in the inter-phase between the two Percoll layers. The supernatant was withdrawn and the mycobacteria washed and further processed to release the DNA.

Lysis of M. paratuberculosis

The cell wall of *M. paratuberculosis* is very rigid and often requires mechanical disruption or prolonged incubation with a set of different enzymes to achieve an efficient lysis. Enzymatic treatment with lipase, lysozyme, and proteinase K (Whipple et al., 1987) was initially used to disrupt the mycobacterial cell wall, but adequate amounts of DNA were not obtained with this method. The most efficient way to release the mycobacterial DNA was to treat extracted mycobacteria with zirconium beads in a bead beater.

PCR constituents (papers I-V)

AmpliTaq Gold™ (Applied Biosystems) is a modified version of AmpliTaq® DNA polymerase (Applied Biosystems) that is inactive at room temperature, which makes the primer less prone to form a duplex with mismatches at suboptimal temperature. The benefit of using AmpliTaq Gold™ is its relative insensitivity to high background DNA, thus making it useful for low copy detection. It is important, though, to keep the pH below 8.3 and the total salt concentration below 60 mM (Rosén, B, personal communication). In the initial PCR analysis of clinical specimes, AmpliTaq® and AmpliTaq Gold™ were compared regarding their ability to affect the sensitivity of the PCR. It was found that AmpliTaq Gold™ yielded a much lower sensitivity than AmpliTaq® and was therefore not chosen in subsequent work. The effects of different amplification buffer compositions were also evaluated. The optimal PCR buffer consisted of 60 or 67 mM Tris-HCl buffer (pH 8.8) and 2 mM $MgCl_2$. Other PCR buffers tested did not perform as well as the above, possibly due to the presence of KCl, which has been reported to impair the amplification efficiency of GC-rich DNA templates. The addition of BSA, a commonly used amplification facilitator, did not improve the sensitivity. Furthermore, the use of carry-over prevention using uracil N-glycosylase, was found in a pre-study to reduce the sensitivity by one order of magnitude and was therefore not included in the present studies.

Hybridisation probes and systems (papers III-V)

Two different hybridisation systems were used in the studies. In the Alk-Phos direct system the DNA probe was labelled with alkaline phosphatase for use in conjunction with the chemiluminiscent substrate CDP-star™. The probe needs to be at least 60 nt (according to the manufacturer) and it is internally labelled, which yields a strong signal in the hybridisation assay. In addition, this systems requires post-hybridisation stringency washes with only two buffers prior to signal generation and detection, making the method rapid and easy to perform. One drawback, though, is the lack of a labelled size marker. In the other hybridisation system, a digoxigenin end-labelled 25-nucleotide probe was purchased from Eurogenetec. The hybridised probe was immunodetected with anti-digoxygenin-AP, Fab fragments and then visualised with the chemiluminescence substrate CSPD®. The dig-system proved less sensitive than the Alk-Phos system and was rather more time consuming in the post-hybridisation handling. However, the available dig-labelled size marker

(Boehringer Mannheim, DNA molecular weight marker VI) made it a suitable method for verifying the PCR results obtained by ERIC-PCR.

Results and discussion

PCR to confirm *M. paratuberculosis* in cultures (paper I)

When a colony suspected to be *M. paratuberculosis* is obtained in a culture, subculturing is performed to verify its mycobactin dependence and to isolate the bacterium for subsequent analysis. This takes a further 6 to 8 weeks before the final identification can be confined. Several diagnostic laboratories apply PCR to identify *M. paratuberculosis* in culture in order to reduce delay in diagnosis, but the quantity of mycobacteria needed to obtain a reliable PCR result has not been established. Furthermore, it has not been proved unambiguously that it is absolutely necessary to lys the mycobacteria prior to the PCR analysis.

The possibility of using one single colony without any pre-treatment as a template in the PCR was evaluated in paper I. Subculturing of the colony suspension was carried out to verify the PCR results. No disparity was found between the PCR results and the subculture results and it was evident that pre-treatment of the mycobacteria prior to analysis by PCR was unnecessary. Furthermore, a single colony was sufficient to obtain a reliable PCR result. This is of importance in cases with a low grade of paratuberculosis infection when only a few colonies may be present in the culture. It also makes it a possible to test suspected colonies as soon as they show up on solid medium and thereby save valuable time.

Detection of *M. paratuberculosis* in clinical specimens by PCR (papers I-III)

One of the main purposes in the present thesis was to develop diagnostic methods for detection of low-grade infection of *M. paratuberculosis* in cattle. *M. paratuberculosis* is shed in minimal quantities in the initial phase of the infection, but in greater numbers as the disease progresses. The primary sites of infection are the lower part of the intestine and the adjacent mesenteric lymph nodes, from where specimens can be collected post mortem. They are of primary interest when investigating suspected cases of paratuberculosis. Fecal samples can be readily sampled from live animals at different intervals and are therefore the first specimens to be analysed. Unfortunately, fecal samples are one of the most difficult types to analyse by PCR due to a variety of amplification inhibitors. To detect the small numbers of the bacterium in clinical specimens, pre-PCR treatments are required. To locate the bottleneck in the detection procedure and to identify factors limiting the overall detection sensitivity, each individual step in the PCR procedure for detection of *M. paratuberculosis* in clinical samples was investigated.

Three different ways to treat samples were investigated and evaluated with respect to their ability to concentrate the mycobacteria and remove amplification inhibitors, and their practicability. In the first study (paper I) mycobacteria were extracted with xylene, followed by lysis with proteinase K. The samples were then analysed by single and nested PCR without further treatment. In the second study (paper II) the mycobacteria were extracted with xylene, followed by disruption by bead beating with zirconium beads. The DNA released was subjected to phenol and chloroform extraction, and ethanol precipitation. Aliquots of the dissolved DNA were analysed by single, fluorescent, and nested PCR. In the third study (paper III), using on fecal samples only, the mycobacteria were extracted by buoyant density centrifugation in Percoll and lysed by bead beating with zirconium beads. DNA sequence capture was applied to concentrate the target DNA and to remove any remaining amplification inhibitors. The processed samples were then analysed by single PCR followed by dot-blot hybridisation. The sensitivity of each PCR system and the subsequent analysis of the amplicons obtained were compared with and evaluated against purified DNA of *M. paratuberculosis*, intact cells of *M. paratuberculosis*, spiked samples and *M. paratuberculosis* positive clinical specimens, treated according to the sampling methods mentioned above. To ensure that the presence of even weak inhibitors of the PCR was detected, a few mimic molecules were added to each individual PCR. A negative PCR result could not exclude the presence of *M. paratuberculosis* in the sample if mimic molecules were not amplified. The use of mimics in the PCR improved its reliability and assisted the interpretation of the negative results. No competitive effects were observed for the IS*900* amplification.

Pre-treatment methods

There was a clear distinction between the possibilities of detecting *M. paratuberculosis* in ileal mucosa, mesenteric lymph nodes, or feces, regardless of the amount of *M. paratuberculosis* in the samples, because of their differing complexity. Ileal mucosa caused the least problems, while fecal samples were the most difficult to analyse.

Extraction of the mycobacteria by means of xylene was to some degree appropriate for the treatment of ileal mucosa and mesenteric lymph nodes, but not for fecal samples, as complete inhibition of the PCR was observed in the latter. It was difficult to recover small amounts of *M. paratuberculosis* with the xylene method and, furthermore, inhibitory substances present in processed samples impaired the PCR sensitivity. The low detection sensitivity of *M. paratuberculosis* in clinical samples, observed in the first study, was probably due to ineffective lysis of the mycobacteria when treated only with proteinase K.

M. paratuberculosis DNA from fecal samples, prepared by buoyant density centrifugation followed by sequence capture, was successfully amplified and detected by dot-blot hybridisation. Buoyant density centrifugation has proved useful to remove the bulk of fecal debris and to concentrate the mycobacteria. However, the loss of *M. paratuberculosis* was to some degree unavoidable. The use of sequence capture made it possible to further remove PCR inhibitors and to concentrate the target DNA from a larger volume, which was necessary after

treating the mycobacteria by bead beating. The results from analyses of the three different pools of spiked feces showed differences in the inhibitory constituents and demonstrated the difficulty of finding one pre-treatment method that could solve all problems with amplification inhibitors.

All the pre-treatment methods were time consuming and are in their present form unsuitable for handling large numbers of samples simultaneously. Nevertheless, these methods do offer a tried and tested way to concentrate relatively small amounts of the target DNA and to remove most amplification inhibitors.

Performance of the PCR

The PCR systems were all very sensitive when applied to pure genomic DNA. As few as 1 to 20 genomes were easily detected. When analysing spiked tissue specimens, single PCR and fluorescent PCR detected 10^3 CFU/g whereas nested PCR detected 10^2 CFU/g, which is close to the detection limit by culture. The results showed nested PCR to be more sensitive than single or fluorescent PCR when analysing spiked specimens. However, when applied to clinical samples, fluorescent PCR and nested PCR were equally sensitive. Most of the culture-positive tissue samples also proved positive by PCR. These samples were estimated by culture to harbour from 20 up to $>10^3$ CFU/g. Samples found to contain fewer than 20 CFU/ g were deemed either negative or inhibited in the PCR analysis.

A more specialised method was needed for PCR analysis of feces than for PCR analysis of tissue specimens. Spiked fecal samples analysed by sequence capture PCR and dot-blot hybridisation showed a detection limit of 10^3 CFU/g, which was only one order of magnitude less sensitive than culture. The combination of buoyant density centrifugation with sequence capture PCR and dot-blot hybridisation allowed detection of the small numbers of *M. paratuberculosis* likely to be found in feces from subclinically infected animals (Chiodini et al., 1984).

The possibility of detecting small numbers of *M. paratuberculosis* seemed to be limited rather by the pre-treatment of the samples than the PCR. The number of handling steps prior to the PCR inevitably contributed to a certain loss of organisms, while low levels of amplification inhibitors, still present in the processed samples, reduced the PCR sensitivity. Furthermore, uneven distribution of *M. paratuberculosis* in clinical samples might have contributed to the failure of PCR to detect the organism in some samples. By comparing the results of the analysis of spiked and clinical specimens it was concluded that nested PCR, is able to some extent to overcome the problems of inhibitory substances present in the pre-treated samples.

Analysis of PCR products

Analysis of PCR products was facilitated by using fluorescent PCR and dot-blot hybridisation, as more PCR-positive samples were detected with these methods than with conventional agarose gel electrophoresis. The dot-blot hybridisation assay using the AlkPhos direct system offered an easy-to-use detection method with high sensitivity, though expensive.

It is concluded that the extraction of mycobacterial DNA from clinical specimens rather than the PCR is the chief bottleneck in diagnostic tests. It is a difficult task to find the balance between extracting small quantities of target DNA and at the same time exclude inhibitory substances of varying composition. The pre-treatment methods used, together with PCR offered a detection sensitivity of *M. paratuberculosis* close to that of culturing. In cases of suspected infection by *M. paratuberculosis*, PCR offers a quick alternative to a preliminary diagnosis. If a sample is found positive by PCR, preventive action such as restricting herd and staff movements can be taken until the diagnosis is confirmed by other tests. However, for routine diagnosis of fecal samples, culture still remains more sensitive than PCR-based detection.

Species specificity of IS*900* PCR (paper IV)

IS*900*-based PCR falsely detected a mycobacterium (strain 2333), isolated from a healthy Swedish dairy cow, as *M. paratuberculosis*. Further investigations, based on mycobactin dependence, AccuProbe test, and sequence analysis of the 16S rRNA gene, proved that the isolated mycobacterium was not *M. paratuberculosis,* but closely related to *M. cookii*, a species isolated from sphagnum vegetation and surface water on moors in New Zealand (Kazda et al., 1990; Monaghan et al., 1991). It was found that strain 2333 possessed a single copy of a sequence 94% identical with IS*900* and that the PCR systems developed for IS*900* detection amplified this new sequence with the same efficiency as for the true IS*900*. Analysis of the PCR products with restriction endonuclease enzymes, as suggested by Cousins et al. (Cousins et al., 1999), did not distinguish between *M. paratuberculosis* IS*900* amplicons and IS*900* amplicons of strain 2333.

Ever since it was discovered in 1989 the insertion sequence IS*900* has been regarded as specific for *M. paratuberculosis* and has formed the basis for diagnostic PCR. The continuing discovery of sequences sharing a close similarity to IS*900* has weakened the detection of IS*900* by PCR as incontrovertible evidence of *M. paratuberculosis*. Little is known about the genome of *M. paratuberculosis* and new alternative targets for species-specific PCR are therefore not ready to hand. The PCR system based on the F57 sequence (Coetsier et al., 2000) in *M. paratuberculosis* was successfully applied to distinguish strain 2333 from *M. paratuberculosis* and could be used as an adjunct to IS*900* PCR.

Strain differentiation (paper V)

When paratuberculosis re-appeared in Sweden in 1993, after being considered eradicated since 1962, a number of surveys were performed to establish the prevalence of the infection in Swedish cattle. It was also of interest to trace the source of infection by epidemiological investigations to ascertain if there existed a domestic chain of transmission of paratuberculosis, as an adjunct to the infection introduced by imported cattle.

The Swedish isolates of *M. paratuberculosis* were investigated by RFLP in collaboration with the veterinary research institute in Brno in the Czech Republic. The isolates were all found to be of subtype B-C1 (Pavlik et al., 1999a) which is the predominant RFLP type of western Europe. The existence of two independent chains of infection could therefore be neither confirmed nor excluded.

Further investigation of the Swedish *M. paratuberculosis* isolates was performed by the RAPD procedure described by Scheibel et al. (Scheibel & Gerlach, 1997). The results obtained with this method were not reproducible and the interpretation of the results was unreliable (unpublished results). Instead, a rep-PCR was developed based on primers targeting the repetitive ERIC element and the insertion sequence IS*900*. The main differences between rep-PCR and RAPD are the use of specific PCR primers, a higher annealing temperature and a longer extension time. The PCR condition used in rep-PCR thereby gives better reproducibility compared with RAPD. The Swedish isolates were analysed together with 37 other *M. paratuberculosis* strains expressing 12 different RFLP types. Except for one strain, all *M. paratuberculosis* strains showed identical fingerprints. *M. paratuberculosis* strain p1850 with RFLP type A-C10, showed one additional band in the analysis by agarose gel electrophoresis, but it shared all the other characteristic bands with the other strains of *M. paratuberculosis*. Both IS*900*/ERIC-PCR and ERIC-PCR failed to distinguish between the *M. paratuberculosis* strains and are, therefore, not regarded as alternatives to RFLP.

There are few genetic differences between *M. paratuberculosis* and *M. avium* and the use of IS*900* has so far been accepted as a reliable way to distinguish these two subspecies. The finding of mycobacterial strains harbouring sequences with close similarity to IS*900* calls for investigation of alternative species specific genes in order to identify *M. paratuberculosis*. A PCR that invariably results in a PCR product has an advantage over any PCR system based on the absence or presence of a specific target. In addition, a PCR system targeting general DNA sequences commonly encountered in diverse bacterial species offers an alternative to a specific PCR to confirm the presence of a specific bacterium. The fact that the rep-PCR developed showed a species-specific band pattern for *M. paratuberculosis* was used to ascertain the potential of the method to distinguish *M. paratuberculosis* from other mycobacteria. Of special interest was the possibility of differentiating *M. paratuberculosis* from *M. avium* and *M. intracellulare*, because these mycobacteria are frequently encountered in the environment, and of course, the mycobacterial strain 2333 which harbours an IS*900*-like sequence. Altogether 16 *M. avium* strains, three *M. intracellulare* strains and 12 other mycobacterial strains, representing both slow growers and

fast growers, were analysed and compared with the *M. paratuberculosis* strains. The fingerprint of *M. paratuberculosis* was clearly distinguishable from those obtained from the other mycobacteria. The IS*900*/ERIC-PCR developed gave reproducible results that successfully distinguished M. *paratuberculosis* from other mycobacterial species and subspecies.

Concluding remarks

Paratuberculosis is a rare disease in Sweden and, when found, it has been exclusively associated with imported of cattle. A voluntary control program to control paratuberculosis has been operating since 1998 by the Swedish Animal Health Service, a private veterinary organisation responsible for conducting health checks on cattle, sheep and pig production in Sweden. The program is based on annual bacteriological culturing of fecal samples and clinical veterinary investigation of participating herds. The molecular tools presented in this thesis offer a rapid aid for the detection of *M. paratuberculosis* in suspected cases of paratuberculosis.

At present, PCR cannot replace conventional microbiological methods, but it does offer a rapid and valuable complement for the diagnosis of *M. paratuberculosis*. Different pre-treatment methods are required prior to PCR and the choice of method depends on the type of clinical specimen to be analysed. Detection of *M. paratuberculosis* is more easily accomplished in tissue specimens than in fecal samples. It is therefore, very encouraging that the method based on buoyant density centrifugation and sequence capture developed for treatment of fecal samples has made it possible to use PCR to detect low numbers of *M. paratuberculosis* in feces.

The application of internal positive PCR controls has substantially improved the possibility of interpreting the outcome of different sample pre-treatment methods regarding their influence of the ultimate PCR result. By using a carefully optimised number of the mimic molecules, the sensitivity of different PCR systems was easily determined and compared. The simple production of the mimic molecules could be adopted for any PCR primers.

The finding of a mycobacterium harbouring an IS*900*-like sequence that was amplified to the same extent as the true IS*900* in *M. paratuberculosis*, has made it impossible to use IS*900* PCR as the definitive method for identification of *M. paratuberculosis*. Alternative PCRs for verifying *M. paratuberculosis* are available, for example the PCR targeting the F57 sequence and the IS*900*/ERIC-PCR reported in this thesis.

Investigation of the Swedish isolates of *M. paratuberculosis* by RFLP showed the strains to belong to the same subtype, namely B-C1. The rep-PCR presented, based on IS*900* and ERIC as target sequences, could not be used to identify any intra-strain variation within the Swedish isolates either. Instead, this method proved valuable to distinguish *M. paratuberculosis* from other closely related mycobacteria, including M*ycobacterium* sp. 2333. The results of the strain characterisation have so far not revealed any evidence of a separate domestic chain of infection. It cannot be ruled out, however, that the subtype of a possible domestic strain is the same as the one found in the imported animals.

Future prospects

The detection methods for *M. paratuberculosis* presented here have a potential for future use in the diagnosis of paratuberculosis. Buoyant density centrifugation treatment of fecal samples together with DNA sequence capture has proved particularly useful. To further verify the applicability of this method, a large number of clinical fecal samples need to be investigated. The possibility of obtaining the required quantity is however limited in Sweden, but collaboration with the veterinary institutes of Denmark and Norway would facilitate this work as the paratuberculosis situation in these countries differs considerably.

In the present work concentration of *M. paratuberculosis* DNA by sequence capture was applied to pre-treated fecal samples. The intention in developing this technique was, however, to be able to adapt it to all kinds of clinical specimens. Studies are already in progress to evaluate sequence capture PCR directly on ileal specimens and lymph nodes. The Norwegian Veterinary Institute has a collection of feces, blood, and tissue specimens from previously performed infection challenge with *M. paratuberculosis* in goats and cattle and this is an excellent basis from which to start.

The major threat of introducing paratuberculosis into Swedish livestock population is the importing of cattle. For breeding purposes, import of semen is a safer option, as transmission of paratuberculosis by contaminated semen is not considered a high-risk source of infection. Nevertheless, imported semen should be free from *M. paratuberculosis* and the donor bulls are therefore serologically tested by ELISA. Whenever this is not practicable a test of the semen is required. To ascertain the presence of *M. paratuberculosis* in semen and to determine its detection limit by PCR, the sequence capture PCR developed presents a straightforward approach.

In a recently described real-time PCR for detection of *M. paratuberculosis,* a sensitivity equal to that of fecal culture was reported (Fang 2002). This method seems to offer several advantages over conventional PCR in the form of an automated PCR with the time-consuming post-detection of amplicons eliminated. The sensitivity of real-time PCR suggests that it could replace nested PCR and thereby reduce the risk of cross-over contamination by previously obtained amplicons. Furthermore, this technique enables us to use additional PCR targets simultaneously and this might be a way to solve the problem of false IS*900* detection.

The sensitivity of PCR could be further improved by using DNA polymerases that are less sensitive to inhibitors frequently encountered in samples such as faeces and blood, for instance *rTth* or *Pwo* (Abu Al-Soud & Radstrom, 1998). Forthcoming new thermostable DNA polymerases with desired function and stability will presumably result from the on-going extensive research in this area.

Further characterisation of the IS*900*-like sequence in strain 2333 and studies on other repetitive sequences found in the *M. avium* complex, sharing a high degree of sequence similarity, may reveal if these elements in mycobacteria have a

common function. For example, it has been suggested that the disruption of genes by insertion sequences may exacerbate the pathogenesis of mycobacteria.

The genome project on *M. paratuberculosis* and *M. avium* will contribute substantially to the discovery of new potential diagnostic target genes (Bannantine et al., 2002). However, we must bear in mind that the differences between *M. paratuberculosis* and *M. avium* may concern expression rather than genome sequence. New techniques in proteomics will therefore equip us with valuable tools for such studies.

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Don't Quit

When things go wrong, as they sometimes will,
When the road you're trudging, seems all uphill,
When the funds are low, and the debts are high,
And you want to smile, but you have to sigh,
When care is pressing you down a bit,
Rest if you must, but don't you quit.

Success is failure turned inside out,
The silver lining of seeds of doubt,
And you can never tell how close you are,
It may be near when it seems so far,
So, stick to the fight when you're hardest hit,
It's when things go wrong that you must not quit.

W. J. A. Rowe