Amplification of 16S rRNA sequences to detect *Mycobacterium paratuberculosis*

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Summary. A probe based on 16S ribosomal RNA (rRNA) sequences was developed to detect *Mycobacterium paratuberculosis*, the causative agent of Johne's disease in cattle. Three universal primers were used to sequence the amplified fragments of the 16S rRNA gene of various species of mycobacteria. When the nucleotide sequences were analysed, a deletion was detected in the sequence of the fast-growing species. An oligonucleotide probe (P) directed to this region was synthesised and hybridised directly with total RNA of various mycobacterial strains in a dot-spot assay. The probe detected *M. paratuberculosis*, some other slow-growing mycobacteria of the *M. avium-intracellulare* (MAI) complex, and one atypical strain, *M. gordonae*. To increase the sensitivity of the probe, a 413-bp fragment of the 16S rRNA gene of *M. paratuberculosis* between P and a second oligonucleotide primer was amplified and hybridised with a *M. paratuberculosis*/*M. avium*-specific probe. When faecal samples of cattle were tested, all culture-positive samples were positive in the PCR assay.

Introduction

Paratuberculosis or Johne's disease, a chronic progressive disease in ruminants, is caused by *Mycobacterium paratuberculosis*.¹ Johne's disease causes substantial economic losses in the cattle industry¹ and its control is hampered by the lack of a rapid and reliable test to detect bacteria shed by individual cattle. Immunologically-based tests to detect Johne's disease have long been used, but these tests are neither sensitive nor specific enough. *M. paratuberculosis* is a slow-growing mycobacterium, closely related to the *M. avium-intracellulare* (MAI) complex, which belong to Runyon group III.^{2–4} Until recently, the only reliable method of detecting *M. paratuberculosis* was to culture the bacteria from faeces.^{5,6} Culture however, requires between 6 weeks and 6 months.

Runyon classified mycobacteria on the basis of their growth rate and pigmentation.⁷ Stahl *et al.*⁸ sequenced 16S ribosomal (r)RNA of many mycobacteria and used the data to classify *M. paratuberculosis*, *M. avium* and *M. intracellulare*, together with *M. scrofulaceum*, in one group. Analyses of the sequences of amplified 16S rRNA genes of several mycobacteria by Rogall *et al.*⁹ confirmed that *M. avium* and *M. paratuberculosis* are closely related; their 16S rRNA sequences showed 99.9% identity. However, they could not confirm the close relatedness of these mycobacteria and *M. scrofulaceum*.

The application of DNA probes offers an alternative approach to detecting bacteria, and in this respect probes directed at rRNA sequences have been shown to be useful.^{10,11} DNA probes directed to the 16S rRNA of bacteria of the MAI complex in culture are available commercially.¹² We detected *M. paratuberculosis* with the Gen-probe kit for the MAI complex¹² (unpublished results). This probe, however, still relies on lengthy culturing of the bacteria. Although DNA probes offer the possibility of detecting micro-organisms directly in clinical material, the sensitivity of these tests is still too low. The sensitivity can be increased enormously by first amplifying the DNA of specific pathogens in clinical material by the polymerase chain reaction (PCR) technique¹³ and then detecting the amplified DNA with a labelled DNA probe.¹⁴

In the present study, we used an oligonucleotide probe (P) complementary to the 16S rRNA of *M. paratuberculosis* isolated from cattle with Johne's disease. The probe was used directly to detect rRNA and to detect *M. paratuberculosis* in faeces of infected animals after amplifying the DNA of the 16S rRNA gene.

Materials and methods

Bacterial strains, faecal samples and growth conditions

All strains used in this study (table I) were grown on Middlebrook 7H10 medium at 37°C. Strains that

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Table I. Source and origin of mycobacteria and control strains used in this study and their specificity to probe P in dot-spot hybridisation assay

Bacterial strains	Source	Origin	Specificity to probe P
<i>M. avium</i> D4	Chicken	Lelystad†	+*
<i>M. avium</i> serotype 6	Human	Lelystad	+
<i>M. avium</i> serotype 19	Pig	Lelystad	+
<i>M. paratuberculosis</i> JII	Lab. strain	Weybridge†	+
<i>M. paratuberculosis</i> J2A	Lab. strain	Weybridge	+*
<i>M. paratuberculosis</i> Teps	Lab. strain	Weybridge	+
<i>M. paratuberculosis</i> 316F	Lab. strain	Weybridge	+
<i>M. paratuberculosis</i> C	Lab. strain	Weybridge	+
<i>M. paratuberculosis</i> Linda	Human	Connecticut	+
<i>M. paratuberculosis</i> 52991	Human	Lelystad	+
<i>M. paratuberculosis</i> B619	Cow	Lelystad	+
<i>M. paratuberculosis</i> H100	Cow	Lelystad	+
<i>M. paratuberculosis</i> B854	Cow	Lelystad	+
<i>M. paratuberculosis</i> H159	Cow	Lelystad	+
<i>M. paratuberculosis</i> C303	Cow	Lelystad	+
<i>M. paratuberculosis</i> Zw95	Cow	Lelystad	+
<i>M. paratuberculosis</i> C538	Cow	Lelystad	+
<i>M. paratuberculosis</i> H92	Cow	Lelystad	+
<i>M. paratuberculosis</i> H109	Cow	Lelystad	+
<i>M. paratuberculosis</i> C456	Cow	Lelystad	+
<i>M. gastri</i>	Pig	Lelystad	-*
<i>M. marinum</i>	Fish	Lelystad	-*
<i>M. triviale</i>	Anteater	Lelystad	-*
<i>M. terrae</i>	Buzzard	Lelystad	-*
<i>M. kansasii</i> 1	Cow	Lelystad	-*
<i>M. gordonae</i>	Fish	Lelystad	+*
<i>M. scrofulaceum</i>	Cow	Lelystad	-*
<i>M. kansasii</i> 2	Cow	Lelystad	-
<i>M. phlei</i> 82	Lab. strain	Weybridge	-
<i>M. fortuitum</i>	Pig	Lelystad	-
<i>M. chelonae</i>	Lion	Lelystad	-
<i>M. smegmatis</i>	Lab. strain	Lelystad	-
<i>Escherichia coli</i>	Lab. strain	Utrecht‡	-

* Hybridisation at 45°C.

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were dependent on mycobactin for growth were cultured on medium supplemented with mycobactin 2 µg/ml. Faecal samples of 10 cattle infected clinically or subclinically with *M. paratuberculosis* were cultured. Faecal samples of 10 culture-negative cattle were tested also.

Preparation of mycobacterial RNA and DNA

Mycobacterial cells (100 mg) were washed three times with phosphate-buffered saline. The pelleted cells were suspended in 500 µl of RNA extraction mixture according to the method of Chomczynski.¹⁵ To the suspension, 500 µl of water-saturated phenol (pH 4.0), 100 µl of chloroform, 50 µl of 2 M sodium acetate (pH 4.4), and 200 µl of 0.1-mm zirconium beads were added. The cells were disrupted in a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA) at high speed for 3 min.¹⁶ The tubes were cooled on ice for 15 min and then centrifuged for 20 min at

4°C at 10 000 *g*. The aqueous phase was shaken with an equal volume of chloroform:isoamylalcohol (24:1) and centrifuged for 10 min at 4°C. RNA was precipitated from the aqueous phase by treatment with an equal volume of ice-cold isopropanol for 1 h at -80°C. Precipitated RNA was pelleted by centrifugation for 20 min at 10 000 *g*. The resulting RNA pellet was dissolved in 300 µl of the RNA extraction mixture and precipitated again. The RNA pellet was resuspended in ethanol 75%, sedimented, vacuum dried, and then dissolved in 40 µl of water. The purity of RNA was checked by agarose gel electrophoresis.

The procedure described for isolating RNA was modified and used to isolate DNA. Pelleted cells (100 mg) were resuspended in 500 µl of 10mM Tris-HCl, 1mM EDTA (TE). To the suspension, 500 µl of TE-saturated phenol (pH 7.5) and 200 µl of zirconium beads were added, and the mycobacteria were disrupted as described above. The tubes were centrifuged for 15 min at 8000 *g*. The aqueous phase was trans-

ferred to a new tube and shaken for 25 min with an equal volume of chloroform:isoamylalcohol:phenol (24:1:25). After centrifugation, the water phase was extracted four times with ether, and DNA was precipitated by adding 200 μ l of 3 M sodium acetate (pH 5.2) and 700 μ l of ice-cold isopropanol. The DNA was pelleted, washed with ethanol 75%, and dried. It was then resuspended in 500 μ l of TE, treated with RNAase A (10 mg/ml) and stored at 4°C.

Isolation of DNA from faecal material

A 1-g sample of faeces was suspended in 10 ml of 0.2 M NaOH.¹⁷ Bacteria were isolated by centrifugation for 30 min at 2000 *g* from the supernate of this suspension obtained by centrifugation for 5 min at 100 *g*. The pellet was resuspended in 0.5 ml of 0.2 M NaOH. An equal volume of TE-saturated phenol:chloroform:isoamylalcohol (25:24:1) and 200 μ l of 0.5-mm zirconium beads or 0.1-mm glass beads were added. The mycobacteria were disrupted in a Mini-Beadbeater as described above. Samples were stored on ice until centrifugation at 16 000 *g* for 10 min. From the aqueous phase, 100 μ l was transferred to a 1.5-ml microcentrifuge tube containing 20 μ l of activated silica (Sigma) and 900 μ l of guanidinisothiocyanate wash buffer.¹⁸ Samples were shaken by hand and left at room temperature for 10 min. They were then centrifuged for 15 s at 16 000 *g*. Supernates were discarded and pellets were washed twice with ethanol 70%, once with acetone, dried for 10 min at 56°C and eluted with 100 μ l of H₂O for 10 min at 56°C. This extract was stored at -80°C until used in the PCR (1 μ l per reaction).

Amplification of DNA

The PCR was performed as described by Saiki¹³ with some modifications. Briefly, 1 μ g of bacterial DNA was incubated in a total volume of 100 μ l containing 200 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, gelatin 0.1 g/L, 200 μ M each of dATP, dCTP, dGTP, dTTP (Boehringer, Mannheim) and 1 μ M of both primers. After heating for 8 min at 95°C, 2.5 units of Taq DNA polymerase (Perkin Elmer/Cetus) were added. Paraffin oil (100 μ l) was added to cover the reaction mixture. After 35 cycles (1 min at 95°C, 2 min at 58°C and 3 min at 72°C), 10 μ l of the amplified DNA was analysed by agarose gel electrophoresis.

Sequencing of amplified DNA fragments

The 16S rRNA gene was amplified by use of two universal primers, located at the 5' and 3' end of the gene, as described by Edwards *et al.*¹⁹ Amplified DNA fragments were directly sequenced according to the instructions of the manufacturer (Taq Track, Q5570, Promega) with labelled primers. The primers (A, B, C) hybridised to conserved regions in the 16S rRNA.²⁰

Synthesis and labelling of oligonucleotide probes

Oligonucleotides were synthesised with a 8600 Biosearch DNA synthesiser. The oligonucleotide probes were labelled at the 5' ends with γ -[³²P]-ATP and T₄ polynucleotide kinase (Boehringer, Mannheim) according to the method of Maniatis *et al.*²¹

Dot-spot hybridisations of rRNA and amplified DNA

RNA was resuspended in 65 μ l of 20 $\times$ SSC (3 M NaCl, 0.3 M trisodium citrate), and 25 μ l of denaturing buffer containing TBE (44.5 mM Tris, 1.5 mM sodium EDTA, 44.5 mM boric acid), formaldehyde 37% and formamide 50%. The mixture was heated for 5 min at 65°C, and 50 μ l was spotted on Hybond-N (Amersham International) with a minifold blotter. The blots were baked for 30 min at 80°C and hybridised with [³²P]-labelled probes for 18 h.

The amplified DNA fragments (4 μ l) were spotted directly on Zetaprobe (BioRad). The blots were treated with 0.5 M NaOH-1.5 M NaCl for 10 min and neutralised with 1.0 M Tris-HCl (pH 8.0)—1.5 M NaCl. They were then hybridised with γ -[³²P]-labelled probes for 2 h and washed in 5 $\times$ SSPE (0.75 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA) containing sodium dodecyl sulphate 0.5 g/L. Fuji RX films were exposed to the blots for various times at -70°C.

Results

Sequencing of PCR fragments

Three oligonucleotides, A, B and C,²⁰ that hybridised to 16S rRNA of mycobacteria at locations corresponding to the 16S rRNA sequence of *Escherichia coli*²²—519–536 (A), 907–920 (B), and 1392–1406 (C)—were used as primers (fig. 1). The 16S rRNA genes of *M. paratuberculosis* laboratory strain J2A, two field strains B854 and C456, and strain Linda, isolated from a human patient with Crohn's disease, were partially sequenced. The 16S rRNA genes of several other atypical mycobacteria were also sequenced (listed in table II). When the sequences from the various mycobacterial strains obtained with primers B and C were aligned, no major differences were found. Alignment of sequences obtained with primer A revealed a deletion in the 16S rRNA of the fast-growing *M. phlei* and *M. fortuitum* strains and in the slow-growing *M. scrofulaceum* (table II). The sequence in this region showed some variation among the slow-growing mycobacteria, and was highly divergent from that of *E. coli*. The sequence of *M. paratuberculosis* in this region was used to derive a complementary oligonucleotide probe (5'CCGAGAAAACCCGAC3'), which was designated probe P, as shown in table II.

Fig. 1. Schematic representation of the 16S rRNA of mycobacteria. The universal primers (A, B and C) were used for direct sequencing. The primer set G and A gave an amplified fragment of 341 bp and the primer set S and P gave a fragment of 413 bp. The sizes of the amplified DNA fragments are indicated as determined by direct sequencing of the amplified *M. paratuberculosis* 16S rRNA gene.

Table II. Sequence alignment of mycobacterial 16S rRNA located at the probe site as determined by sequencing the amplified fragments; numbering corresponds to the *E. coli* 16S rRNA sequence²²

Mycobacteria	Sequence
	433
<i>M. paratuberculosis</i> J2A	CCUCUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGAUUG-ACGG
<i>M. paratuberculosis</i> B854	CCUCUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGAUUG-ACGG
<i>M. paratuberculosis</i> C456	CCUCUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGAUUG-ACGG
<i>M. paratuberculosis</i> Linda	CCUNUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGAUUG-ACGG
<i>M. avium</i> D4	CCUCUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGAUUG-ACGG
<i>M. avium</i> serotype 6	CCUCUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGANNG-ACGG
<i>M. gordonae</i>	CCUCUUUCACCAUCGACGAAGGUCCGGUUUUUCUCGGGCUG-ACGG
<i>M. gastri</i>	CCUCUUUCACCAUCGACGAAGGUCCGGUUGUCUCGGAUUG-ACGG
<i>M. scrofulaceum</i>	CCUNUUUCGCCAGGGACGAAG-----CGCAAGUGGACGG
<i>M. phlei</i>	CCNCUUUCAGUAGGGACGAAG-----CGUGAGUG-ACGG
<i>M. fortuitum</i>	CCUCUUUCAAUAGGGACGAAG-----CGCAAGUG-ACGG
	probe P 3' CAGGCCCAAAGAGCC 5'

N, undetermined nucleotide.

Dot-spot hybridisation of rRNA

Probe P was tested in dot-spot hybridisation assays for its specificity for rRNA of several mycobacteria and *E. coli*. One of the universal sequence primers (A), which hybridises to all rRNA tested, was used as a control. Probe P reacted with all 10 field strains of *M. paratuberculosis* (fig. 2A), all *M. paratuberculosis* laboratory strains tested, and two human isolates, strain Linda and strain 52991 (fig. 2B); rRNA of *M. avium* strains also reacted in dot-spot hybridisation assays (fig. 2B), but rRNA of *M. scrofulaceum* did not (fig. 2C).

The probe clearly differentiated the fast-growing mycobacteria—*M. chelonae*, *M. fortuitum*, *M. phlei*—from the slow-growing *M. paratuberculosis* (fig. 2B). The specificity of the oligonucleotide probe was further tested in dot-spot assays with rRNA of several slow-growing mycobacteria not belonging to the MAI complex. The rRNA of *M. kansasii*, *M. gastri*, *M.*

marinum and *M. gordonae* hybridised weakly with the probe at 40°C. When the hybridisation temperature was raised to 45°C, *M. paratuberculosis*, bacteria of the MAI complex and the atypical mycobacterium, *M. gordonae*, were the only species that reacted positively (fig. 2C and data not shown). The specificity of probe P for all bacteria tested is summarised in table I.

With probe P in dot-spot assays it was possible to detect 5 ng of RNA, corresponding to about 10⁷ bacteria.

Amplification of chromosomal DNA

The PCR was used to increase both the sensitivity and the specificity of the assay. One of the sequence primers (A) was used together with primer G (5'-CTCAAGACGCATGTCTTCTGGTGGAA-AGCTTTTGCGGT3'), which corresponds to nu-

Fig. 2. Dot-spot hybridisation assays (A) Probe P and a positive control probe (A) were hybridised to serial dilutions of RNA from *M. paratuberculosis* laboratory strains 316F and JII and field strains of *M. paratuberculosis* isolated from cattle with paratuberculosis. (B) The same experiment with *M. avium* serotype 6, 18 and 19; two *M. paratuberculosis* strains Linda and 52991; *M. avium* D4; *M. paratuberculosis* Teps and J2A; fast-growing mycobacteria *M. chelonae*, *M. phlei*, *M. fortuitum*. (C) Assay with atypical slow-growing mycobacteria, *M. paratuberculosis* J2A and *M. avium* D4 at an increased hybridisation temperature of 52°C.

cleotides 185–223 of the *E. coli* 16S rRNA sequence²² and which is used as a DNA probe in a commercial kit to detect *M. avium* in culture,²³ to amplify a fragment of 341 bp. In fig. 1, the location of the primers and the size of the amplified fragments are shown schematically. Primers G and A were combined in the PCR on chromosomal DNA of several mycobacteria. Apart from *M. paratuberculosis*, the other slow-growing mycobacteria (*M. avium*, *M. kansasii*, *M. gordonae* and *M. gastri*) and the fast-growing *M. phlei* yielded a product of the appropriate size (fig. 3A). These amplified fragments were spotted on nylon filters and hybridised to probe P at 52°C. This resulted in the same specificity as with the direct hybridisation assay (fig. 3A). However, when this primer set was tested in the PCR on faeces of culture-negative cattle, an amplified fragment was also seen in the agarose gel (fig. 4A). After hybridising these amplified fragments to probe P, these culture-negative faecal samples gave

negative results (fig. 4A). It seems likely that the primers hybridised non-specifically to DNA of saprophytic mycobacteria present in faeces. Apparently, this non-specific hybridisation interfered with specific hybridisation to *M. paratuberculosis* DNA. Only weak positive or negative reactions were observed after hybridisation to probe P (fig. 4B).

To solve this problem, primer P, which did not react with saprophytic mycobacteria in the direct dot-spot hybridisations, was used in the PCR together with another primer, S (5'GTCTGAACGGAAAGGCC-T3'), which was derived from the 16S rRNA sequence (8) at position 63–79 (fig. 1). When we tested this primer set on chromosomal DNA of several mycobacteria, DNA of fast-growing mycobacteria was not amplified (fig. 3B). After hybridisation of these amplified fragments to probe G at 72°C, *M. gordonae* also gave negative results (fig. 3B). When these primers were tested in the PCR on faeces of two culture-

Fig. 3. Agarose gel electrophoresis of amplified DNAs of different mycobacteria. Amplification was done between (A) primers G and A (lanes 1–9), and (B) primers S and P (lanes 1–9). Lane 1, *M. paratuberculosis* J2A; 2, *M. avium* D4; 3, *M. kansasii*; 4, *M. gordonae* 1; 5, *M. gastri*; 6, *M. phlei*; 7, *E. coli*; 8, negative control (PCR mixture without additions); 9, positive control (1 µg of *M. paratuberculosis* genomic DNA); M, phage λ PstI marker. Dot-spot hybridisation assay of amplified DNAs of *M. paratuberculosis* J2A, *M. avium* D4, *M. kansasii*, *M. gordonae*, *M. gastri* and *M. phlei*; amplification was between (A) primers G and A hybridised with probe P at 52°C, and (B) primers S and P hybridised with probe G at 72°C.

Fig. 4. Agarose gel electrophoresis of the amplified fragments of two culture-positive faecal samples (lanes 1 and 2) and the two culture-negative faecal samples (3 and 4). Amplification was between (A) primers G and A and (B) primers S and P; lanes 5 and 6, negative and positive controls; M λ PstI marker. Dot-spot hybridisation of the culture-positive (1 and 2) and culture-negative (3 and 4) faecal samples (A) after amplification with primers G and A and hybridisation with probe P at 52°C and (B) after amplification with primers S and P and hybridisation with probe G at 72°C (5, negative controls; 6, positive controls).

negative cattle, no amplification was seen (fig. 4B), whereas faecal samples of two culture-positive animals yielded the expected amplified fragment of 413 bp

hybridising to probe G (fig. 4B). Another 10 culture-positive and 10 culture-negative faecal samples of cattle were tested in the PCR with primers S and P. All culture-positive cattle gave a positive hybridisation signal with probe G (fig. 5I and II), whereas the culture-negative cattle did not give such a signal (fig. 5III and IV).

Fig. 5. Dot-spot hybridisation of 10 culture-positive faecal samples (II-5 and III-5), and 10 culture-negative faecal samples (III-5 and IV-5) after amplification with primer S and P and hybridisation to probe G at 72°C; V1 negative control; V2, positive control.

Discussion

The 16S rRNA genes of various mycobacteria were amplified and partially sequenced. When the sequences were aligned, a highly diverged region was found. The *M. paratuberculosis* sequence of this region was used to synthesise a complementary oligonucleotide probe, designated P. All 16S rRNA sequences of *M. paratuberculosis*, laboratory strains and field strains, isolated from cattle, were identical. Stahl *et al.*⁸ also reported that the 16S rRNA sequences of *M. paratuberculosis* and *M. avium* were similar. However, their conclusions were based on *M. paratuberculosis* strain

18. This strain is peculiar in not requiring mycobactin for growth and in having a restriction fragment length polymorphism identical to that of *M. avium*, but different from that of other *M. paratuberculosis* strains.²⁴ Our partial 16S rRNA sequences and the data of Rogall *et al.*⁹ clearly demonstrate that all *M. paratuberculosis* strains, *M. avium*, and the Crohn's disease isolate are identical. Unlike Stahl *et al.*,⁸ we found that *M. scrofulaceum* was different from the bacteria of the MAI complex with respect to the 16S rRNA sequences in the region of primer A.

Some slow-growing mycobacteria were more difficult to discriminate from *M. paratuberculosis*, since their sequences differed by only one nucleotide from the sequence of *M. paratuberculosis* in the region of probe P. Nevertheless, we were able to differentiate these mycobacteria from *M. paratuberculosis* in a dot-spot hybridisation assay of rRNA with probe P under stringent conditions. *M. avium* and *M. gordonae* could not be discriminated from *M. paratuberculosis* with probe P, since their sequences were identical in this region.

When probe P was hybridised directly to RNA, the detection limit was 10⁷ bacteria/sample. This is not sensitive enough to be used to detect cattle infected subclinically with *M. paratuberculosis*. To increase the sensitivity of the assay, the 16S rRNA gene of mycobacteria was amplified and these amplified fragments were hybridised with labelled probe P. When this assay was applied to faecal material, DNA

in faeces of *M. paratuberculosis*-negative animals was also amplified. After hybridising these amplified fragments to probe P, samples from all animals gave negative results, even culture-positive animals. This suggests that the primers G and A hybridised non-specifically with other DNA present in the samples. Selection of other primers (S and P) in the PCR on faecal material resulted in the exclusive and complete detection of culture-positive animals. Although probe G is not absolutely specific for *M. paratuberculosis*, we and Hurley *et al.*²⁵ detected no false-positive results from animals in a limited number of samples.

A more specific probe to detect *M. paratuberculosis*, based on an insertion element, has been reported.²⁷ However, it is not known whether this insertion element is present in all *M. paratuberculosis* strains. Collins *et al.*²⁶ described some caprine *M. paratuberculosis* strains which did not carry the *M. paratuberculosis* specific insertion element. More extensive testing has been done on *M. tuberculosis* with a similar insertion element. From 34 *M. tuberculosis* strains tested, five strains did not carry the insertion element.¹⁴ Since rRNA is present in all organisms, rRNA-derived detection methods for *M. paratuberculosis* are likely to prove useful in detecting *M. paratuberculosis*-infected animals. A more detailed field study is required to evaluate the sensitivity and the specificity of the assay.

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References

- Chiodini RJ, Van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* 1984; **74**: 218-262.
- Hurley SS, Splitter GA, Welch RA. Deoxyribonucleic acid relatedness of *Mycobacterium paratuberculosis* to other members of the family Mycobacteriaceae. *Int J Syst Bacteriol* 1988; **38**: 143-146.
- Saxegaard F, Baess I. Relationship between *Mycobacterium avium*, *Mycobacterium paratuberculosis* and "wood pigeon mycobacteria." *APMIS* 1988; **96**: 37-42.
- Yoshimura HH, Graham DY. Nucleic acid hybridization studies of mycobactin-dependent mycobacteria. *J Clin Microbiol* 1988; **26**: 1309-1312.
- Merkal RS, Larsen AB, Kopecky KE, Ness RD. Comparison of examination and test methods for early detection of paratuberculous cattle. *Am J Vet Res* 1968; **29**: 1533-1538.
- Merkal RS. Diagnostic methods for detection of paratuberculosis (Johne's disease). Proceedings of the 74th Annual Meeting of the US Animal Health Association 1970; **7**: 620-623.
- Runyon EH, Wayne LG, Kubica GP. Mycobacteriaceae. In: Buchanan RE, Gibbons NE (eds) *Bergey's Manual of determinative bacteriology*, 8th edn. Baltimore, Williams and Wilkins Co. 1974: 681-701.
- Stahl DA, Urbance JW. The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *J Bacteriol* 1990; **172**: 116-124.
- Rogall T, Wolters J, Flohr T, Böttger EC. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int J Syst Bacteriol* 1990; **40**: 323-330.
- Kohne DE. Application of DNA probe tests to the diagnosis of infectious disease. *Am Clin Prod Review* 1986; **5**: 20-29.
- Kohne D, Hogan J, Jonas V, Dean E, Adams TH. Novel approach for rapid and sensitive detection of microorganisms: DNA probes to rRNA. *Microbiology* 1986; **110**: 112.
- Drake TA, Hindler JA, Berlin OGW, Bruckner DA. Rapid identification of *Mycobacterium avium* complex in culture using DNA probes. *J Clin Microbiol* 1987; **25**: 1442-1445.
- Saiki RK, Scharf S, Faloona F *et al.* Enzymatic amplification of β -globulin genomic sequence and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; **230**: 1350-1354.
- Hermans PWM, Schuitema ARJ, Van Soolingen D *et al.* Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. *J Clin Microbiol* 1990; **28**: 1204-1213.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156-159.
- Hurley SS, Splitter GA, Welch RA. Rapid lysis technique for mycobacterial species. *J Clin Microbiol* 1987; **25**: 2227-2229.
- Vary PH, Andersen PR, Green E, Hermon-Taylor J, McFadden JJ. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J Clin Microbiol* 1990; **28**: 933-937.
- Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME *et al.* Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; **28**: 495-503.

19. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 1989; **19**: 7843–7853.
20. Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* 1985; **82**: 6955–6959.
21. Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory. 1982.
22. Stern S, Powers T, Changchien L-M, Noller HF. RNA-protein interactions in 30S ribosomal subunits: folding and function of 16S rRNA. *Science* 1989; **244**: 783–790.
23. Hogan JJ, Smith RD, Kop JA, McDonough SH. European patent application. Nucleic acid probes for detection and/or quantification of non-viral organisms. 1987. Number 873110363.4
24. McFadden JJ, Butcher PD, Thompson J, Chiodini R, Hermon-Taylor J. The use of DNA probes identifying restriction-fragment-length polymorphisms to examine the *Mycobacterium avium* complex. *Mol Microbiol* 1987; **1**: 283–291.
25. Hurley SS, Splitter GA, Welch RA. Development of a diagnostic test for Johne's disease using a DNA hybridization probe. *J Clin Microbiol* 1989; **27**: 1582–1587.
26. Collins DM, Gabric DM, de Lisle GW. Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. *J Clin Microbiol* 1990; **28**: 1591–1596.