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Species-specific identification of *Mycobacterium bovis* by PCR

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The Random Amplified Polymorphic DNA (RAPD) technique was used in the identification of a species-specific fragment of *Mycobacterium bovis*. A fragment of approximately 500 bp was amplified from the genome of 15 different *M. bovis* strains, including *M. bovis* BCG Pasteur, but was shown to be absent in 26 different mycobacteria and 20 different clinical isolates of *Mycobacterium tuberculosis*. When the fragment was used as a probe in a Southern blot analysis, several radioactive bands common to *M. tuberculosis* and *M. bovis* were observed. However, this fragment hybridized specifically to a 2900 bp *Eco*RI fragment in the *M. bovis* genome, but failed to hybridize in either *M. tuberculosis* or *M. avium* chromosomal DNA. Based on a partial nucleotide sequence of the 500 bp fragment, two oligonucleotide primers were designed and a PCR assay was developed. Using purified mycobacterial DNA samples, only *M. bovis* and *M. bovis* BCG rendered a unique amplification band. This PCR assay is able to detect down to 10 fg purified *M. bovis* DNA, which corresponds roughly to two bacilli. The assay is also useful for identifying the bacilli directly from uncultured biological samples, such as milk.

Keywords: RAPD, PCR, diagnosis, *Mycobacterium bovis*

INTRODUCTION

Bovine tuberculosis is currently an important zoonosis worldwide, and the possibility of human infection with *Mycobacterium bovis* cannot be ignored. Although scarce epidemiological information is available, *M. bovis* has been reported to have caused between 6 and 30% of the cases of human tuberculosis (TB) in the USA before milk pasteurization (Karlson & Carr, 1970). It is also the cause of 6·3% of the bacteriologically confirmed cases of tuberculosis in Western Ireland (Cormican & Flynn, 1992). Brett & Humble (1991), in a study conducted in New Zealand, showed an increase of bovine TB cases between 1983 (3·7%) and 1989 (14·6%). Despite the fact that in some regions in Latin America human TB diagnosis is still based on smear examination, a report has estimated that approximately 7000 new TB cases per year are caused by infection from *M. bovis* (PAHO, 1991).

To prevent human disease, a successful scheme of control and eradication of *M. bovis* infection in cattle is essential. However, eradication campaigns have been hampered by the low sensitivity and specificity of the bovine skin test currently used for the detection of infected animals (Radford *et al.*, 1988; Collins & Grange, 1983). The lack of an accurate diagnostic method for the detection of the causative agent in cattle leads not only to an enhanced risk in public health programmes, but also to economical loss for the cattle industry.

Speciation of mycobacteria for diagnostic purposes, among them *M. bovis*, has always been difficult. Attempts to develop more accurate serological diagnostic methods have been carried out (Fifis *et al.*, 1989; Harboe *et al.*, 1990; Griffin *et al.*, 1991), but have failed to show sufficient sensitivity and specificity, due mainly to the high antigenic cross-reactivity among mycobacteria (Daniel & Janicki, 1978; Harboe & Nagai, 1984). Recently, molecular biology techniques have proved to be of great value for the diagnosis of mycobacteria and also to distinguish between mycobacterial species (Franco *et al.*, 1989; Zainuddin & Dale, 1989; Haas *et al.*, 1993); the polymerase chain reaction (PCR) has been particularly useful for these purposes (Cook *et al.*, 1994; Noordhoek *et al.*, 1994; Mizrahi *et al.*, 1993; Telenti *et al.*, 1993). Most of

Abbreviation: RAPD, Random Amplified Polymorphic DNA.

these PCR assays amplify fragments from the so-called *Mycobacterium tuberculosis* complex, and thus cannot distinguish between infections caused by *M. tuberculosis* and those caused by *M. bovis*. Although specific identification of *M. bovis* has been reported, the procedure requires the amplification of fragments common to all mycobacteria and the subsequent specific identification using radiolabelled probes or restriction endonuclease patterns (Barry *et al.*, 1993; Hance *et al.*, 1989; Collins & de Lisle, 1985).

As a consequence of the high antigenic cross-reactivity and the close genomic relationship among mycobacteria, the identification of a species-specific sequence is not an easy task (Eisenach *et al.*, 1986; Baess, 1979, 1983). We have previously reported a PCR assay for the diagnosis of human tuberculosis (Del Portillo *et al.*, 1991) based on a *M. tuberculosis* species-specific sequence (Parra *et al.*, 1991). In the present paper, we describe the use of the Random Amplified Polymorphic DNA (RAPD) technique (Williams *et al.*, 1990; Welsh & McClelland, 1990) for the identification of a species-specific *M. bovis* genomic fragment, and the development of a single-step PCR assay that can be used as a powerful tool in epidemiological studies, which would allow an efficient control of bovine tuberculosis.

METHODS

Bacterial strains. The sources of mycobacterial strains used are listed in Table 1. Mycobacteria were grown in Sauton or Lowenstein-Jensen medium, harvested and stored at 4 °C until used. *M. bovis* strains were isolated from infected animals in different regions around the country. *M. tuberculosis* clinical isolates were obtained from the Bacteriology Laboratory, Hospital San Juan de Dios. All strains were typed by conventional microbiological and biochemical tests.

Chromosomal DNA extraction. Large-scale DNA extraction from both fast- and slow-growing mycobacterial species was performed as previously described by Del Portillo *et al.* (1991). For small-scale purification of DNA, a loopful from a grown colony was resuspended in TE (10 mM Tris/HCl, pH 8·0, 1 mM EDTA) and washed three times with the same buffer. The bacilli were incubated for 1 h at 37 °C in the presence of lysozyme (2 mg ml^{-1}). Bacterial membranes were disrupted by increasing the temperature to 60 °C and the addition of SDS and proteinase K (Bethesda Research Laboratory) to a final concentration of 1 % (w/v) and 250 μg ml^{-1}, respectively. After 1·5 h, DNA was extracted with phenol/chloroform and precipitated with 0·6 vol. 2-propanol. The pellet was washed with 70 % (v/v) ethanol and resuspended in 30–50 μl distilled water; 10 μl was used for the RAPD technique, or for the specific PCR assay.

RAPD primers. The oligonucleotide sequence of the primers used for the RAPD technique are listed in Table 2. The oligonucleotide sequences were designed according to the GC content of mycobacterial genomes, and using the Oligo version 4.0 program (National Biosciences). The primers were synthesized by the solid phase phosphite triester method on a Pharmacia LKB Gene Assembler Special. Purification and concentration of each primer was carried out as published elsewhere by Maniatis *et al.* (1982).

Amplification by the RAPD technique. The reactions were performed in a final volume of 50 μl containing 10 μl DNA from the different mycobacterial strains, 1 × reaction buffer (Gene amp kit, Perkin Elmer Cetus), 2·5 U *Taq* polymerase, 0·2 mM of each deoxynucleoside triphosphate, and 75 pmol of each primer. The reaction was carried out on a Perkin Elmer DNA thermal cycler. The reaction was subjected to 30 cycles of amplification with a denaturation step of 94 °C for 1 min, an annealing step of 30 s at the melting temperature (T_m, Table 2) of the primer used, and an extension step of 1 min at 72 °C.

Analysis of the RAPD products. An aliquot (5 μl) from the RAPD assay was analysed by gel electrophoresis in a 1 % (w/v) agarose gel. DNA was visualized by staining with ethidium bromide (0·5 μg ml^{-1}; Sigma) and gels were photographed on a UV transilluminator. The amplified products were blotted onto Z-probe membranes (Bio-Rad) for hybridization analysis.

The specific fragment amplified by the RAPD technique, approximately 500 bp long, was purified from agarose gels

Table 1. **Reference strains used for the RAPD and PCR**

Strain	Source*
M. bovis	ATCC 19210
M. bovis BCG Pasteur	ATCC 27291
M. bovis	Cow isolate (Colombia)
M. bovis	Clinical isolate (Spain)
M. tuberculosis H37Rv	TMC 102
M. tuberculosis H37Ra	ATCC 25177
M. simiae	ATCC 25275
M. scrofulaceum	ATCC 19981
M. gordonae	ATCC 14470
M. nonchromogenicum	TMC 1481
M. triviale	ATCC 23290
M. intracellulare	ATCC 13950
M. phlei	TMC 1548
M. vaccae	ATCC 15483
M. diernhoferi	ATCC 19340
M. flavescens	TMC 1541
M. fortuitum	ATCC 6842
M. ulcerans	ATCC 19423
M. chelonae subsp. *chelonae*	ATCC 35752
M. chelonae subsp. *abscessus*	ATCC 19977
M. avium	ATCC 25291
M. kansasii	TMC 1204
M. africanum	ATCC 25420
Actinomyces sp.	ATCC 15214
Rhodococcus sp.	ATCC 12485
Nocardia asteroides	ATCC 3308
M. gastri	TMC 1456
M. smegmatis	ATCC 19420
M. szulgai	TMC 1328
M. terrae	TMC 1450
M. parafortuitum	ATCC 19686
M. marinum	TMC 1218
M. microti	ATCC 19422

* ATCC, American Type Culture Collection; TMC, Trudeau Mycobacterial Culture Collection.

Table 2. Primers used for RAPD assay and *M. bovis* detection

Primer	Nucleotide sequence	T_m (°C)*	%GC at T_m†
JB1	5′ CGTCCGCCGA	36	46·8
JB2	5′ CGTCCGCTGA	34	42·7
JB3	5′ CCTCTCAGCA	32	38·6
JB4	5′ CCTATCAGCA	30	34·5
JB5	5′ CCTATCATCA	28	30·4
JB21	5′ TCGTCCGCTGATGCAAGTGC	64	68
JB22	5′ CGTCCGCTGACCTCAAGAAG	64	68

* T_m (°C), Melting temperature 2(A + T) + 4(G + C).

† %GC at T_m, melting temperature %GC (Baldino *et al.*, 1989).

using a Geneclean kit (Bio 101). Amplified fragment (10 ng) was labelled with the Rediprime kit (Amersham) using [α-^{32}P]dCTP (Amersham).

The Z-probe membranes containing the RAPD products from the different mycobacterial strains were hybridized with the radiolabelled probe (1×10^6 c.p.m. ml^{-1}) at 42 °C overnight in a solution containing 50% (v/v) formamide, 0·12 M Na_2HPO_4, pH 7·2, 0·25 M NaCl, and 7% (w/v) SDS. After hybridization, the membrane was washed for 15 min in 2 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate)/0·1% SDS at 42 °C; 30 min in 1 × SSC/0·1% SDS at 42 °C, and finally for 30 min in 0·1 × SSC/0·1% SDS at 65 °C, and exposed overnight to Kodak X-Omat film at −70 °C.

For a genomic Southern blot analysis, 5 μg *M. tuberculosis*, *M. bovis* and *M. avium* DNAs were digested to completion with *Eco*RI. The hybridization was carried out as described above.

Cloning and sequencing of the specific fragment. The purified fragment was cloned into the pCR1000 cloning plasmid (Invitrogene) and nucleotide sequencing of clone pLD 1 was carried out using T7 and M13 forward primer with the Sequenase kit from USB (Sanger *et al.*, 1977).

Amplification by PCR. Based on the specific 500 bp fragment partial nucleotide sequence, a pair of primers was designed for use in a specific PCR assay (Table 2). Reaction conditions were optimized for 30 cycles to be performed as follows: a denaturation step at 94 °C for 1 min, an annealing step at 68 °C for 1 min, and a synthesis step at 72 °C for 1 min.

Specificity and sensitivity of the PCR. In order to determine the reaction specificity, 50 ng different mycobacterial DNAs were used as a target for the reaction. The amplified products were analysed by ethidium-bromide-stained gels and by Southern blot analysis. Studies regarding the specificity of the reaction with non-related mycobacterial species were performed in spiked milk as follows. A loopful from a grown colony was resuspended in 1 ml crude milk. The samples were heated at 80 °C for 10 min, in order to inactivate the micro-organisms, and centrifuged at 12500 ***g*** for 15 min. The supernatant was discarded and the pellet treated in the same manner as described for small-scale DNA purification. The amplified products were analysed by electrophoresis in 1% (w/v) ethidium-bromide-stained agarose gels.

In order to establish the limit of sensitivity of the PCR assay, serial dilutions of *M. bovis* DNA were used as targets for the reaction. The amplification products were analysed as described above.

RESULTS

Identification of the species-specific fragment

With the aim of recognizing a *M. bovis*-specific genomic fragment, RAPD assays were conducted using DNA from both *M. bovis* and the closely related species *M. tuberculosis*. The RAPD primers were used alone or in different combinations (Table 2). Although several amplification products were visualized in ethidium-bromide-stained agarose gels, the majority were common to both mycobacterial species (data not shown). When primer JB2 was used alone, an amplification band of approximately 500 bp long was obtained, which was clearly present in three different *M. bovis* isolates but absent in the *M. tuberculosis* H37Rv strain (Fig. 1). To determine if the fragment was specific to *M. bovis* and absent in the genomes of different mycobacterial strains, DNA isolated from 22 mycobacteria and 3 different *M. bovis* isolates was used in the RAPD assay with primer JB2. As can be seen in Fig. 2(a), several RAPD amplification products are obtained from the diverse genomic DNAs tested, thus making it difficult to clearly recognize the presence or absence of the specific 500 bp fragment. The amplification products shown in Fig. 2(a) were therefore blotted onto Z-probe membranes and hybridized with the radiolabelled 500 bp fragment obtained from the RAPD assay of *M. bovis* DNA. A positive hybridization signal was observed

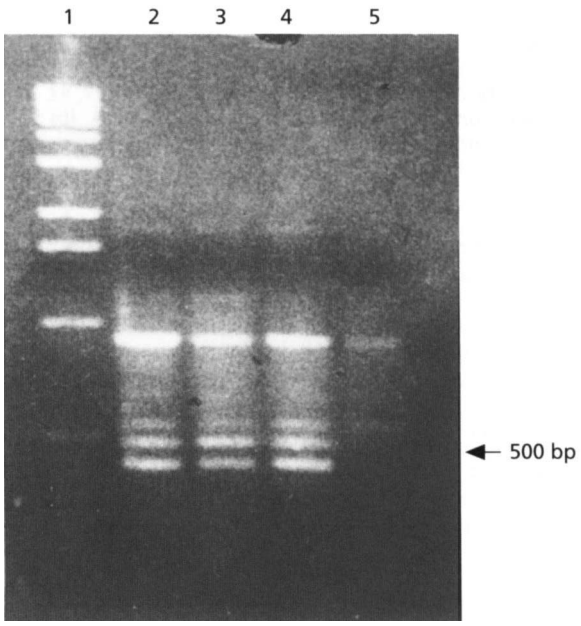

Fig. 1. Identification of the *M. bovis*-specific fragment. Agarose gel electrophoresis of the RAPD assay products of genomic DNA from *M. bovis* and *M. tuberculosis* using primer JB2. Lanes: 1, 1 kb ladder molecular size markers (Gibco-BRL); 2, *M. bovis* ATCC 19210; 3, *M. bovis* BCG Pasteur substrain ATCC 27291; 4, *M. bovis* wild-type; 5, *M. tuberculosis* H37Rv TMC 102.

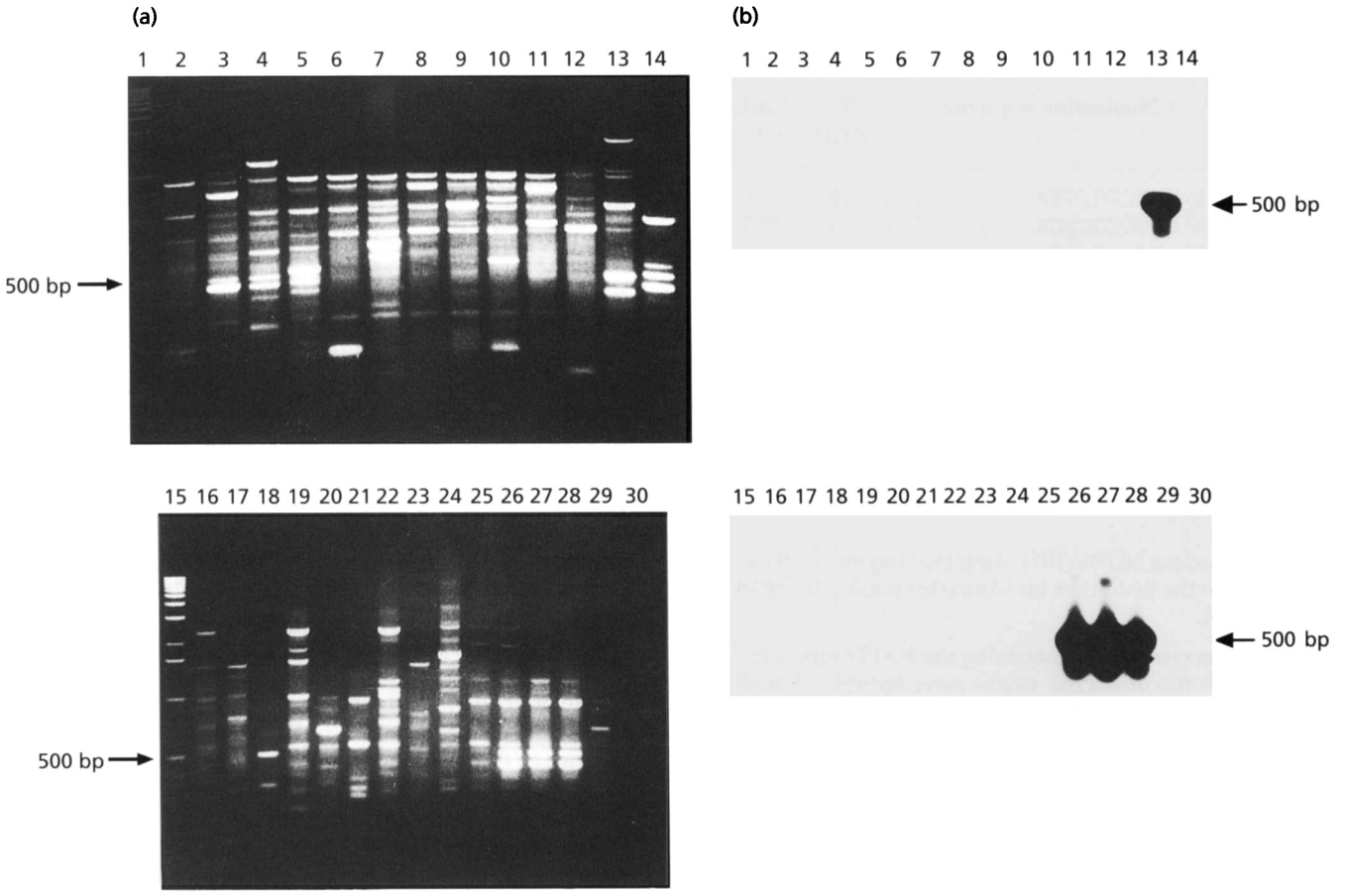

Fig. 2. RAPD analysis of different mycobacterial strains. (a) Agarose gel electrophoresis (1% gel). Lanes: 1, 1 kb ladder molecular size markers; 2, *M. simiae*; 3, *M. scrofulaceum*; 4, *M. gordonae*; 5, *M. nonchromogenicum*; 6, *M. triviale*; 7, *M. intracellulare*; 8, *M. phlei*; 9, *M. vaccae*; 10, *M. diernhoferi*; 11, *M. flavescens*; 12, *M. fortuitum*; 13, *M. ulcerans*; 14, *M. bovis*; 15, molecular size markers; 16, *M. chelonae* subsp. *chelonae*; 17, *M. chelonae* subsp. *abscessus*; 18, *M. avium*; 19, *M. kansasii*; 20, *M. gastri*; 21, *M. smegmatis*; 22, *M. szulgai*; 23, *M. terrae*; 24, *M. parafortuitum*; 25, *M. tuberculosis* H37Rv TMC 102; 26, *M. bovis* ATCC 19210; 27, *M. bovis* BCG Pasteur substrain ATCC 27291; 28, *M. bovis* wild-type; 29, *M. marinum*; 30, negative control without DNA. (b) Southern blot analysis using DNA transferred from gels in Fig. 2(a) and hybridized with the 500 bp radiolabelled fragment obtained from the RAPD assay using *M. bovis* DNA.

only with the genomes of the *M. bovis* strains and not with any of the other DNAs used (Fig. 2b).

The radiolabelled fragment was also used as a probe in a Southern blot using *Eco*RI-digested genomic DNA from *M. bovis*, *M. tuberculosis* and *M. avium*. The autoradiography revealed homologous regions between *M. bovis* and *M. tuberculosis*. However, the 500 bp fragment allowed the identification of a polymorphic region of 2900 bp in the *M. bovis* genome which was not homologous in the genomes of *M. tuberculosis* and *M. avium* (Fig. 3).

Development of the *M. bovis*-specific PCR

The nucleotide sequences of the primers used in this study are shown in Table 2. The *M. bovis*-specific 500 bp fragment was cloned in the pCR1000 cloning vector and the ends of the insert sequenced. Based on the partial nucleotide sequence of the recombinant clone pLD 1, primers JB21 and JB22 were synthesized. A PCR assay using these two primers was optimized using purified *M. bovis* and *M. tuberculosis* H37Rv DNA, such that after 30 amplification cycles a fragment of approximately 495 bp was detected exclusively from *M. bovis* DNA. In this assay, no other amplification products were observed from either the *M. tuberculosis* or the *M. bovis* genomes (data not shown).

Specificity and sensitivity of the PCR

The specificity of the PCR assay was examined using DNA isolated from 14 different reference mycobacterial species. The 495 bp amplification product was found only with *M. bovis* DNA. Representative samples tested by PCR amplification and Southern blot analysis of the products are shown in Fig. 4. To demonstrate that this PCR assay could be useful in distinguishing between *M. tuberculosis* and *M. bovis*, 20 *M. tuberculosis* clinical isolates and 11 *M. bovis* isolates were amplified with the JB21 and

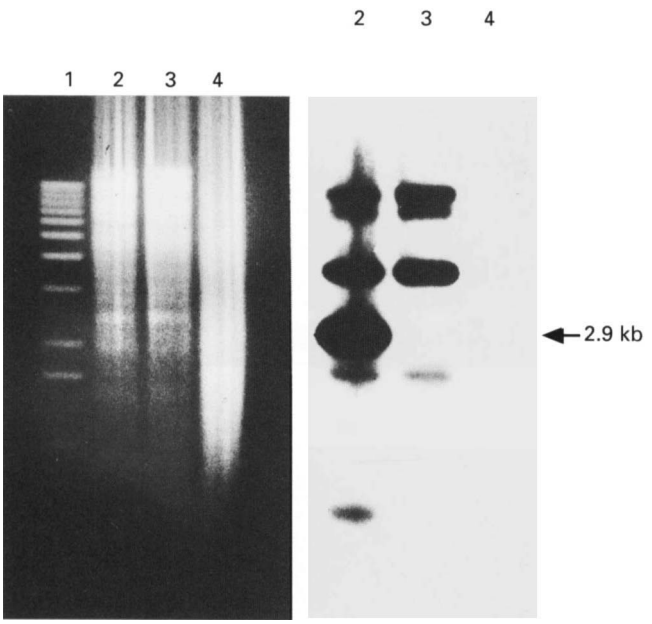

Fig. 3. Agarose gel electrophoresis and corresponding Southern blot analysis of *Eco*RI restriction of *M. bovis, M. tuberculosis* and *M. avium* genomic DNAs. Lanes: 1, 1 kb ladder molecular size markers; 2, *M. bovis* ATCC 19210; 3, *M. tuberculosis* H37Rv TMC 102; 4, *M. avium* ATCC 25291.

JB22 primers. As can be seen in Fig. 5, the PCR assay is shown to amplify a fragment exclusively from the *M. bovis* genomes, confirming the results obtained with the reference strains. These same strains were assayed by a *M. tuberculosis*-specific PCR test (Del Portillo *et al.*, 1991), which, as expected, rendered a 396 bp amplification band with the 20 *M. tuberculosis* strains (data not shown).

To demonstrate that the PCR assay could be carried out directly on biological samples, 1 ml crude milk was spiked with *M. bovis* or unrelated bacteria commonly isolated from milk (Table 1). PCR amplification of the 495 bp fragment was obtained only from milk spiked with *M. bovis* but not of that spiked with other bacteria, demonstrating not only that the assay is specific for *M. bovis*, but also that milk components do not inhibit the PCR reaction (data not shown).

The sensitivity of the PCR assay with primers JB21 and JB22 was determined by adding decreasing amounts of *M. bovis* DNA to the reaction vials. Down to 10 fg DNA could be amplified reproducibly to give a clearly detectable band in ethidium-bromide-stained agarose gels (data not shown).

DISCUSSION

A rational approach of preventing human infection by *M. bovis* and to reduce its economical impact due to diseased cattle involves the implementation of eradication programmes. In order to achieve this objective, a rapid, specific and reliable diagnostic method for the timely detection of *M. bovis* in infected animals is essential. In spite of the high DNA homology among genomes of the various mycobacterial species, and in particular of those belonging to the *M. tuberculosis* complex, which include

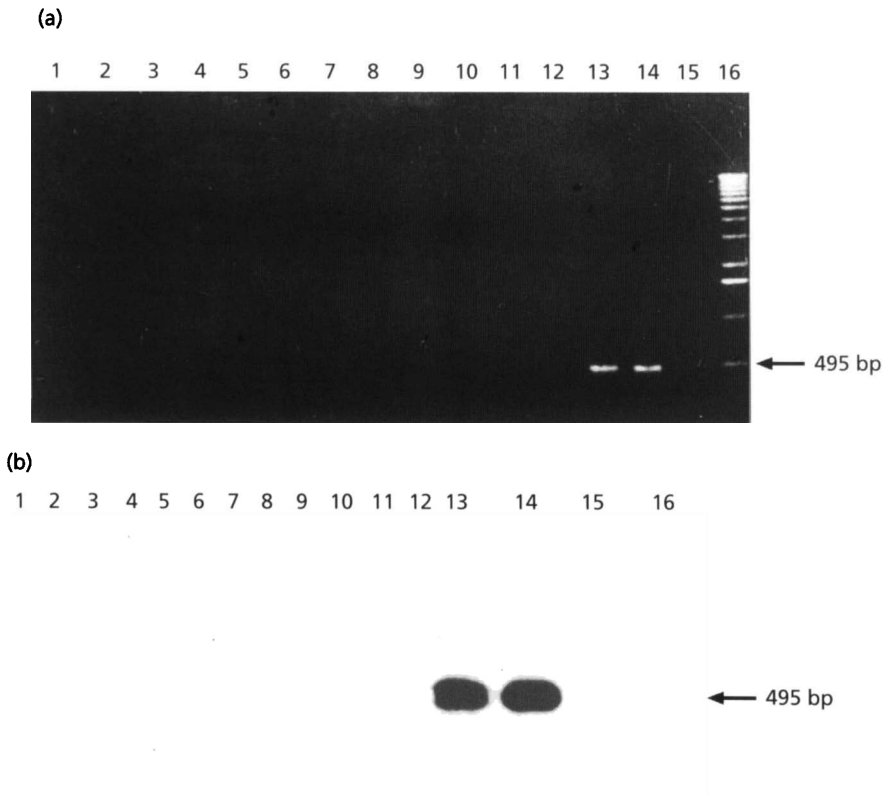

Fig. 4. Specificity of the PCR assay. (a) Amplification of DNA from various mycobacterial strains. Lanes: 1, *M. microti*; 2, *M. smegmatis*; 3, *M. scrofulaceum*; 4, *M. intracellulare*; 5, *M. marinum*; 6, *M. africanum*; 7, *M. avium*; 8, *M. fortuitum*; 9, *M. gordonae*; 10, *M. phlei*; 11, *M. tuberculosis* H37Ra; 12, *M. tuberculosis* H37Rv TMC 102; 13, *M. bovis* ATCC 19210; 14, *M. bovis* BCG Pasteur substrain ATCC 27291; 15, negative control without DNA; 16, 1 kb ladder molecular size markers. (b) Autoradiograph of samples from Fig. 4(a) hybridized with the 500 bp radiolabelled fragment.

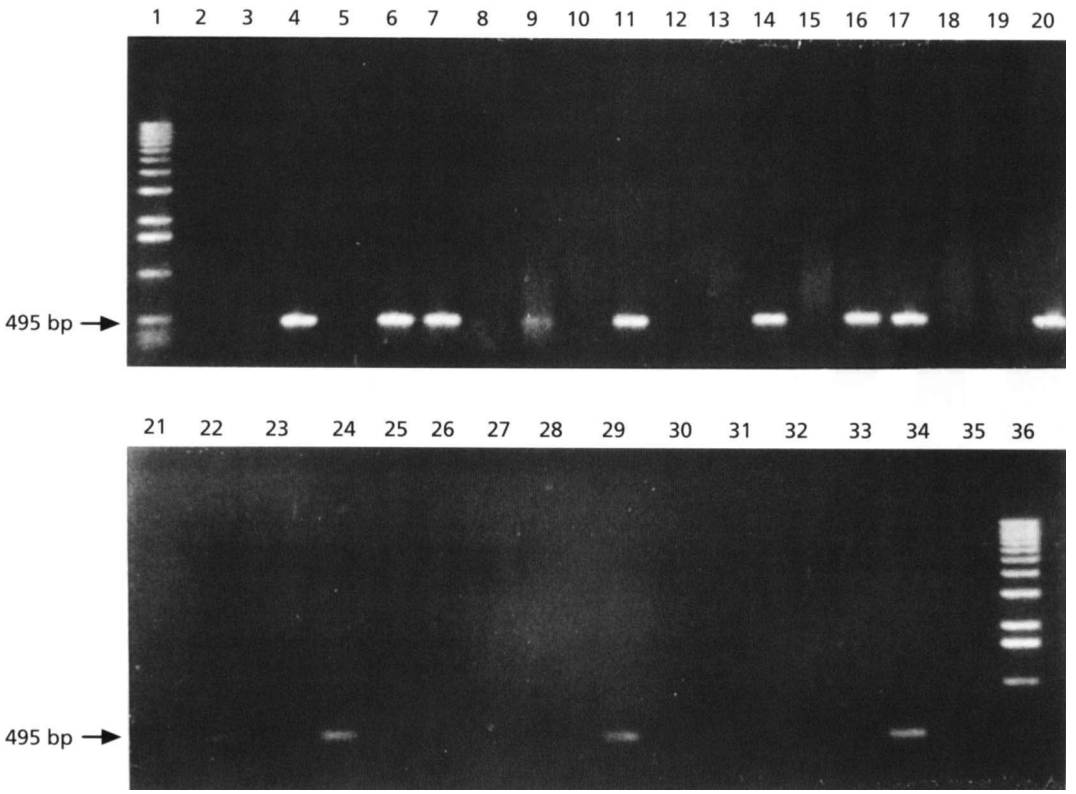

Fig. 5. **PCR specificity with the different *M. bovis* and *M. tuberculosis* clinical isolates. Analysis of the PCR products obtained from cultured clinical isolates DNAs. Lanes: 1 and 36, 1 kb ladder molecular size markers; 20 and 34, positive control *M. bovis* ATCC 19210; 35, negative control without target DNA; 2, 3, 5, 8, 10, 12, 13, 15, 18, 19, 21, 23, 25, 26, 27, 28, 30, 31, 32 and 33, DNA from different *M. tuberculosis* isolates amplified with JB21-JB22 specific primers; 4, 6, 7, 9, 11, 14, 16, 17, 22, 24, 29, DNA from different *M. bovis* isolates amplified with JB21-JB22 specific primers, showing the 495 bp amplification fragment.**

M. tuberculosis, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti* (Collins & de Lisle, 1984, 1985), we were able to develop a new species-specific PCR assay capable of exclusively detecting *M. bovis* DNA.

In this study, a combination of two commonly used molecular biology techniques were applied for the identification of a species-specific genomic fragment of the *M. bovis* bacillus: the RAPD technique was used to obtain a fragment unique to *M. bovis*, which was then used to design primers for the highly sensitive DNA amplification technique of PCR (Saiki *et al.*, 1988). The RAPD technique has been used previously for strain typing of several micro-organisms (Bassam *et al.*, 1992; Jayarao *et al.*, 1992), including *M. tuberculosis* (Palittapongarnpim *et al.*, 1993). The approach described here allowed the differentiation of *M. bovis* from other mycobacterial species, and more importantly, from the strains belonging to the *M. tuberculosis* complex, which share more than a 90% homology at the DNA level (Imaeda, 1985).

Southern blot analysis using *Eco*RI-digested genomic DNA hybridized to the radiolabelled 500 bp fragment obtained from *M. bovis* RAPD amplification revealed four fragments common to both the *M. bovis* and the *M. tuberculosis* genomes, indicating possible partial homologies, which confirms the close relationship between these two species. A strong 2900 bp hybridization signal was observed only in the *M. bovis* genome, suggesting the presence of a polymorphic region that could also be used in a restriction pattern analysis. Whether this 500 bp fragment is part of previously reported repetitive sequences (Thierry *et al.*, 1990; Hermans *et al.*, 1991) or is part of an intergenic region between these repeats is not yet known, but is under active investigation by our group.

The PCR assay using primers JB21 and JB22 clearly amplifies a fragment exclusively in the *M. bovis* genome. Detection of *M. bovis* by this assay can be carried out in a single step and thus presents an advantage over previous reports which involve an initial amplification step and the subsequent use of differential probes (Barry *et al.*, 1993; Collins & de Lisle, 1985) or restriction enzyme patterns (Hance *et al.*, 1989). Due to the clumping phenomena, characteristic of mycobacteria, quantification of the number of mycobacteria in a test sample or spiked milk has not yet been performed. However, we were able to detect down to 10 fg DNA, which corresponds roughly to two bacilli, accordingly to previous reports (Baess, 1984).

The specific PCR reported here can distinguish *M. bovis*

from other mycobacteria, including the closely related species *M. tuberculosis*. On the other hand, this method can be used directly on biological samples such as milk making this a good assay for the rapid, specific and sensitive diagnosis of *M. bovis*. Early detection of *M. bovis* by PCR could be useful either as an epidemiological tool for field trials in cattle or in human clinical follow-up programmes, in order to establish the real magnitude of bovine tuberculosis.

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