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Newly developed primers for the detection of *Mycobacterium avium* subspecies *paratuberculosis*

Elke Vansnick [a,d,*], Pim de Rijk [b], Francis Vercammen [c], Dirk Geysen [a], Leen Rigouts [b], Françoise Portaels [b]

[a] *Veterinary Department, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium*
[b] *Mycobacteriology Unit, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium*
[c] *Centre for Research and Conservation, Royal Zoological Society of Antwerp, K. Astridplein 26, 2018 Antwerp, Belgium*
[d] *Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium*

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Abstract

Recent publications reported the existence of IS*900* like sequences in mycobacteria different from *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). The primers used for IS*900* detection of *Map* have amplified these sequences causing false positive results. In this study, we have developed two new PCR assays for the detection of *Map*. The first assay is based on the IS*900* sequence using primers different from the ones previously reported, the second assay on the f57 sequence. The specificity of the tests was checked by analysis of 190 mycobacterial isolates (74 *Map* and 116 non-*Map* isolates). All *Map* strains were positive and all non-*Map* strains were negative. Serial dilutions of *Map* bacteria were used to assess the sensitivity of the assays. We achieved a sensitivity of 1 CFU per PCR for both assays. In addition, a PCR-simulating computer programme was used to evaluate the specificity of the new IS*900* primers.

The combination of the two PCR assays has proven to be useful for the identification of *Map* but validation on a large range of clinical samples still needs to be done.

Keywords: Paratuberculosis; PCR; IS*900*; *Mycobacterium avium*; f57

1. Introduction

Paratuberculosis or Johne's disease is a chronic and incurable disease of domestic and wild animals (in particular ruminants) caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). The disease is characterised by chronic diarrhoea and the symptoms of a general infectious process. Emaciation, decreased milk production, oedema, anaemia, infertility and eventually death are the dominant signs during the late stage of the disease. Large numbers of *Map* organisms may be shed in the faeces, thereby contaminating the environment (Cocito et al., 1994; Harris and Barletta, 2001).

The application of molecular methods for the diagnosis of paratuberculosis is under constant de-

* Corresponding author. Tel.: +32-3-2476278; fax: +32-3-2476268.
E-mail address: evansnick@itg.be (E. Vansnick).

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velopment and evaluation. A number of genes and sequences unique to *Map* have been identified over the years. The IS*900* sequence was discovered in the late 1980s (Stevenson and Sharp, 1997). This insertion sequence is a 1451 bp segment that lacks inverted terminal repeats and is repeated 15–20 times in the *Map* genome (Green et al., 1989). The f57 sequence was shown to be specific for the Johne's disease agent and is not found in any other mycobacterial species or *M. avium* subspecies *avium*. It is a 620 bp segment with a G + C content of 58.9% (Coetsier et al., 2000; Harris and Barletta, 2001).

The insertion element IS*900* has been routinely used to detect the presence of *Map* as it was shown to be present only in this subspecies (Collins et al., 1989; Green et al., 1989). The use of PCR for diagnostics is widespread: detection of IS*900* in formalin-fixed paraffin-embedded tissues (Whittington et al., 1999), after BACTEC 12B radiometric culture (Whittington et al., 1998), in commercial samples of bulk milk (Grant et al., 2000) and in human intestinal biopsies (Collins et al., 2000). However, *Map* is very closely related to common environmental organisms of the *M. avium* complex, particularly *M. avium* subspecies *avium* (Hurley et al., 1988). A 97% DNA homology amongst most isolates of *Map* and *M. avium* subspecies *avium* has been reported (Bannantine et al., 2002). Sequences related to IS*900* were also found in Wood Pigeon mycobacteria (IS*902*) and *M. avium* subspecies *avium* (IS*901*). The insertion sequence IS*1626* (found in *M. avium* subspecies *avium* and *M. intracelluare*) is another closely related insertion sequence of IS*900* (Puyang et al., 1999). It has been shown that some *Mycobacterium* species contain DNA sequences with considerable homology to the sequence of IS*900* (Cousins et al., 1999; Englund et al., 2002). The primers reported in the paper by Vary et al. (1990) are widely used in several variants of the IS*900* PCR test. Using these primers, Cousins et al. (1999) reported an IS*900* like PCR amplicon from environmental mycobacteria, thus demonstrating that false positive results could be obtained from strains other than *Map*. Englund et al. (2002) characterised a *Mycobacterium* isolate harbouring one copy of a sequence showing 94% identity to IS*900* at the nucleic acid level. These data indicated that PCR results based on the IS*900* sequence alone should be interpreted with caution. The currently used IS*900* primers may not be specific for *Map* as IS*900* (like) elements may be present in other closely related mycobacteria (Cousins et al., 1999; Englund et al., 2002). Cousins et al. (1999) recommended that restriction digestion should be used to confirm the profile of the internal sequence of the amplified product. However Englund et al. (2002) identified a *Mycobacterium* sp. (strain 2333) with an IS*900* like sequence of which the restriction sites after amplification with the original primers p90/91 and 150/921 were identical to the restriction sites in amplified DNA of *Map*. Therefore, restriction endonucleasis analysis did not to solve the problem of false positives. Englund et al. (2002) recommended that a positive IS*900* PCR should be confirmed by subsequent sequencing or by a PCR assay targeting another gene in *Map*.

In this study, two sets of newly developed PCR primers based on the insertion sequence IS*900* and the unique sequence f57 were developed and evaluated. The nucleotide sequences of these primers are characteristically different from primers previously reported.

2. Materials and methods

2.1. Bacterial isolates

Mycobacterial isolates from the collection of the Belgian *Mycobacterium* Reference Laboratory (Institute of Tropical Medicine, Antwerp, Belgium) were used. The *Map* isolates are listed in Table 1 , the non-*Map* isolates in Table 2 . *Map* isolates were grown on slopes of modified Löwenstein-Jensen medium supplemented with mycobactin J (1 mg/l), PANTA plus (40 ml/l) and sodium pyruvate (4 g/l), and cultured at 37 °C. The non-*Map* isolates were cultured on Löwenstein-Jensen slants or on Stonebrink medium (*M. bovis*), Ogawa supplemented with Fe ammonium citrate (1.5%) (*M. Haemophilum*) and Middlebrook 7H11 (*M. genavense*, *Corynebacterium* sp.).

All mycobacterial and bacterial species were identified with classical biochemical and/or molecular tests.

2.2. Extraction of genomic DNA

One loop of bacterial cells was suspended in 600 μl of disruption buffer (4 M guanidine thiocyanate (ICN biochemicals, Costa Mesa, CA), 0.025 M sodium cit-

Table 1
Map isolates used in the study

Strain	Source	Country
M02-0904	*Cervus elaphus*	Belgium
M02-0909	*Cervus elaphus*	Belgium
M02-1466	*Cervus elaphus*	Belgium
M02-1444	*Cervus elaphus*	Belgium
M02-1461	*Cervus elaphus*	Belgium
M02-1463	*Cervus elaphus*	Belgium
M02-1445	*Cervus elaphus*	Belgium
M02-0910	*Cervus elaphus*	Belgium
M02-1462	*Cervus elaphus*	Belgium
M02-1465	*Cervus elaphus*	Belgium
M02-1447	*Cervus elaphus*	Belgium
M02-1451	*Cervus elaphus*	Belgium
M02-1458	*Cervus elaphus*	Belgium
M02-1663	*Cervus elaphus*	Belgium
M02-1448	*Cervus elaphus*	Belgium
M02-1441	*Cervus elaphus*	Belgium
M02-0706	Cattle	Belgium
M02-0806	Cattle	Belgium
M02-0702	Cattle	Belgium
M02-0710	Cattle	Belgium
M02-1696	Cattle	Belgium
M02-1717	Cattle	Belgium
M02-0700	Cattle	Belgium
M02-1715	Cattle	Belgium
M02-0699	Cattle	Belgium
M02-1680	Cattle	Belgium
M02-0705	Cattle	Belgium
M02-1675	Cattle	Belgium
M02-1679	Cattle	Belgium
M02-1673	Cattle	Belgium
M02-0900	Cattle	Belgium
M02-0708	Cattle	Belgium
M02-0711	Cattle	Belgium
M02-0697	Cattle	Belgium
M02-1041	Cattle	Belgium
M02-0808	Cattle	Belgium
M02-1042	Cattle	Belgium
M02-0698	Cattle	Belgium
M02-1678	Cattle	Belgium
M02-0701	Cattle	Belgium
M02-0807	Cattle	Belgium
M02-1040	Cattle	Belgium
M02-0811	Cattle	Belgium
M02-0810	Cattle	Belgium
M02-0370	Cattle	Belgium
M02-0369	Cattle	Belgium
2666	Cattle	ATCC 19698
9403	Cattle	Burundi
M99-0901	Cattle	Belgium
M99-0912	Cattle	Belgium
M99-0932	Cattle	Belgium
M99-0933	Cattle	Belgium
M99-0937	Cattle	Belgium
M99-0938	Cattle	Belgium
M99-0941	Cattle	Belgium
M99-0942	Cattle	Belgium
M99-0943	Cattle	Belgium
M99-0946	Cattle	Belgium
M99-0947	Cattle	Belgium
M99-0948	Cattle	Belgium
M99-2678	Cattle	Australia
M99-2679	sheep	Australia
M99-2681	Cattle	Australia
M00-0010	Cattle	Belgium
M00-0012	Cattle	Belgium
M00-1671	Cattle	Belgium
M00-0002	Cattle	Belgium
M00-0003	Cattle	Belgium
M00-0324	Cattle	Belgium
M00-0004	Cattle	Belgium
M00-0451	Cattle	Belgium
M00-0452	Cattle	Belgium
M00-0453	Cattle	Belgium
M00-0005	Cattle	Belgium
Total number of *Map* strains		74

Table 2
Non-*Map* isolates used in the study

Species	No. of isolates tested
M. abscessus	2
M. africanum	1
M. avium	4
M. bovis	2
M. canetti	1
M. celatum	2
M. chelonae	5
M. chitae	1
M. duvalii	1
M. flavescens	1
M. fortuitum	2
M. haemophilum	5
M. heidelbergense	1
M. gadium	1
M. gastri	1
M. genavense	4
M. gordonae	5
M. interjectum	1
M. intermedium	1
M. intracellulare 1	5
M. intracellulare 2	5
M. kansasii	2
M. mageritense	1
M. malmoense	6
M. marinum	5
M. microti	1

Table 2 (*Continued*)

Species	No. of isolates tested
M. nonchronogenicum	1
M. parafortuitum	1
M. peregrinum	6
M. phlei	1
M. scrofulaceum	2
M. simiae	4
M. simiae like	1
M. smegmatis	6
M. szulgai	1
M. terrae	1
M. triplex	1
M. triviale	1
M. tuberculosis	1
M. ulcerans	5
M. vaccae	1
M. xenopi	2
Nocardia asteroides	1
Nocardia brasiliensis	1
Nocardia nova	1
Nocardia farcinica	1
Rhodococcus equi	1
C. flaccum faciens	1
C. huayni	1
C. equi	5
C. pyogens	1
C. xerosis	1
C. diphteriae	1
Total number of isolates	166

rate (pH 7), 0.5% sarkosyl (Sigma, St. Louis, MO), 0.1 M 2-mercapto-ethanol (Sigma), 20 mM EDTA (pH 8) (Sigma)). DNA was isolated out of the disruption buffer by a phenol:chloroform:isoamylalcohol (PCI) extraction. Six hundred microlitres of a PCI mixture (25:24:1; Acros Organics, Geel, Belgium) was added to the disruption buffer and mixed with a vortex for 5 s. After centrifugation at 12,000 × *g* for 5 min, the upper aqueous phase was transferred to a new tube and mixed with an equal volume of PCI mixture. The sample was mixed with a vortex and centrifuged at 12,000 × *g* for 5 min. The upper aqueous phase was transferred to a new tube and mixed with 1 vol.% of chloroform:isoamylalcohol mixture (24:1). After centrifugation, the aqueous phase was transferred to a new tube followed by a DNA precipitation in 0.1 vol.% of 3 M sodium acetate and 1 vol.% ice-cold isopropanol. After centrifugation during 10 min at 12,000 × *g*, the supernatant was discarded. The pellet was first dried in an exsiccator and then dissolved in 1× TE (10 mM Tris–HCl (pH 8), 1 mM EDTA). The concentration of the extracted DNA was measured with GeneQuant (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.3. PCR

Oligonucleotides were designed on the basis of the f57 (Genbank accession no. X70277) and IS*900* sequences. The sequences, their locations within the respective genes and the predicted size of the PCR products for these primers are summarised in Table 3. The amplification of both loci was based on the nested PCR approach. Primers IS*900* S1 and IS*900* R3 (2) were used for the first run of the IS*900* amplification and primers F57 and R57 for the f57 PCR. IS*900* S2 and IS*900* R1 were used as nested primers for IS*900* and F57 and F57n for the f57 sequence.

Primary PCR was performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.6 mM $MgCl_2$, 200 μM of each dNTP, 20 pM of each primer, 0.5 U Taq polymerase (Silverstar, Eurogentec, Seraing, Belgium) and 5 μl bacterial DNA extract. The tubes were placed in a thermocycler (PTC 100; MJ Research, Massachusetts, USA) and amplification was as follows: one cycle of denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 68 °C for 45 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min.

The secondary PCR was performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.6 mM $MgCl_2$, 200 μM of each dNTP, 20 pM of each primer, 0.5 U Taq polymerase and 1 μl of the first PCR solution. The tubes were placed in a thermocycler (PTC 100; MJ Research, Massachusetts, USA) and amplification was as follows: one cycle of denaturation at 94 °C for 4 min followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 68 °C for 45 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. A PCR mixture with water as template was used as negative control. Bacterial DNA of the *Map* reference strain ATCC 19698 was used as positive control. PCR results were analysed by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide and visualised by ultraviolet trans-illumination.

Table 3
Sequence, position and amplicon length of *Map* primers used in the study

PCR primers	Nucleotide sequence	Target gene	Annealing positions	Product length
IS*900* S1	5′-GGGTTGATCTGGACAATGACGGTTA-3′	IS*900*	202–226	572
IS*900* R3 (2)	5′-AGCGCGGCACGGCTCTTGTT-3′	IS*900*	751–771	
IS*900* S2	5′-GGAGGTGGTTGTGGCACAACCTGT-3′	IS*900*	228–251	452
IS*900* R1	5′-CGATCAGCCACCAGATCGGAA-3′	IS*900*	657–677	
F57	5′-CCTGTCTAATTCGATCACGGACTAGA-3′	f57	151–176	432
R57	5′-TCAGCTATTGGTGTACCGAATGT-3′	f57	558–580	
F57Rn	5′-TGGTGTACCGAATGTTGTTGTCAC-3′	f57	549–572	424

2.4. Sensitivity of the PCR

A serial dilution of the *Map* isolate 02-0904, chosen at random, ranging from 10^6 to 0.1 CFU per PCR was used to estimate the sensitivity of the PCR. The serial dilution was confirmed with solid phase cytometry (ChemScan; Chemunex, Ivry-sur-Seine, France), which allows rapid enumeration of micro-organisms. It is difficult to obtain single cell suspensions of *Map* due to their very hydrophobic cell wall. For this reason, the sensitivity will be expressed in colony forming units instead of single *Map* cells whereas 1 CFU can contain several organisms. Several negative controls were always included in PCR. A strict procedure was followed to avoid cross-contamination between samples or carry-over of PCR products.

2.5. In silico analysis of the PCR assays

Computer analysis of the PCR assays with the Vary, the Millar and the newly developed primers on IS*900*-related sequences were done using the PCR software Amplify (Engels, 1993). The primers (p90/91 and 150/921) described by Millar et al. (1995) and Vary et al. (1990) are both widely used for IS*900* PCR identification and direct detection of *Map*.

In the past, these primers gave false positive results when applying on IS*900* like sequences. IS*900* like and closely related insertion sequences were selected for "*in silico*" analysis. The following sequences were used: IS*900* (Genbank nos. AF416985 and AF305073), IS*900* like (Genbank no. AF455252 (Englund et al., 2002), IS*900* like WA-1 and IS*900* like WA-2 (Cousins et al., 1999), IS*1613* (Genbank no. AJ011837), IS*901* (Genbank no. X59272) and IS*902* (Genbank no. X58030).

The PCR software Amplify allows simulating and testing polymerase chain reactions. This program was used because the organisms containing these IS elements could not be tested with our PCR. Amplify uses two measures of the quality of a primer match. Primability is the measure of how easily the DNA polymerase will be able to extend the sequence at the 3′ end of the primer and stability is the measure of how tightly the primer and target are bound. The primability and stability need to be above a given cut-off point to give a primer match.

3. Results

3.1. Specificity of the PCR assay

The specificity of the PCR assays was confirmed by the analysis of DNA from a panel of 190 mycobacterial isolates (74 *Map* isolates and 116 non-*Map* isolates). Results show that paratuberculosis-specific primer pairs amplify only DNA from *Map* and not from the non-paratuberculosis strains. The amplicons were of the expected length, according to the sequence published and no secondary bands have been detected. Negative results with the IS*900* and the f57 PCR assay for DNA other than *Map* were not attributable to the inadequacy of the PCR assay, as positive controls were always amplified efficiently.

3.2. Sensitivity of the PCR assay

Sensitivity was determined for both paratuberculosis primer sets. The IS*900* PCR assay could detect 1 CFU per PCR. For the f57 PCR assay also the detection limit was 1 CFU per PCR.

Table 4
In silico analysis with Amplify (Amplify scoring: 5 = expected results, no mispriming; 1 = aspecific bands, mispriming)

	New primers	Vary primers	Millar primers
IS*900* (AF416985)	5	3	4
IS*900* (AF305073)	5	2	4
IS*900* like (AF455252)	3	2	2
IS*900* like (WA-1; Cousins et al. (1999))	4	1	1
IS*900* like (WA-2; Cousins et al. (1999))	5	1	1
IS*901* (X59272)	5	4	5
IS*1613* (AJO11837)	5	1	4
IS*902* (X58030)	5	4	5
IS*1626* (AF071067)	5	5	4

3.3. *In silico analysis*

A summary of the results of the "*in silico*" analysis with the PCR-simulating programme Amplify is given in Table 4.

The newly developed primers should give very good results on the IS*900* sequences (only bands of the expected size). The Vary and Millar primers would be expected to give rise to many aspecific bands. For the IS*900* like sequence (AF455253), amplification may be obtained under suboptimal PCR conditions with the new primers. For the WA-1 and WA-2 IS*900* like sequences (Cousins et al., 1999), there should be no annealing of the new primers to these sequences whereas they should give an amplification product with the Vary and Millar primers. The Millar primers and the new primers should give no amplification for IS*901* and IS*1613*. With the Vary primers, the IS*1613* will be amplified. All primers should give expected results for IS*902*.

4. Discussion

Our in silico analysis showed that the Millar and Vary primers should give rise to many aspecific bands and that the primers would amplify the IS*900* like sequences. These results clearly demonstrated that this IS*900* assay could not be used as a diagnostic tool.

In this study, new primers were developed based on the sequence of IS*900* and the unique sequence f57. The PCR assay on the f57 sequence was chosen as a complementary test to confirm or exclude the presence of *Map* after a positive IS*900* PCR test.

The specificity of the new PCR assay was tested for both primer pairs with DNA extracted from 190 mycobacterial isolates. All of the *Map* isolates generated a PCR product of the expected size. Non-*Map* isolates, including *M. avium*, did not generate any false positive amplification. The results of this study indicated that the newly developed assays are a useful tool for the identification of *Map*. Nevertheless we did not test strains containing IS*900* like sequences as described by Englund et al. (2002) and Cousins et al. (1999). However, computer simulations indicate that the new primers should not amplify the IS*900* like sequences with optimal PCR conditions.

Although the IS*900* primers were specific in our hands, we also recommend including the f57 PCR assay to confirm the presence of *Map* after a positive IS*900* PCR. In silico analysis with Amplify showed that it should be possible to amplify the IS*900* like sequence described by Englund et al. (2002) with the new primers if PCR conditions are suboptimal. The latest information regarding the specificity of IS*900* suggests that it is recommended to use another sequence as characteristic. The f57 sequence has no homology with any known sequence (Poupart et al., 1993; Coetsier et al., 2000) and can replace IS*900* as characteristic sequence but further investigation of this gene is necessary.

The next step would be the validation of the PCR assay on clinical samples as we suspect that the sensitivity will not be as high as the one reported on bacterial cultures, most likely due to the presence of inhibitors to the PCR reaction or the difficulty in extracting mycobacterial DNA. The first experiments in our lab on DNA extracted from spiked and clinical faecal samples gave promising results (personal communication).

Over the last few years, multiplex PCR-based assays have been designed to co-identify distinct mycobacterial strains in the same PCR tube (Yeboah-manu et al., 2001). The duplex PCR procedure offers an easy, rapid and inexpensive way of identifying a bacterial species. We think that it should be possible to develop such a duplex PCR (targeting IS*900* and f57) for the identification of *Map*. Bannantine et al. (2002) revealed by genome-scale comparison of *Map* and *M. avium* subspecies *avium*, 21 potential diagnostic candidate sequences that could be used in a multiplex PCR assay. However, each of these sequences needs to be validated for their specificity using a complete panel of *Mycobacterium* sp. DNA.

5. Conclusion

The combination of two PCR assays (IS*900* PCR assay and f57 assay) with newly developed primers has proven to be superior in the identification of *Map* but validation on clinical samples needs to be done.

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