

Rapid differentiation of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* by amplification of insertion element IS901

P. SVAŠTOVÁ, I. PAVLÍK, M. BARTOŠ

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: The aim of this study was to examine the specificity of primers designed to detect the insertion element IS901 commonly used in differentiation of *Mycobacterium avium* complex strains. This study shows that one of these primers non-specifically anneals to a sequence inside insertion element IS900, specific IS of *M. avium* subsp. *paratuberculosis* and to another sequence flanking this element. The resulting non-specific amplicon can be a product of amplification from some *M. avium* subsp. *paratuberculosis* strains and can simulate the presence of insertion element IS901 in these strains. However size difference between specific and non-specific amplicons allows such false-positive results to be distinguished. In addition the single PCR allows a rapid and simple differentiation between IS901+ *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* strains.

Keywords: IS901; IS900; PCR; Johne's diseases; avian tuberculosis

Members of the *Mycobacterium avium* species are a family of intracellular bacteria that includes *M. avium* subsp. *avium* (*M. avium*), *M. avium* subsp. *silvaticum* (*M. silvaticum*) and *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*). These organisms cause significant disease in domestic and wild animals; *M. paratuberculosis* is the causative agent of paratuberculosis (Johne's disease) in ruminants and primates and is proposed as a causative agent of Crohn's disease in humans (Chiodini, 1989; Hermon-Taylor, 2000; Hermon-Taylor and Bull, 2002). Avian tuberculosis caused by IS901+ *M. avium* strains is a serious disease of birds and other vertebrates including human beings (Thorel *et al.*, 1997; Pavlik *et al.*, 2000).

The most common conserved gene used for identification of *M. paratuberculosis* is the insertion element IS900 (Roiz *et al.*, 1995). However IS900-related insertion IS1626 from *M. avium* and *M. intracellulare* was identified (Puyang *et al.*, 1999). Harris and Barletta (2001) report that some primers designed for amplifi-

cation from IS900 can non-specifically amplify from IS1626. This indicates that PCR results based on the IS900 sequence alone should be interpreted with caution and that some IS900 primers use may not be specific for *M. paratuberculosis*.

Another specific insertion sequence, IS901, was found in pathogenic *M. avium* complex strains isolated from patients with AIDS in 1991 (Kunze *et al.*, 1991). It is associated with virulence infections of *M. avium* serotype 1, 2 and 3 strains in birds and other animals (Collins and Stokes 1987; Pavlik *et al.*, 2000).

The element has a nucleotide sequence of 1 472 bp. Primers P1-IS901 and P2-IS901 derived from positions 76 and 1 184 by Kunze *et al.* (1992) are commonly used for identification of IS901 in genome of mycobacteria (Pavlik *et al.*, 2000).

The aim of the study was to examine the specificity of IS901 specific primers on selected *M. paratuberculosis* strains.

MATERIAL AND METHODS

Mycobacterial strains and growth conditions

The mycobacterial species and strains used in the study were from the collection of our laboratory. The *M. paratuberculosis* strains were cultured on Herrold egg yolk medium (HEYM) with mycobactin J at 37°C for 3 months. A total of 88 *M. paratuberculosis* strains of different origin from our laboratory collection were used in this study. As a control the collection strain *M. avium* 5889 (cultivated on HEYM without mycobactin J for 3 weeks), which contains the specific insertion element IS901, was used.

DNA preparation

To prepare a DNA sample for PCR amplification, a loopful of a single bacterial colony was taken from HEYM and resuspended in 50 µl distilled water in a screw-cap micro centrifuge tube. The samples were then boiled for 20 min prior to being centrifuged for 5 min/14 000 g to settle cell debris. A 2 µl of supernatant, containing the genomic DNA, was used for subsequent PCR amplification.

PCR amplification

PCR was carried out with Taq PCR Master Mix Kit (QIAGEN, USA) to a final volume of 20 µl. An approximately 10 ng of genomic DNA in bacterial lysate and 10 pmol of each primer were used in the reaction. Amplification for IS901 was done with the primers P1-IS901 (5'-GCA ACG GTT GTT GCT TGA AA-3') and P2-IS901 (5'-TGA TAC GGC CGG AAT CGC GT-3') based on insertion sequence IS901 of *M. avium* (Kunze *et al.*, 1992), resulting in 1 108 bp PCR product. A positive control, 250 fg DNA from *M. avium* strain containing specific sequence and a negative control, sterile water, were always included in each run.

DNA samples were first denatured completely by incubation at 94°C for 3 min before the amplification cycle; then DNA was amplified subjecting it to 33 cycles of (i) denaturation at 94°C for 1 min, (ii) primer annealing at 66°C for 45 s, and (iii) elongation at 72°C for 4 min, using a PTC200 thermocycler (MJ Research, USA). After the last amplification cycle, the samples were incubated further at 72°C for 3 min for complete elongation of the final PCR products. After the PCR, the amplification results were visualised by performing 0.8% agarose gel electrophoresis in TBE buffer and

ethidium bromide staining. As a marker a 100 bp ladder was used.

Control amplification for IS900 was done with the own designed primers IS900-P3N (5'-GGG TGT GGC GTT TTC CTT CG-3') and IS900-P4N (5'-TCC TGG GCG CTG AGT TCC TC-3') specific for insertion sequence IS900 (GeneBank Acc. No. X16293). The amplification conditions were the same as described for IS901, except the annealing temperature was set at 67°C and the elongation at 72°C for 2 min. The resulting amplicon was 257 bp.

Cloning and sequencing of amplification product

Amplification products were purified by QIAQuick PCR purification kit (QIAGEN, USA) according to the manufacturer's instructions and cloned to T-vector pCR2.1 using a TA-Cloning Kit (Invitrogen, Corp., USA). In short, 10 µl ligation mixture of TA-vector and amplicon was incubated overnight at 14°C and 3 µl of the mixture were then heat-shock transformed into bacterial cells *Escherichia coli* TOP10F', which were used for propagation of plasmid constructs. Mini-scale isolation of plasmid DNA was used for the preparation of recombinant plasmid for sequencing. The sequencing reactions with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., USA) were performed with approximately 250 ng of PCR-amplified DNA as a template and 3.2 pmol of both forward and reverse sequencing primer on the plasmid pCR2.1. The unincorporated dye terminators and primers were separated from the extension products by spin column purification (QIAGEN, USA). The products were dried, resuspended in loading buffer (deionised formamide and 50 mM EDTA [5 : 1], pH = 8.0), heat denatured for 3 min at 90°C and immediately loaded onto capillary electrophoresis column in an automated DNA sequencer (ABI PRISM 310 GENETIC ANALYSER, APPLIED BIOSYSTEMS, USA). Sequencing data were assembled and edited by GeneBase software (Applied Maths, Belgium).

RESULTS AND DISCUSSION

The primers P1-IS901 and P2-IS901 applied on *M. avium* strains produce a specific amplicon 1 108 bp long. The same primers used for *M. paratuberculosis* strains give a non-specific amplicon, named pseudoIS901, more than 1 200 bp long (Figure 1).

In 86/88 (97.7%) *M. paratuberculosis* strains used in this study we have confirmed the presence of pseudo IS901. The identification of *M. paratuberculosis* strains was verified by cultivation on mycobactin J and the presence of specific insertion element IS900 confirmed by PCR.

To eliminate the presence of such non-specificity we have optimised the condition of amplification by changing the annealing temperature in the extension from 50 to 72°C. The increase of annealing temperature to 70°C led to elimination of the pseudoIS901 amplicon, but simultaneously resulted in decreased sensitivity of the amplification and false negative results in amplification of the control *M. avium* strain. The decrease of annealing temperature led to an increase in the number of non-specific fragments. Similar effects were observed when concentrations of MgCl₂ in the amplification mix were changed from 1.5 mM to 4.0 mM. An annealing temperature of 66°C and original composition of the Taq PCR Master Mix Kit (QIAGEN, USA) remained the best for specific and most sensitive amplification.

The non-specific amplicon pseudoIS901 from three strains were cloned to the vector pCR2.1. Screening of selected bacterial colonies was performed by specific PCR. Presence of insert in the vectors was indicated

by PCR showing an amplicon 1 400 bp in length. The resulting recombinant plasmid was designated pCR2.1-pseudoIS901.

From sequencing data (Figure 2b) we have found the amplicon pseudoIS901 is a 1 250 bp long product of amplification where only one primer – P2-IS901 is utilised (Figure 2a). The primer anneals non-specifically in position 1 174–1 193 of *M. paratuberculosis* insertion element IS900 (GeneBank Acc. No. X16293). A difference in sequence of the primer P2-IS901 and its complementary site on IS900 is 2 bp only. The second annealing site for the primer P2-IS901 is position 680–688 (from 5') of the flanking sequence of IS900: (locus 1 – gene ORF3891c, GeneBank Acc. No. AJ250015; Bull *et al.*, 2000). The distance of this flanking site from the beginning of IS900 is 47 bp. In this second annealing site only 9 bp of 3'-end region (45% of homology) of the P2-IS900 recognise their target sequence, the rest 11 bp is non-complementary.

Our results show that the non-specificity during amplification is not correlated with the presence of IS901 in *M. paratuberculosis* genome, but non-specific amplicons occur as a result of weak annealing conditions in PCR reaction. The presence of the non-specific amplicon pseudoIS901 during routine analysis of *M. avium* complex strains can complicate interpreta-

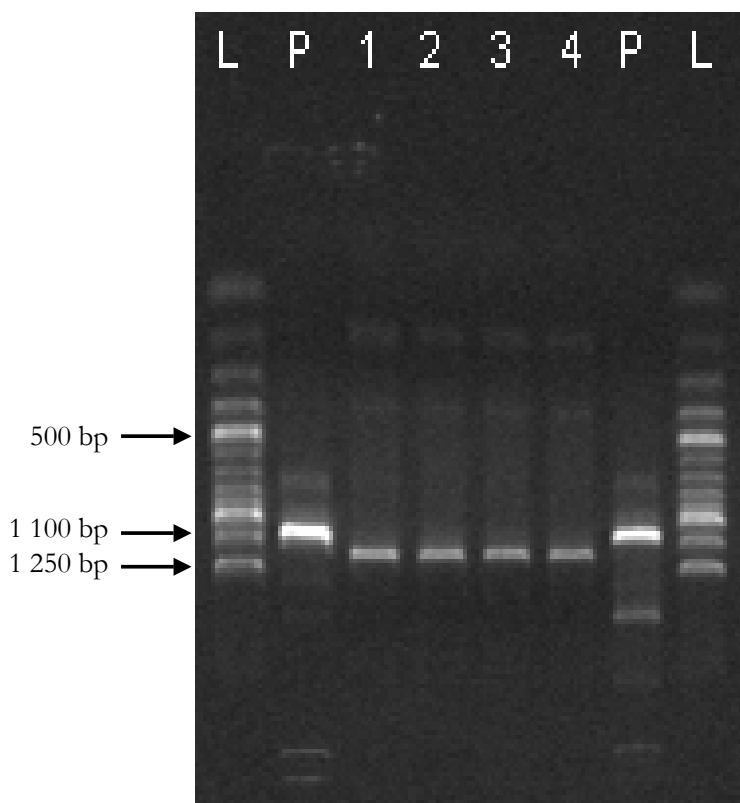
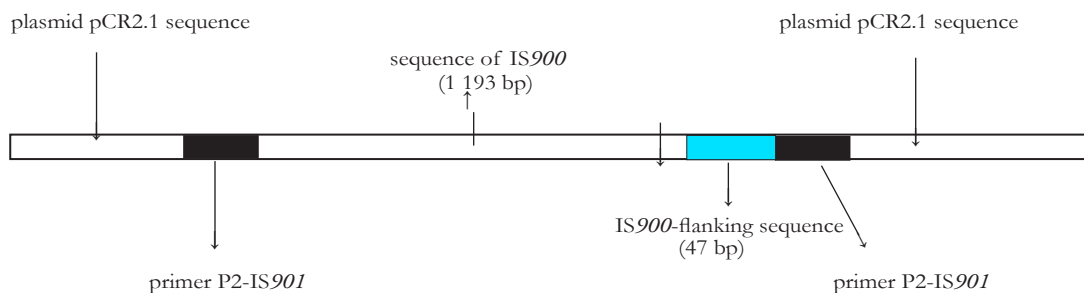


Figure 1. Example of real samples amplification

The result of amplification by the primers P1-IS901 and P2-IS902 on 0.8% agarose gel: L – 100 bp ladder, P – collection strain *M. avium* 5889, 1 – *M. paratuberculosis* strain 7217, 2 – *M. paratuberculosis* strain 20/383, 3 – *M. paratuberculosis* strain 8768, 4 – *M. paratuberculosis* strain 7095. A strong amplicon, 1 108 bp long specific for IS901 is amplified from *M. avium*. Only weak non-specific 1 250 bp long band, pseudoIS901, is amplified from *M. paratuberculosis*

a) The scheme of the clone pCR2.1-pseudoIS901.



b) The sequencing data

pseudoIS901 – sequenced from left arm of plasmid vector (only part of the sequence is shown)

1–25 = plasmid pCR2.1 part

26–45 = underlined is primer P2-IS901 (**bold underlined** are nucleotides which are different on primer and target sequence)

26–170 = part of the sequence of IS900

1	11	21	31	41	51	61
CCGCCAGTGT	GCTGGAATTC	GGN <u>TT</u> TGATA	CGGCCGGAAT	CGCGT GGTAC	CGGCGCCAGG	CCGGCGACGC
71	81	91	101	111	121	131
CGGCGAGGCG	GTCGGCGGAG	GCGAATGCGG	CCATGTCCCC	GCCGGTGGNA	GGCGAGGAAC	TCAGCGCTCC
141	151	161				
AGNANGACGC	CCGAAATCCG	GGCATGCTCA...				

pseudoIS901- sequenced from right arm of plasmid vector (only part of the sequence is shown)

1–25 = plasmid pCR2.1 part

26–45 = underlined is primer P2-IS901 (**bold underlined** are marked nucleotides which are different on primer and target sequence)46–92 = underlined is IS900-flanking sequence

93–213 = part of the sequence of IS900

1	11	21	31	41	51	61
GATGGATATC	TGCAGAATTC	GGCT <u>TT</u> TGATA	CGGCCGGAAT	CGCGT TGGAA	...GTATCGCGAA	...GCATTTGCGG
71	81	91	101	111	121	131
<u>NGTCACCTTG</u>	<u>ATTGTCACGG</u>	<u>AATTCCTTAC</u>	<u>CITTCCTGAA</u>	<u>GGGTGTTCCG</u>	<u>GNGCCGTCGG</u>	<u>CCTTANCCTC</u>
141	151	161	171	181	191	201
GAATTNCCA	GNGACGTCGG	GTATGGNTTN	ATGTGGTTGC	NGTGNTGGAC	GGCCNAAGGA	GATTGGCCCG
211						
NCC ...						

Figure 2. Structure of pseudoIS901 amplicon

tion of the PCR results. However the size difference between specific amplicon for IS901 in *M. avium* (1 108 bp) and for the pseudo-IS901 amplicon in *M. paratuberculosis* (1 250 bp) is easily differentiated on common agarose gels. In addition this size difference can rapidly differentiate between *M. avium* and *M. paratuberculosis* in a single PCR reaction.

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Corresponding Author

Mgr. Petra Švástová, Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel. +420 5 41 32 12 41, fax +420 5 41 21 12 29, e-mail: svastova@vri.cz
