Rapid differentiation of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* by amplification of insertion element IS*901*

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ABSTRACT: The aim of this study was to examine the specificity of primers designed to detect the insertion element IS*901* 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 IS*900*, 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 IS*901* 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 IS*901*+ *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* strains.

Keywords: IS*901*; IS*900*; 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 IS*901*+ *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 IS*900* (Roiz *et al.*, 1995). However IS*900*-related insertion IS*1626* from *M. avium* and *M. intracellulare* was identified (Puyang et al., 1999). Harris and Barletta (2001) report that some primers designed for amplification from IS*900* can non-specifically amplify from IS*1626*. This indicates that PCR results based on the IS*900* sequence alone should be interpreted with caution and that some IS*900* primers use may not be specific for *M. paratuberculosis*.

Another specific insertion sequence, IS*901*, 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-IS*901* and P2-IS*901* derived from positions 76 and 1 184 by Kunze *et al.* (1992) are commonly used for identification of IS*901* in genome of mycobacteria (Pavlik *et al.*, 2000).

The aim of the study was to examine the specificity of IS*901* specific primers on selected *M. paratuberculosis* strains.

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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 IS*901*, 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 IS*901* was done with the primers P1-IS*901* (5'-GCA ACG GTT GTT GCT TGA AA-3') and P2-IS*901* (5'-TGA TAC GGC CGG AAT CGC GT-3') based on insertion sequence IS*901* 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 IS*900* was done with the own designed primers IS*900*-P3N (5'-GGG TGT GGC GTT TTC CTT CG-3') and IS*900*-P4N (5'-TCC TGG GCG CTG AGT TCC TC-3') specific for insertion sequence IS*900* (GeneBank Acc. No. X16293). The amplification conditions were the same as described for IS*901*, 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-IS*901* and P2-IS*901* 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 pseudoIS*901*, 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 IS*901*. The identification of *M. paratuberculosis* strains was verified by cultivation on mycobactin J and the presence of specific insertion element IS*900* 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 pseudoIS*901* 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 $MgCl_2$ 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 pseudoIS*901* 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-pseudoIS*901*.

From sequencing data (Figure 2b) we have found the amplicon pseudoIS*901* is a 1 250 bp long product of amplification where only one primer – P2-IS*901* is utilised (Figure 2a). The primer anneals non-specifically in position 1 174–1 193 of *M. paratuberculosis* insertion element IS*900* (GeneBank Acc. No. X16293). A difference in sequence of the primer P2-IS*901* and its complementary site on IS*900* is 2 bp only. The second annealing site for the primer P2-IS*901* is position 680–688 (from 5') of the flanking sequence of IS*900*: (locus 1 – gene ORF3891c, GeneBank Acc. No. AJ250015; Bull *et al.*, 2000). The distance of this flanking site from the beginning of IS*900* is 47 bp. In this second annealing site only 9 bp of 3'-end region (45% of homology) of the P2-IS*900* 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 IS*901* 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 pseudoIS*901* during routine analysis of *M. avium* complex strains can complicate interpreta-

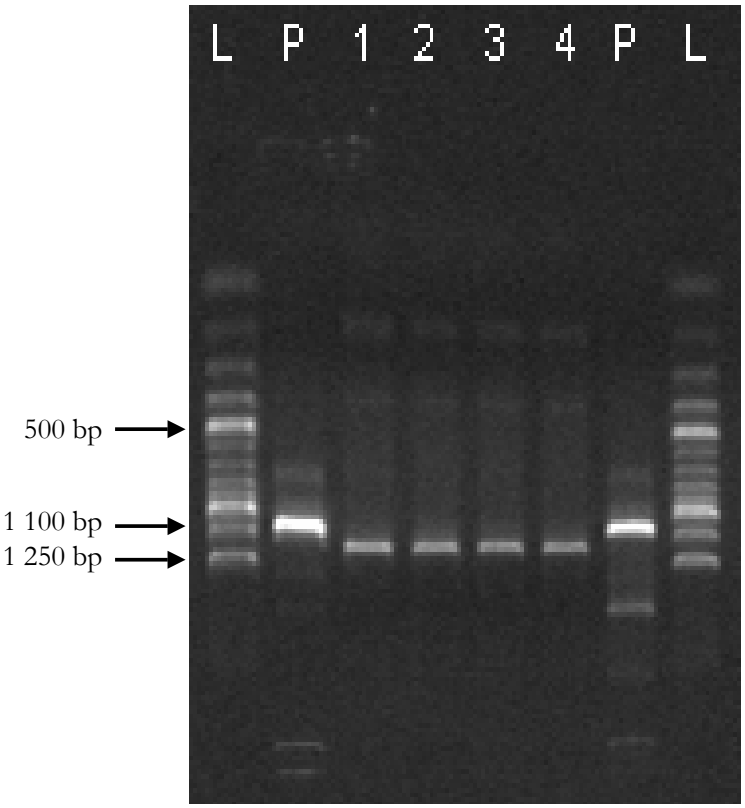
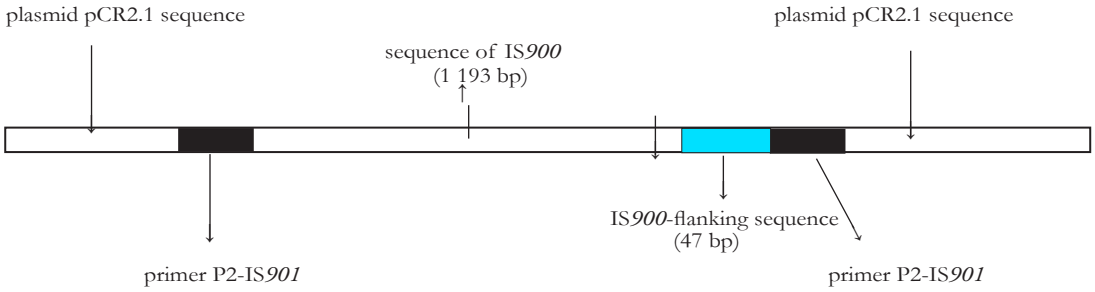


Figure 1. Example of real samples amplification

The result of amplification by the primers P1-IS*901* and P2-IS*902* 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 IS*901* is amplified from *M. avium*. Only weak non-specific 1 250 bp long band, pseudoIS*901*, is amplified from *M. paratuberculosis*

a) The scheme of the clone pCR2.1-pseudoIS*901*



b) The sequencing data

pseudoIS*901* – 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-IS*901* (**bold underlined** are nucleotides which are different on primer and target sequence)

26-170 = part of the sequence of IS*900*

1	11	21	31	41	51	61
CCGCCAGTGT	GCTGGAATTC	GGNTTTGAT**A**	CGGCCGGAAT	C**G**CGTGGTAC	CGGCGCCAGG	CCGGCGACGC
71	81	91	101	111	121	131
CGGCGAGGCG	GTCGGCGGAG	GCGAATGCGG	CCATGTCCCC	GCCGGTGGNA	GGCGAGGAAC	TCAGCGCTCC
141	151	161				
AGNANGACGC	CCGAAATCCG	GGCATGCTCA…				

pseudoIS*901*- 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-IS*901* (**bold underlined** are marked nucleotides which are different on primer and target sequence)

46–92 = underlined is IS*900*-flanking sequence

93–213 = part of the sequence of IS*900*

1	11	21	31	41	51	61
GATGGATATC	TGCAGAATTC	GGCTT**TGATA**	**CGGCCG**GAAT	CGCGTTGGAA	GTATCGCGAA	GCATTTGCGG
71	81	91	101	111	121	131
NGTCACCTTG	ATTGTCACGG	AATTCCTTAC	CTTTCTTGAA	GGGTGTTCCG	GNGCCGTCGG	CCTTANCTTC
141	151	161	171	181	191	201
GAATTNNCCA	GNGACGTCGG	GTATGGNTTN	ATGTGGTTGC	NGTGNTGGAC	GGCCNAAGGA	GATTGGCCCG
211						
NCC …						

Figure 2. Structure of pseudoIS*901* amplicon

tion of the PCR results. However the size difference between specific amplicon for IS*901* in *M. avium* (1 108 bp) and for the pseudo-IS*901* 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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