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IS*900*-PCR-based detection and characterization of *Mycobacterium avium* subsp. *paratuberculosis* from buffy coat of cattle and sheep

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Abstract

Johne's disease is one of the main causes of economic losses in ruminants and a major health hazard in the developing and developed world. Up till now, many microbiological, serological and molecular methods have been tried for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In this study, we attempt a PCR-based detection of IS*900*, distinct insertion sequences of MAP from the buffy coat of cattle ($n = 262$) and sheep ($n = 78$), and direct genotyping by single strand conformational polymorphism (SSCP). A total of 30 (11.45%) cattle and one sheep (1.28%) were positive for MAP-IS*900*. This IS*900*-based PCR detection proved highly specific, particularly when tested on other non-MAP strains. SSCP analysis grouped the MAP-IS*900* into four distinct clusters based on different band patterns. Nucleotide sequence variability between MAPs detected from sheep (GenBank accession AY974348) and cattle (GenBank accession AY974345–AY974347) was noticed in the study. Although, in recent years IS*900*-PCR-based detection of MAP from WBCs is being used in human, its use in animals is still limited. Our work not only supports its use in animals but also suggests further IS*900*-SSCP-based MAP-genotyping, coupled with DNA sequencing, as a promising tool for rapid and effective Johne's disease surveillance.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; IS*900*; Cattle; Sheep; PCR; Johne's disease

1. Introduction

Johne's disease affects ruminants worldwide. Due to its long incubation period, mainly subclinical or hidden forms of Johne's disease are noticed. However, after a long subclinical phase, clinical signs can be

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recorded, such as cachexia and diarrhoea. Paratuberculosis in livestock causes significant economic losses due to reduced production, increased treatement cost and culling. Herd prevalence of Johne's disease in Europe is reported to be between 7 and 55%, in the United States nearly 40% and in Australia it ranges between 9 and 22% (Manning and Collins, 2001). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) may also be a cause of Crohn's disease in human (Gaya et al., 2004; Greenstein and Collins, 2004; Romero et al., 2005). Recently, MAP has been detected in blood from patients with Crohn's disease (Naser et al., 2004). In animals, MAP infection can also be systemic (Gwozdz et al., 2000), due to extraintestinal infiltration in blood stream, occasionally leading to the presence of the pathogen into milk (Sweeney et al., 1992). Research works of Grant et al. (2002) and Ayele et al. (2005), have confirmed the presence of MAP in retail milk (pasteurized as well as raw) supplies suggesting consumer exposure to the pathogen. This may explain why Crohn's disease is moderately prevalent in the developing as well as the developed world, where milk is consumed (Greenstein and Collins, 2004).

Disease control is hampered due to the hidden nature of Johne's disease and ineffective diagnosis, particularly in the subclinically infected animal population. Diagnostic tests, such as ELISA, agar gel immunodiffusion (AGID) test and fecal culture, are being used commonly (Buergelt et al., 2004). Though fecal culture is considered the gold standard, the method is time-consuming (6–8 weeks). Serological tests, coupled with apparent clinical signs, can be considered reliable for Johne's disease diagnosis. However, in apparently healthy or subclinically infected animals, tests aimed at antigen or antibody detection frequently give rise to false negative results (Buergelt and Williams, 2004). PCR methods, targeting MAP specific insertion sequence (IS*900*) or other species-specific genes, have been developed to increase specificity and sensitivity of diagnosis, as well as shorten the time required (Millar et al., 1995; Englund et al., 1999; Djonne et al., 2003; O'Mahony and Hill, 2004; Ellingson et al., 2005). IS*900* belongs to the same family of insertion sequences as IS*901* (*M. avium* subsp. *avium*), IS*902* (*M. avium* subsp. *silvaticum*) and IS*1110* (*M. avium* subsp. *avium*) (Englund et al., 2002). IS*900* is a 1451 bp segment that lacks inverted terminal repeats and does not generate direct repeats in target DNA (Green et al., 1989; Englund et al., 2002). IS*900*-based RT-PCR detection was also used to differentiate the viable MAP infection in patients with Crohn's disease (Mishina et al., 1996; Naser et al., 2004).

To date, studies have focused on the PCR-based detection of MAP from feces, milk or culture. IS*900*-PCR-based MAP detection directly from peripheral blood of animal is a recent concept. In this study, we used this notion to investigate the presence of MAP in buffy coat of cattle and sheep. The study also proposes further IS*900*-SSCP-based MAP genotyping.

2. Materials and methods

2.1. Mycobacterial strains

Different MAP and non-MAP strains were obtained from Slovakia, Czech Republic and India. The details of the cultures are presented in Table 1. DNA from the mycobacterial cultures was isolated by using a commercial DNA isolation kit (Aplicor, Roche).

2.2. Animals

The cattle population ($n = 262$) from the south-western part of India, used in this study, included different *Bos indicus* (mainly Gir, Red Sindhi, Sahiwal and Goan breeds) and *Bos indicus–taurus* animals (Jersey or Holstein–Friesian crossed with the earlier cited breeds). The sheep population ($n = 78$) consisted of pure ($n = 40$) and improved Valachian ($n = 38$) breeds. During blood collection, animals were examined for their health status and grouped accordingly (Table 2); 3–4 ml of blood was collected in sterile tubes containing 2 ml of 1.5% EDTA. Buffy coat was separated as described earlier (Sambrook et al., 1989) and used for DNA extraction. Then, 2–3 ml of blood from the same animals was collected for serological diagnosis. Blood from known paratuberculosis-positive cattle was included in the experiment as a positive control.

2.3. DNA extraction from buffy coat of cattle and sheep

DNA from buffy coat was released and extracted using proteinase K (Sambrook et al., 1989). For

Table 1
Different mycobacterial species used to test specificity of primers

Strain	Species	Host	Locality	Cultured from
P4919	MAP	Cattle	Benesov (CR)	Feces
P5153	MAP	Cattle	Benesov (CR)	Feces
P4894	MAP	Cattle	Benesov (CR)	Feces
P5197	MAP	Cattle	Benesov (CR)	Feces
P4822	MAP	Cattle	Benesov (CR)	Feces
P271	MAP	Cattle	Rychnov (CR)	Feces
P227	MAP	Cattle	Rychnov (CR)	Feces
P202	MAP	Cattle	Rychnov (CR)	Feces
P2859/02	MAP	Cattle	Nitra (SR)	Feces
P3423/02	MAP	Cattle	Nitra (SR)	Feces
P648/03	MAP	Cattle	Nitra (SR)	Feces
MAP456	MAP	Cattle	Mumbai (India)	Feces
MAP457	MAP	Cattle	Mumbai (India)	Feces
13/03	*M. avium*	NA	Tatra (SR)	NA
56/03	*M. avium*	NA	Tatra (SR)	NA
T964	*M. avium*	Ostrich	Domazlice (CR)	Intestine
T63	*M. avium*	Wild turkey	Nachod (CR)	Intestine
T61	*M. avium*	Poultry	Nachod (CR)	Intestine
T64	*M. avium*	Wild turkey	Nachod (CR)	Intestine
T62	*M. avium*	Poultry	Nachod (CR)	Intestine
T970	*M. avium*	Pig	Nymburk (CR)	Intestine
Tbc a 3/50	*M. avium*	Poultry	Nitra (SR)	NA
D4ER0067	*M. avium*	Poultry	Nitra (SR)	NA
T75	*M. avium*	Poultry	Pisek (CR)	Intestine
T76	*M. avium*	Poultry	Pisek (CR)	Intestine
10/03	*M. kansasii*	Human	Tatra (SR)	NA
33/03	*M. chelonae*	Human	Tatra (SR)	NA
29/03	*M. chelonae*	Human	Tatra (SR)	NA
19/03	*M. chelonae*	Human	Tatra (SR)	NA
45/03	*M. fortuitum*	Human	Tatra (SR)	NA
54/03	*M. fortuitum*	Human	Tatra (SR)	NA
20/03	*M. gordonae*	Human	Tatra (SR)	NA
15/03	*M. gordonae*	Human	Tatra (SR)	NA
5/03	*M. gordonae*	Human	Tatra (SR)	NA
8/03	*M. gordonae*	Human	Tatra (SR)	NA
Ti150/30/02	*M. gordonae*	Human	Nitra (SR)	NA
Ti98/30/02	*M. gordonae*	Human	Nitra (SR)	NA
22/03	*M. tuberculosis*	Human	Tatra (SR)	NA
29/03	*M. tuberculosis*	Human	Tatra (SR)	NA
26/05	*M. tuberculosis*	Human	Tatra (SR)	NA
23/05	*M. tuberculosis*	Human	Tatra (SR)	NA
B2331	*M. tuberculosis*	Human	Mumbai (India)	Sputum

MAP: *Mycobacterium avium* subsp. *paratuberculosis*; NA: information not available; CR: Czech Republic; SR: Slovakia.

Table 2
Number of MAP positive cases in apparently healthy and unhealthy cattle population

Health status	Total number of animals tested	Number of negative samples	%	Number of positive samples	%
Apparently healthy	136	128	94.12	8	5.88
Unhealthy[a]	126	104	82.54	22	17.46

[a] Unhealthy animals showed different sings, symptoms or disease like—diarrhea, weakness, abortion, retention placenta, mastitis, cystic ovary, metabolic disorder or other infections like brucellosis.

purification, 500 μl of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added, mixed vigorously and then centrifuged at 13 000 rpm for 15 min. Nucleic acid was precipitated, washed, dried and dissolved in 50 μl of ultra-pure sterile water. The concentration and purity of the DNA were then determined spectrophotometrically.

2.4. Nested PCR

Primers (outer) were designed using insertion sequences (IS*900*) from the GenBank database. Outer primers (M.paraFor/out: 5′-AGGGTGTTCGGGGC-CGTCGCTTAG and M.paraRev/out: 5′-TGAGGTC-GATCGCCCACGTGACCT) were constructed to amplify the 406 bp fragment. PCR was performed on 0.1–0.15 μg of DNA in a reaction mixture containing 1× PCR buffer, 3 mmol $MgCl_2$, 0.2 mM dNTP, 2.5 μM primers and 2.0 U of Taq polymerase (BioLabs). PCR cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 31 cycles of 95 °C for 1.25 min, 56.5 °C for 50 s, 72 °C for 1.0 min with final extension at 72 °C for 10 min. Sensitivity and specificity of the detection method was increased by a second round of amplification using internal primers, as described by Naser et al. (2004), (M.paraForward/int: 5′-ATGTGGTTGCTGTGTTG-GATGG and M.paraRevrse/int: 5′- CCGCCGCAAT-CAACTCCAG), which produced an amplicon of 298 bp. The reaction mixture for the second round of amplification was the same, except that 1 μl of amplified product from the first PCR was used as template. PCR-cycling conditions were also similar, except the annealing temperature was kept at 63 °C for 45 s.

To judge the specificity of primers, the PCR was first performed on DNA extracted from different mycobacterial species (Table 1). Sensitivity of PCR was assessed by reducing the template DNA isolated from MAP by a 10-fold dilution series. After confirmation of sensitivity and specificity of the primers, PCR was used for MAP detection from buffy coat of animals.

2.5. Single strand conformational polymorphism analysis (SSCP)

SSCP was performed to assess MAP genotype variability. In short, 5 μl of amplified product was mixed with equal amount of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene–cyanol), subjected to denaturation at 95 °C for 10 min and cooled rapidly on the ice. Denaturated single-stranded amplimers were loaded on 14% acrylamide/bisacrylamide (37.5:1, v/v; Bio-Rad) gels containing 10% glycerol. Electrophoresis was performed using 200 V at 5 °C in 0.5× TBE buffer for 20 h in the electrophoresis chamber (Ingeny, Goes, The Netherlands). Gels were silver-stained according to the method described by Bassam et al. (1991).

2.6. Sequencing

Representative samples from each SSCP profile were sequenced. The sequences were aligned and checked for nucleotide variations. Sequences were submitted to the GenBank (USA) under the accession numbers AY974345–AY974348.

2.7. Serological testing

Serum samples were tested with indirect ELISA. The test was performed as described by Wood et al. (1992) and the manual of diagnostic tests and vaccines for terrestrial animals (Office International des Epizooties, 2000). PPD (purified protein derivative absorbed with *M. phlei*) was used as antigen.

2.8. Statistical analysis

Animal cohorts were compared by χ^2-test. A P value less than 0.05 was judged to be significant. The agreement between ELISA and PCR was tested by a κ test (Win-Episcope Software). A κ value more than 0.5 was considered as moderate agreement between two tests.

3. Results

None of the mycobacterial species, except MAP, showed positive result, confirming the specificity of IS*900*-PCR. Amplicons after the first and second rounds of PCR were of expected length. When both outer and inner primers were tested on the DNA isolated from the buffy coat of known MAP negative animals no secondary bands or false positive reaction

Table 3
Positivity of MAP in different age groups of cattle

Age groups	Total number of animals tested	Number of negative samples	%	Number of positive samples	%
3–5 years	29	29	100	0	0
6–10 years	217	192	88.48	25	11.52
11 years and above	16	11	68.75	5	31.25
Total	262	232	88.55	30	11.45

was observed. The IS*900*-PCR was able to amplify MAP DNA diluted up to 0.01 ng when tested for its sensitivity by 10-fold serial dilution. None of the PCR internal negative controls, or DNA extraction controls, was positive, indicating no laboratory contamination.

A total of 13 samples were found positive by indirect ELISA. These seropositive samples were confirmed positive for MAP infection by IS*900*-PCR. Agreement between indirect ELISA and PCR was moderate (κ: 0.59). A total of 30 of 262 cattle were found positive for MAP by IS*900*-PCR and majority of cases were from the age group 6–10 years (Table 3), however, the percentage of positive individuals in older (11 years and above) animals was significantly higher (χ^2, $P < 0.05$). MAP DNA was detected in eight of 136 (5.88%) apparently healthy and 22 of 126 (17.46%) unhealthy cattle (Table 2). The sheep population was apparently healthy without any noticeable clinical symptoms. One of 38 (2.63%) Improved Valachian sheep was positive for MAP, while no sheep from pure Valachian group showed the presence of MAP in their buffy coat.

When amplified PCR products were subjected to SSCP analysis four different profiles (band patterns) were observed indicating nucleotide variability in the amplified IS*900* gene fragment (Fig. 1). Thus, SSCP also enabled us to distinguish the genotype of MAP that infected a particular animal. In the cattle population, three different genotypes were observed, whereas fourth genotype was detected in sheep. Ten of 30 positive samples from cattle were grouped under SSCP profile-*a* and, when sequenced, two consecutive substitutions (GC instead of CG; GenBank accession AY974345) were observed (Fig. 2). SSCP profile-*b* (accession number AY974346) and profile-*c* (accession number AY974347) included four and 16 MAP positive cattle samples, respectively. The fourth SSCP profile (*d*) belonged to MAP-IS*900* (accession number

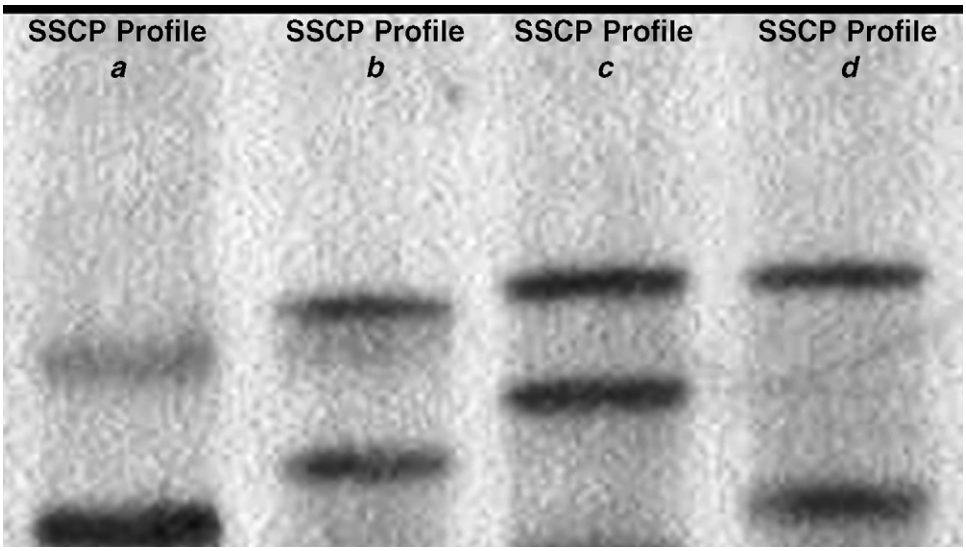

Fig. 1. Single strand conformational polymorphism (SSCP) analysis of the MAP-IS*900* gene. Representative profiles of each SSCP group are illustrated. SSCP profiles *a*, *b* and *c* belongs to MAP-IS*900* detected from buffy coat of cattle. SSCP-profile *d* belongs to MAP-IS*900* amplified from the sheep buffy coat.

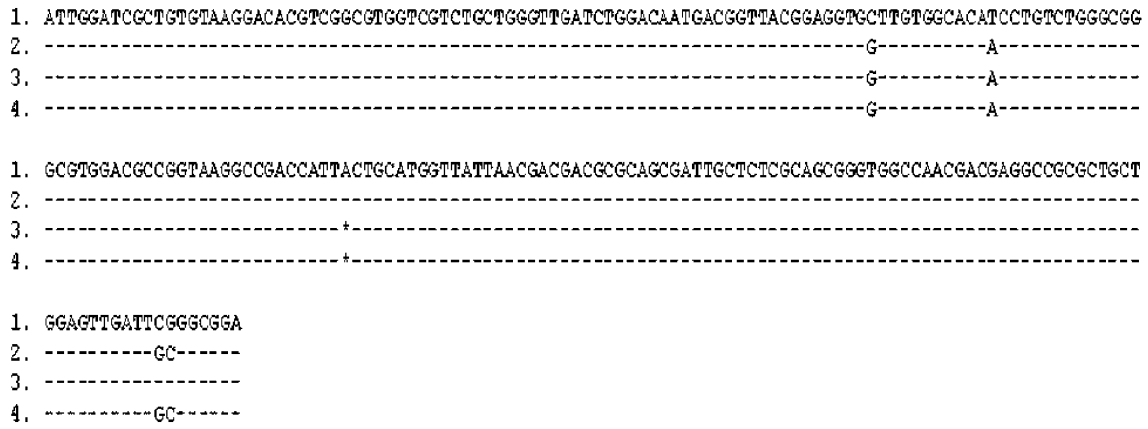

Fig. 2. Nucleotide sequence alignment of MAP-IS*900* gene fragment amplified from buffy coat of cattle and sheep. Nucleotide sequence 1 (AY974348) corresponds to SSCP profile-*d*; the IS*900* was detected from buffy coat of sheep. Sequences 2 (AY974347, SSCP profile-*c*), 3 (AY974346, SSCP profile-*b*), and 4 (AY974345, SSCP profile-*a*) are of MAP-IS*900* gene detected from buffy coat of cattle. Genbank accession number are indicated in parenthesis. (*) indicates nucleotide deletion.

AY974348) detected from buffy coat of sheep (Figs. 1 and 2).

4. Discussion

he introduction of IS*900*-dependent PCR has reduced the time and labor required for the MAP diagnosis. IS*900* is present in 15–18 copies in MAP genome (Bull et al., 2000). Owing to the extremely slow progression of Johne's disease, infected animals appear healthy, without shedding MAP in milk or feces, while harboring potential infection in phagocytic cells, such as macrophages. Such animals pose a real threat for the herd. Hitherto, with the help of PCR, MAP has been identified from milk, feces and other farm wastes. In the present study, we successfully detected MAP with the help of the IS*900*-PCR technique from peripheral blood leucocytes of cattle and sheep. Some authors (Naser et al., 1999; Englund et al., 2002) have indicated the presence of a IS*900*-like sequence in MAP-related mycobacteriae, which increases the risk of false positives results. The results of our IS*900*-PCR, performed on different MAP and non-MAP, have eliminated the possibility of false positives.

The prevalence of MAP in cattle was 11.45%, reflected in the risk of MAP infection in older animals. This observation can be correlated with the exceptionally long incubation period of Johne's disease, as well as an age-dependent susceptibility to MAP infection. The higher occurrence of MAP positive cases in unhealthy cattle (Table 2) indicates the chances of either mixed infections or increased susceptibility to MAP infection in stressed animals. Although very low positivity (1.28%) in sheep was observed in the study, a single positive case in this cohort confirms the MAP risk.

Difference in MAP strains isolated from cattle and sheep have been demonstrated earlier. Genotyping of MAP strains from different countries has proved the heterogeneity between ovine (S-type or I type) and bovine strains (C type or II type) (Collins et al., 1990; Whipple et al., 1990; Thoresen and Olsaker, 1994; Choy et al., 1998; Moreira et al., 1999; Pavlik et al., 1999; Cousins et al., 2000). In humans, C type is demonstrated most frequently, indicating the close association between bovine strains and Crohn's disease (Pavlik et al., 1999; Whittington et al., 2000). Results of our study also present heterogeneity of MAP in cattle and sheep. The possible explanation for this heterogeneity is: (1) host-pathogen interaction, (2) specificity or host preference of MAP strains, (3) factors governing the route of infection, susceptibility and pathogenesis, and (4) pathogen adaptation to the environmental factors (Whittington et al., 2000).

The majority of research studies have used RFLP, pulse-field gel electrophoresis or related techniques for MAP genotyping. These techniques are laborious, time-consuming and need large amount of genomic DNA from cultured bacteria (Collins et al., 1990;

Whipple et al., 1990; Pavlik et al., 1999; Stevenson et al., 2002; De Juan et al., 2005). Recent introduction of PCR-based genotyping has enabled scientists to identify mycobacterial strain variation within a short time (Cousins et al., 2000; Whittington et al., 2000; Stevenson et al., 2002). Different genes, especially rRNA (16S, 23S, 5S), have been targeted to establish a taxonomic relationship between *Mycobacterium avium* complexes. However, these genes are highly conserved within the *M. avium* complex (Rogall et al., 1990; Stahl and Urbance, 1990; van der Giessen et al., 1994). The diverse nature of IS*900* has enabled differentiation between *M. avium* complex and strain identification within MAP. Using IS*900* as a hybridization probe, around 20 different RFLP types have been identified, showing MAP strain heterogeneity (Collins et al., 1990; Whipple et al., 1990; De Lisle et al., 1992; Thoresen and Olsaker, 1994). RFLP analysis using IS*1311* has also been promising for differentiation between/within the *M. avium* and MAP strains (Collins et al., 1997; Whittington et al., 1998). Moreover, point mutations in IS*1311* have allowed differentiation of isolates originating from sheep and cattle (Marsh et al., 1999; Whittington et al., 1998). Although the earlier cited techniques can detect strain variation within MAP, DNA sequencing is considered the gold standard for detection of polymorphism or pinpoint divergence detection between strains. Clustering of the samples by inexpensive SSCP techniques, as described in this study, followed by sequencing of the representative samples from each group is a promising confirmative way for MAP genotyping.

The direct detection of MAP from buffy coat of animal and its genotyping is a novel concept, which enables effective Johne's disease surveillance, and strengthens the epidemiological aspects of MAP infection.

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