Characterization of genetic diversity of animal and human *Mycobacterium avium* strains by IS*1245*-IS*1311* spacer typing

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ABSTRACT: A PCR method previously developed for typing *Mycobacterium avium* was used to characterize the genetic diversity of *M. avium* strains isolated from swine (n = 90) and humans (n = 24). The strains were identified with IS*901* PCR and IS*1245* PCR: 38 strains were of IS*901*+ and IS*1245*+ genotype (*M. avium* subsp. *avium*) and 76 strains were of IS*901*– and IS*1245*+ genotype (*M. avium* subsp. *hominissuis*). All human isolates were IS*901* negative. IS*1245*-IS*1311* spacer typing was successfully accomplished for 59 isolates while 55 isolates gave no amplification signal. The isolates with negative typing results were additionally tested for the presence of IS*1311* and all with the exception of one gave positive results. IS*1245*-IS*1311* spacer typing failed in all IS*901*+ isolates as they yielded no bands. A high degree of heterogeneity among isolates was observed: 59 isolates demonstrated 43 different patterns comprising up to 6 bands.

Keywords: mycobacteria; genotyping; insertion sequences; banding patterns; swine; humans

Mycobacterium avium and *Mycobacterium intracellulare* are members of *Mycobacterium avium* complex (MAC). MAC isolates are divided into 28 serotypes (Wolinsky and Schaeffer, 1973). Serotypes 1 to 6, 8 to 11 and 21 are classified as *M. avium* and serotypes 7, 12 to 20 and 22 to 28 are classified as *M. intracellulare*. Based on biochemical and DNA analysis, *M. avium* was first subdivided into subspecies corresponding to pathogenicity and host-range characteristics: *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis* (Thorel et al., 1990). In order to clarify the epidemiology of *M. avium*-related infections, Mijs et al. (2002) proposed that *M. avium* strains most frequently isolated from pigs with tuberculous changes in lymph nodes (Guerrero et al., 1995; Komijn et al., 1999) and from humans with respiratory or disseminated MAC infection (Kunze et al., 1992; Bono et al., 1995; Guerrero et al., 1995; Bauer and Andersen, 1999) should be classified as *M. avium* subsp. *hominissuis* (*M. a. hominissuis*; serotypes 4 to 6, 8 to 11 and 21, genotype IS*901*–, IS*1245*+) while *M. avium* isolates originating from birds should remain considered as a separate, evolutionarily conserved taxon designated *M. avium* subsp. *avium* (*M. a. avium*; serotypes 1 to 3, genotype IS*901*+, IS*1245*+). The majority of *M. a. avium* isolates induces generalised tuberculosis in birds (Thoen and Steele, 1995) and can be transmitted from this reservoir to humans and various animal species. *M. a. hominissuis* strains do not cause avian tuberculosis; however, they can cause pathological changes in pullets at the site of intramuscular inoculation.

M. avium genome contains several insertion sequences (IS), short DNA fragments which are capable of transposition. ISs can be species-specific, strain-specific or specific for a certain group of

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strains and represent a suitable tool in epidemiological research as genetic markers (Grange, 1996). IS*901* is present in all *M. a. avium* isolates virulent for mice (Kunze et al., 1992). All field isolates of serotypes 1, 2 and 3, which are fully virulent for birds, also contain IS*901* (Pavlik et al., 2000). IS*901* is present also in *M. a. silvaticum* (Moss et al., 1992). Supposedly the most frequent IS found in human *M. avium* isolates is IS*1110*, which is related to IS*900* and IS*901* (Hernandez Perez et al., 1994). IS*1245* and IS*1311*, sharing 85% similarity at the DNA level, are present in the majority of *M. avium* isolates (Guerrero et al., 1995; Roiz et al., 1995). IS*900* is characteristic for *M. avium* subsp. *paratuberculosis* (Green et al., 1989).

The methods that allow differentiation of strains within a species add to our understanding of epidemiology of *M. avium* infections. Several genotyping procedures can be used to type *M. avium* (e.g. pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis, sequencing etc). Genotyping techniques are considered to be more technically complex than phenotyping methods but their reproducibility and discriminatory power yield better results. In the need for a simple, rapid and inexpensive means of genotyping that would be suitable for large-scale epidemiological studies Picardeau and Vincent (1996) introduced a PCR-based typing method with the primers designed for the inverted repeats of the insertion sequences IS*1245* and IS*1311* and therefore amplifying DNA fragments between the copies of both insertion elements (IS*1245*-IS*1311* spacer typing).

The aim of our study was to test this PCR typing method on a variety of *M. avium* strains isolated from animals and humans in order to characterize the genetic diversity among Slovenian *M. avium* strains and to assess the suitability of the method for routine typing of *M. avium* isolates.

MATERIAL AND METHODS

M. avium isolates

The strains (n = 114) were isolated from pigs (n = 90) and humans (n = 24). All swine strains belonged to different animals. Human strains were isolated from 23 patients as 2 strains represented sequential isolates (isolated in 2002 and 2003, respectively) from one patient. Animal strains were isolated at the Institute of Microbiology and Parasitology of Veterinary Faculty, Ljubljana. Human strains were provided by the University Clinic of Respiratory and Allergic Diseases, Golnik (UC Golnik). The cultures were maintained on conventional solid media (Middlebrook 7H10, Stonebrink, Löwenstein – Jensen) at 37°C.

Identification of animal isolates

IS*1245* PCR and in-house IS*901* PCR were used to assess the molecular characteristics of the strains. IS*1311* PCR was performed only in strains with negative typing results. Template DNA was extracted with the simplified boiling method: a few colonies were suspended in 50 µl PCR-grade distilled water, incubated at 100°C for 15 min and centrifuged for 2 min at 11 000×g. The supernatant was used for PCR with the primer sets described previously (Kunze et al., 1992; Guerrero et al., 1995; Roiz et al., 1995). Amplification reactions were performed in a thermocycler (Applied Biosystems, Foster City, CA, USA) with 20-µl volumes containing 10 µl *Taq* PCR Master Mix (Qiagen, Hilden, Germany), 7.8 µl UltraPure® water (Invitrogen, Carlsbad, CA, USA), 10 pmols each oligonucleotide primer (Invitrogen, Carlsbad, CA, USA) and 2 µl of template DNA. For IS*901* PCR, the first 5 cycles consisted of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C. The next 27 cycles, consisting of 30 s at 94°C, 30 s at 64°C and 30 s with the addition of 2 s in every cycle at 72°C, were followed by a final extension for 5 min at 72°C. IS*1245* and IS*1311* PCRs were performed following the amplification protocols described previously (Guerrero et al., 1995; Roiz et al., 1995). Amplification products were analyzed by electrophoresis on 2% agarose gels and detected by ethidium bromide staining.

Identification of human isolates

The strains were identified at the UC Golnik on the basis of their physical and biochemical characteristics and on the basis of AccuProbe assays (*Mycobacterium avium* complex assay and/or *Mycobacterium avium* assay and/or *Mycobacterium intacellulare* assay, GenProbe, San Diego, CA, USA). The strains were subsequently identified also with IS*901* and IS*1245* PCRs.

IS*1245*-IS*1311* spacer typing

A small loop of mycobacteria was suspended in 100 µl TE containing 1% Triton-X, incubated at 100°C for 30 min and centrifuged for 2 min at 11 000×g. The supernatant was used as a DNA source without further purification. The amplification protocol was a variation of a procedure described elsewhere (Picardeau and Vincent, 1996). Amplification was performed in a thermocycler (Biometra, Göttingen, Germany) with 50-µl volumes containing 25 µl *Taq* PCR Master Mix (Qiagen, Hilden, Germany), 22 µl UltraPure® water (Invitrogen, Carlsbad, CA, USA), 50 pmols each oligonucleotide primer (Invitrogen, Carlsbad, CA, USA) described previously (Picardeau and Vincent, 1996) and 2 µl of template DNA. Amplification protocol started with 5 cycles consisting of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C. The next 30 cycles, consisting of 30 s at 94°C, 30 s at 52°C and 30 s with the addition of 2 s in every cycle at 72°C, were followed by a final extension for 5 min at 72°C. Amplification products were analyzed by electrophoresis on 2% agarose gels and detected by ethidium bromide staining. The results were analysed with BioNumerics software (Applied Maths, version 4.0, Sint-Martens-Latem, Belgium). Dendrogram was created using an UPGM (Dice coefficient) algorithm made by means of BioNumerics software.

RESULTS

The results of PCR identification and typeability of IS*1245*-IS*1311* spacer typing of the strains are shown in Table 1. The majority of investigated strains belonged to *M. a. hominissuis* (66.7%); 33.3% strains were *M. a. avium*. In pigs, 57.8% strains were *M. a. hominissuis* and 42.2% *M. a. avium*. All human isolates lacked IS*901*.

IS*1245*-IS*1311* spacer typing was successfully accomplished for 59 (51.8%) isolates while 55 (48.2%) isolates gave no amplification signal. All IS*901*+ strains and 17 *M. a. hominissuis* were not typeable. The isolates with negative typing results were tested for the presence of IS*1311* and all except one gave positive results.

A high degree of genetic diversity among isolates was observed: 59 isolates demonstrated 43 different patterns comprising up to 6 bands (Figure 1). The discrimination index of the method (Hunter and Gaston, 1988) was 0.985.

All isolates were subjected to 2 IS*1245*-IS*1311* spacer typing tests in order to assess the reproducibility of the method. One isolate expressed different PCR profiles in different PCR tests while in one case the isolate was positive in the first test and negative in the second. All other isolates gave identical results in both tests; the reproducibility rate was 96.6%.

DISCUSSION

IS*1245*-IS*1311* spacer typing introduced by Picardeau and Vincent (1996) was described as a simple, rapid and reproducible method as discriminating as IS*1245* RFLP and providing a relevant characterization of *M. avium* strains. So far it was used to characterize human *M. avium* strains from HIV positive and HIV negative patients (Picardeau and Vincent, 1996; Picardeau et al., 1997; Pestel-Caron et al., 1999), to compare the levels of relatedness of clinical and food *M. avium* isolates (Yoder et al., 1999) and to characterize *M. avium* isolates in an epidemic among farmed lesser white-fronted geese (Kauppinen et al., 2001). PCR profiles of *M. avium* strains isolated from humans were found to be relatively diverse with the patterns consisting of less than 10 bands (Picardeau and Vincent, 1996; Pestel-Caron et al., 1999). Identical

Table 1. Results of PCR identification and typeability of IS*1245*-IS*1311* spacer typing of *Mycobacterium avium* strains isolated from animals and humans

Identification	Typing positive		Typing negative		Total
	animal	human	animal	human	
M. avium avium (IS*901*+, IS*1245*+)	0	0	38	0	38
M. avium hominissuis (IS*901*–, IS*1245*+)	38	21	14	3	76
Total	38	21	52	3	114

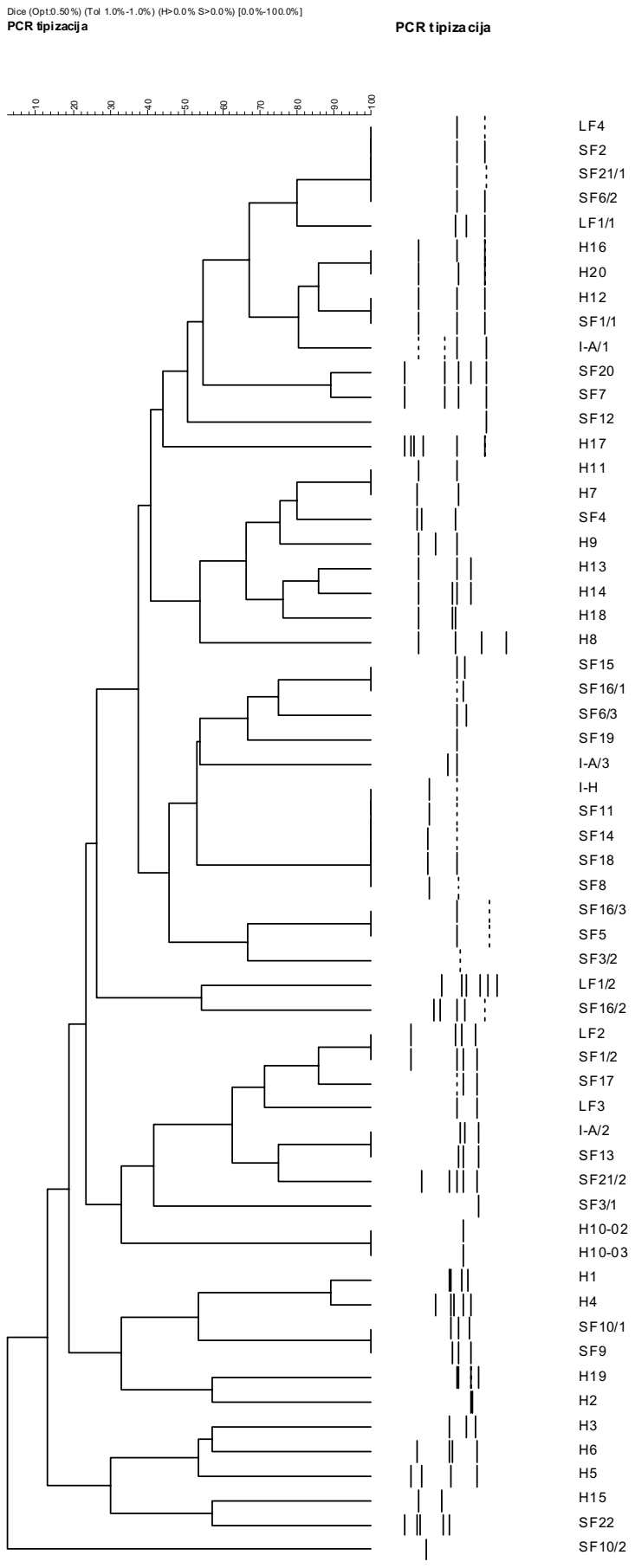

Figure 1. Dendrogram of IS*1245*-IS*1311* spacer typing patterns of human and swine *Mycobacterium avium* isolates. Origin of the isolates is designated as follows: I-H: imported from Hungary; I-A: imported from Austria; SF1-22: small farms (less than 1000 animals); LF1-4: large farms (more than 1 000 animals); H1-20: human isolates (H10-02 and H10-03: strains, isolated from the same patient in the year 2002 and 2003, respectively)

and closely related profiles were found in comparisons between clinical and food samples (Yoder et al., 1999). *M. avium* strains isolated from lesser white-fronted geese showed mainly one-band patterns in comparison to the environmental isolates that yielded multi-banded patterns (Kauppinen et al., 2001). No data on presence of IS*901* in the investigated strains were found in previous reports on *M. avium* IS*1245*-IS*1311* spacer typing.

This is the first study of *M. avium* isolates in Slovenia based on IS*1245*-IS*1311* spacer typing. *M. avium* isolates were found to be very heterogeneous which is in agreement with the findings reported previously (Pestel-Caron et al., 1999; Yoder et al., 1999). We found only two clusters (consisting of 4 and 5 strains, respectively) and 9 duplicates of the same pattern, which cannot be correlated with the origin of the strains. The isolates from human and animal sources were indistinguishable in only one case. The two isolates from the same patient, obtained in two different time periods, were identical. The discrimination index of the method (0.985) is high but a high proportion of one-band (11.9%) and two-bands (33.9%) patterns among our isolates should be taken into account. Frequent occurrence of one-band patterns, associated with low IS copy number, is a critical limitation as such patterns are poorly discriminatory for epidemiologically unrelated isolates. Apart from PFGE and RFLP, random amplified polymorphic DNA (RAPD) analysis might provide a solution in these cases as it was described as a useful and more discriminating rapid method for fingerprinting *M. avium* strains (Kaupinnen et al., 2001).

Furthermore, all IS*901*+ isolates in the present study yielded no bands. This might be due to low IS*1245* copy number as *M. a. avium* isolates carry only 3 copies of IS*1245* and 1 or 2 copies of IS*1311* (O'Grady et al., 2000). One of the possible reasons for this outcome could also be that IS*901* copies are located between the copies of IS*1245* and IS*1311*, increasing the distance between them and disabling the amplification of the DNA fragment between both insertion elements. If IS*901*+ strains indeed cannot be typed, then the usefulness of the method is significantly reduced, particularly in veterinary medicine.

As proposed previously, the method would be suitable for preliminary screening or to investigate small numbers of isolates collected over a short period of time (Pestel-Caron et al., 1999). As it seems from the results of our study, only IS*901* negative strains are typeable with this method, which is a considerable drawback when testing field samples. To our belief, tedious and time-consuming PFGE or RFLP should still remain the reference techniques for *M. avium* strain characterization.

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