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ORIGINAL ARTICLE

Genotyping of *Mycobacterium avium* subsp. *avium* isolates from naturally infected lofts of domestic pigeons in Ahvaz by *IS901* RFLP

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ABSTRACT

Background and Objectives: Avian tuberculosis is one of the most important infections affecting most species of birds. *Mycobacterium avium* can not only infect all species of birds, but also infect some domesticated mammals.
The most crucial aspect of control and eradication scheme is identification of infection sources and transmission routs. Molecular techniques such as restriction fragment length polymorphism and pulse field gel electrophoresis have been shown to be much more discriminatory and suitable for use in the epidemiological study.
Materials and Methods: Eighty suspected pigeons to avian tuberculosis based on their clinical signs, were subjected to the study. Forty *Mycobacterium avium* subsp. *avium* isolates out of a total of 51 identified isolates were subjected to the test.
Results: *IS901*-RFLP using *Pvu* II was successfully conducted and produced 7 patterns. The majority of isolates (60%) were RFLP type PI.1. This type was the most similar type to standard strain. However, all the patterns obtained in this study were different from the standard strain.
Conclusion: The result of this study indicate that these isolates probably are limited to Khuzestan region. We recommend DNA fingerprinting differentiation of non tuberculous Mycobacteria particularly *Mycobacterium avium* complex isolated from infected birds and human to possibly find source of infections.

Keywords: *Mycobacterium avium,* RFLP, polymorphism, avian tuberculosis.

INTRODUCTION

Avian tuberculosis is one of the most important infections affecting most species of birds (1, 2). Several mycobacterial species have been identified causing avian tuberculosis, but the organisms confirmed most frequently are *Mycobacterium avium* belonging to serotypes 1, 2, and 3 (genotypes *IS901*+ and *IS1245*, respectively), and *Mycobacterium genavense* (3, 4).

All species of birds can be infected with *Mycobacterium avium*. Generally speaking, domesticated fowl or captive wild birds are affected more frequently than those living in a wild state (4). *Mycobacterium avium* can not only infect all species of birds, but can also infect some domesticated mammals to cause the disease, usually with localized lesions. Disseminated tuberculosis caused by *Mycobacterium avium* has also been reported in rabbits and swine (4). In immune-competent humans, *Mycobacterium avium* complex (MAC) isolates produce localized soft tissue infections including chronic pulmonary infections in the elderly and cervical lymphadenitis in children, but rarely any disseminated disease. In

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HIV infected and AIDS patients, or in other immuno-compromised persons, MAC isolates frequently cause severe systemic infections (5).

The most crucial aspect of control and eradication of the disease is identification of infection sources and transmission routs. The use of serotyping for epidemiological studies of virulent *Mycobacterium avium* subsp. *avium* (MAA) isolates in birds is limited by the prevalence of only three serotypes (5) and untypable auto-agglutinating isolates (6). Molecular techniques such as restriction fragment length polymorphism (RFLP) and pulse field gel electrophoresis (PFGE) have been shown to be much more discriminatory and therefore suitable for use in the epidemiological study of MAC infections (5). Although PFGE has been useful, it requires a greater amount of DNA than restriction fragment length polymorphism (RFLP) (7). MAA genome contains insertion sequence *IS901*, which is used as markers for identification and typing (8). MAA genome contains up to 14 copies of insertion sequence *IS901* in different localizations in the genome. The number of copies ensures that resulting patterns are easy to read and they can be easily compared with the patterns of other isolates (8).

The aim of this study was to characterize MAA isolates from naturally infected of domestic pigeons (Columba livia var. domestica) using *IS901* RFLP analysis and comparison of the detected *IS901* RFLP types with each other and with the standard type.

MATERIALS AND METHODS

Samples. Eighty pigeons (Columba livia var. domestica), out of a total of about 600 pigeons from 10 lofts, based on their clinical signs and poor health condition were selected and were gradually transferred to the avian disease section of Shahid Chamran's University of Ahvaz. Smears were prepared from lesions of the affected organs of some of the pigeons and stained with ZN technique and were examined by light microscopic for the presence of AFB, to ensure the pigeons being infected with TB (9). Tissue samples from visible lesions and liver samples from birds without gross lesions were collected aseptically as previously described (10), stored in 50-mL screw-cap containers and shipped on dry ice to the Reference Mycobacteriology Department at Razi Vaccine and Serum Research Institute, Karaj, Iran for definitive identification.

Mycobacterial isolation. At the tuberculosis reference laboratory, approximately 4 g of thawed tissues of each bird was pooled and ground in a mortar containing sand using a pestle (using sterile materials and equipments). The homogenized mixtures were decontaminated according to the NALC (*N*-acetyl-l-cysteine)-NAOH method (11). The inoculums were cultured on 4 culture slopes including glycerinated Lowenstein-Jensen (LJG) medium, pyruvate-enriched Lowenstein-Jensen medium (LJP), mycobactin J-supplemented Herrold-egg yolk medium and plain Herrold-egg yolk medium. The inoculated slopes were incubated at 41 ºC for 8 to 12 weeks. After incubation all isolates were stained by ZN technique, to make sure they were acid-fast bacteria.

Molecular identification. All AFB isolates were sub-cultured onto two fresh mycobactin J-supplemented Herrold-egg yolk slants in order to achieve bacterial growth enough for extraction of chromosomal DNA. Genomic DNA of all isolates from each infected pigeon was extracted according to the Van Soolingen method (12). All isolates were tested by the PCR assays targeting the 16S rRNA gene for identification of mycobacterium members, (Amplicon size 543 bp), specifically *IS1245* for MAC (Amplicon size 427 bp), and *IS901* for identification of MAA (Amplicon size 1108 bp), (13-15) (Table 1). PCRs were conducted with incorporation of positive controls (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713), negative species controls (*Mycobacterium bovis* AN5 strain, ATCC number 35726) and negative controls (distilled water) (16, 17). PCR products were analyzed on ethidium bromide-stained agarose gels (2%) in a submerged electrophoresis system.

Forty MAA isolates originated from 80 euthanized naturally infected of domestic pigeons (Columba livia var. domestica), with MAA (from more than 10 lofts) were submitted for this study. Genomic DNA was extracted and RFLP analysis was performed according to the method of van Soolingen et al (18). Approximately 3 µg of the purified Mycobacterial DNA for RFLP analysis was digested by *Pvu* II (Fermentas) and incubated at 37 ºC overnight. DNA fragments were separated by gel electrophoresis and transferred onto a positively charged nylon membrane. The digoxigenin tail-labelled *IS901* probes,

Table 1: Characteristics of the PCR assays used for the detection and identification of mycobacterial isolates collected from pigeons.
F: forward, R: reverse

Genomic marker	Species	Amplicon size (bp)	Primer sequences*(5′→3′)	References
16S rRNA	*Mycobacterium* spp	543	F: ACGGTGGGTACTAGGTGTGGGTTTC R: TCTGCGATTACTAGCGACTCCGACTTCA	Huard et al (2003)
IS1245	*M. avium* complex	427	F: AGGTGGCGTCGAGGAAGAC R: GCCGCCGAAACGATCTAC	Guerrero et al (1995)
IS901	M. avium subsp. avium	1108	F: GCAACGGTTGTTGCTTGAAA R: TGATACGGCCGGAATCGCGT	Kunze et al (1991)

consisted of a 1108-bp PCR product and purified using a Gel Extraction Kit (Roche, Germany), were used for hybridization which was performed at 65 ºC using a rolling bottle method. The hybridized membrane was exposed to alkaline phosphatase-conjugated anti-dig antibody solution and the hybridization signals were detected using the substrate BCIP/NBT. Photography was performed with a scanner (Cannon Laser Base MF3110, Japan).

RESULTS

Fifty one AFB were isolated from pigeons. All isolates were positive for *IS1245* and *IS901* in PCR (Figs. 1 and 2). Clinical symptoms, necropsy findings, ZN staining and molecular identification confirmed that the pigeons were infected with MAA. *IS901*-RFLP using *Pvu* II was successfully conducted on 40 isolates and resulted in seven RFLP types (Fig. 3). The majority of isolates (60%) were of RFLP type PI.1. This type was related but slightly different from the standard control (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713) (Fig. 3).

DISCUSSION

The most crucial aspect of control and eradication of the disease is identification of infection sources and transmission routs. Molecular techniques such as RFLP and PFGE for epidemiological studies of *Mycobacterium avium* complex have been shown to be much more discriminatory than serotyping (5, 6). MAA genome contains insertion sequence *IS901*, which is used as a marker for identification and typing (8). MAA contains up to 14 copies of insertion sequence *IS901* interspersed through the genome. The number of copies ensures that resulting patterns are easy to read and they can be easily compared with the patterns of other isolates (8).

In this study *IS901*-RFLP typing using *Pvu* II was successfully conducted on 40 MAA isolates from naturally infected of domestic pigeons (Columba livia var. domestica) and produced 7 patterns. The majority of isolates (60%) were RFLP type PI.1 and in comparison this type was the most similar type to standard strain, however all the patterns obtained in this study, were different from the standard strain (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713). In addition no common pattern between this study and the only another similar study in Tabriz-Iran (which is not published) was found and it indicates that the sources of their infection were not same.

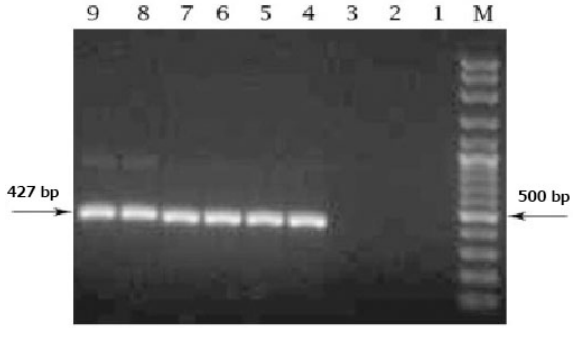

Fig 1. PCR analysis. The 427 bp specific fragment from IS1245. Lane M, DNA size marker (100 base pair ladder). Lane 1 and 2, negative controls (distilled water). Lane 3, negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726). Lane 4, positive control (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713). Lane 5 to 9 samples tested for *Mycobacterium avium* subsp. *avium*

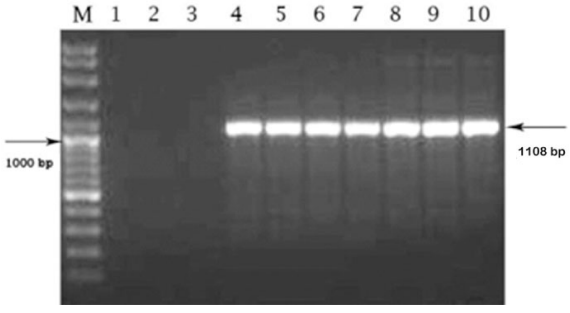

Fig 2. PCR analysis. The 1108 bp specific fragment from *IS901*. Lane M, DNA size marker (100 base pair ladder). Lane 1 and 2, negative controls (distilled water). Lane 3, negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726). Lane 4, positive control (*Mycobacterium avium* subsp. avium D4 strain, ATCC number 35713). Lane 5 to 10 samples tested for *Mycobacterium avium subsp*. *avium*

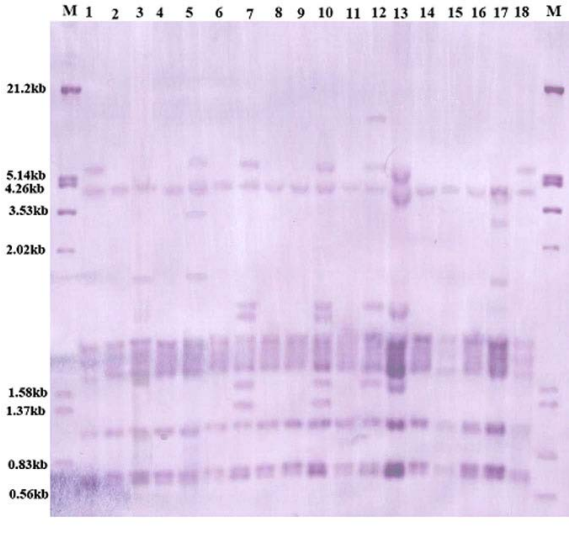

Fig. 3. *IS901* RFLP types discovered in 40 *Mycobacterium. avium* subsp. *avium* isolates after using RE *PvuII*. Lane M, DNA size marker (number 3 Roche Germany). Lane 1 and 18, standard control (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713). PI. 1 types include: 2, 4, 6, 8, 9, 11, 14, 15 and 16. PI. 2 types include: 7 and 10. PI. 3 types include: 5. PI. 4 types include: 3. PI. 5 types include: 13. PI. 6 types include: 12. PI. 7 types include: 17.

In conclusion, DNA fingerprinting demonstrated that pigeons in Iran were infected with different strains of *M. avium*. Because of proximity between human and domesticated pigeons, we suggest that genetic relationships between human and animal isolates of *M. avium* be investigated.

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