

Avian Tuberculosis in Naturally Infected Lofts of Domestic Pigeons, Isolation, Molecular Identification and Study of Necropsy Findings

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KEY WORDS: *Mycobacterium avium* subsp. *avium*, pigeon, Acid fast basil, lesion, PCR

Abbreviations: AFB = Acid fast basil, MAA = *Mycobacterium avium* subsp. *avium*, MAC = *Mycobacterium avium* complex, PCR = polymerase chain reaction. ZN = Ziehl-Neelsen

ABSTRACT

The purpose of the present study was to investigate the occurrence of *Mycobacterium avium* subsp. *avium* in naturally infected lofts of domestic pigeons, clinical signs, necropsy findings, differential susceptibility of male and female pigeons to infection and disease and vertical transmission. Eighty out of more than 600 pigeons were selected based on their clinical signs and poor health conditions and under standard condition were euthanized, necropsied and followed by bacterial culture on specific media for

MAA. Further 10 pigeon eggs, which were laid by these birds, were also individually cultured in search for *Mycobacterium avium* subsp. *avium*. Fifty one *Mycobacterium avium* subsp. *avium* isolated from pigeons and 1 from eggs. All acid fast basil isolate were tested by the PCR assays targeting the 16S rRNA, IS1245 and IS901 genes. Swollen joints in legs and wings consequent lameness and inability to fly were the most important clinical signs. In necropsy findings liver was the most affected organ which had lesion. The least affected organ was lung also no macroscopic necropsy lesion was found in the gonads, kidneys and CNS. Based on clinical and necropsy findings and isolation of acid fast bacteria, no significant difference between male and female pigeons in susceptibility to infection and disease was found. Based on necropsy findings four forms of disease manifestations were

described, classic tuberculosis, intestinal form, liver form and non TB form. It was concluded that on the basis of frequency of clinical signs, necropsy lesions, isolation and identification of MAA from suspected pigeons, frequency of avian tuberculosis in the pigeons was high, but it needs further investigation, especially molecular epidemiology studies.

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 HIV infected and AIDS patients, or in other immuno-compromised persons, MAC isolates frequently cause severe systemic infections.⁵ Symptoms of mycobacteriosis in birds include chronic illness characterized by weight loss, diarrhea, dyspnea, lameness and poor feathering, however a significant number of birds die acutely without exhibiting any recognized symptoms.² The most common route of infection for susceptible birds is via the alimentary tract; however, pulmonary avian tuberculosis and egg transmission have also been described.^{3,6} Lesions are most frequently seen in liver,

spleen, intestines and bone marrow and less frequently in the other organs, while central nervous system is rarely affected.⁶ Stress factors appear to enhance the development of avian tuberculosis in birds living in captivity.³ The importance of avian tuberculosis and the risk of its zoonotic spread motivated our interest to investigate the occurrence, molecular epidemiology, clinical signs, necropsy findings, differential susceptibility of male and female pigeons to infection and disease and vertical transmission of *Mycobacterium avium* subsp. *avium* in naturally infected lofts of domestic pigeons (*Columba livia* var. *domestica*).

MATERIAL AND METHODS

Samples

Following complaints from owners of some private pigeon lofts which many of their birds were exhibiting clinical signs such as swollen joints, lameness, emaciation, tubercle formation under the skin and granulomas in the conjunctival sac. The affected lofts were visited and all the birds were closely examined. The bio-security conditions of the lofts were poor. 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. Further 10 pigeon eggs, which were laid by the birds, were also collected, though it was not possible to determine as to which egg belonged to which bird. The birds were numbered and their clinical signs were registered in the working sheets before euthanizing them for subjecting to necropsy examinations. Gross lesions observed in the internal organs were noted on the working sheets. 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.⁷ Tissue samples from visible lesions and liver samples from birds without gross lesions were collected aseptically as previously described,⁸ stored in 50-mL screw-

Figure 1: Soft tissue swelling around the joint, caused by *Mycobacterium avium* subsp. *avium*.



cap containers and shipped on dry ice to the tuberculin department of the reference laboratory, the 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.⁹ 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 °C 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.¹⁰ 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),^{11,12,13} (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

Figure 2: Tubercle granuloma in the conjunctiva, caused by *Mycobacterium avium* subsp. *avium*.



Table 1: Characteristics of the PCR assays used for the detection and identification of mycobacterial isolates collected from pigeons.

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)

F: forward, R: reverse

Table 2: Distribution of external lesions in 35 pigeons with clinical or external lesions out of 80 suspected pigeons.

Tissue	Joints	Muscle	Conjunctiva	Subcutaneous
Pigeon Number				
35	26	3	8	3

Table 3: Distribution of lesions in the internal organs of 58 pigeons with necropsy lesions out of 80 suspected pigeons.

Organ	Liver	Heart	Spleen	Intestines	Oviduct	Lung	Gizzard	Proventriculus	Abdominal cavity
Pigeon Number									
58	54	7	9	18	3	1	2	2	16

Table 4: The results of lesions and cultures of male and female suspected pigeons

Sex	Number	Lesion	Culture positive
Male	32	21	20
Female	48	39	31

According to analysis by Chi Square test, no significant different was found. $P < 0.05$

Table 5: Four forms of disease manifestations based on necropsy findings in suspected pigeons

Disease form	classic	intestinal	liver	non TB
Pigeon number	28	4	26	5

Figure 3: Multifocal granulomatous hepatitis in infected pigeons with *Mycobacterium avium* subsp. *avium*.



(distilled water).^{14,15} PCR products were analyzed on ethidium bromide-stained 2% agarose gels in a submerged electrophoresis system.

Statistical analysis: Data were analyzed by Chi-squared test. P value less than 0.05.

RESULTS

Clinical signs

Among the eighty pigeons selected on the basis of their clinical signs and poor health condition, 35 pigeons exhibited external lesions, out of which swollen joints in legs and wings (and consequent lameness and inability to fly) (Figure 1) were the most important. The other less frequently recorded external lesions included granuloma within the conjunctival sac (Figure 2) and tubercle formation on breast muscle and subcutaneous tissue (Table 2). However, most of the affected birds had good appetite.

Necropsy findings

Necropsy revealed lesions in the internal organs of the 58 pigeons. Liver was the most frequently affected organ (Figure 3) and followed (in descending order) by the intestines, abdominal cavity, spleen, heart, oviduct, Gizzard, proventriculus and lungs, lesions were seen (Table 3), (Figure 4 and 5). Lungs were the least affected internal organ, while no macroscopic lesion was found in the gonads, kidneys and CNS. Clinical signs, necropsy findings and isolation of acid fast bacteria did not reveal any significant difference in susceptibility to infection and disease between male and female pigeons ($p < 0.05$) (Table 4). The size of tuberculous granuloma varied from organ to organ and ranged from 1mm to 20 mm, the largest size of TB lesions being located in

the abdominal cavity. Four forms of disease manifestations were described: The classic form a disease with widespread tubercle formation in most organs; the intestinal form:

Figure 4: Nodular granulomatous lesions in the liver and heart of infected pigeons with *Mycobacterium avium* subsp. *avium*.



In which only intestines had the typical macroscopic lesions; the liver form: In which only liver had the typical macroscopic lesions; and the non-TB form: In which the cultures were positive without the presence of any gross TB lesion. The most common form was the classic form and the next was the liver form. The least common form of the disease was non-TB form (Table 5). In 17 pigeons out of 80 suspected pigeons no external and internal mycobacterial lesions were observed, also no mycobacterial were isolated. Based on clinical and necropsy signs it seemed that they infected with Newcastle virus and parasitic worms, and because of this they were selected wrongly. Also some of the infected pigeons with MAA had Newcastle and parasitic diseases symptoms. In the necropsy examinations no macroscopic mineralization was found. No Clinical signs and necropsy lesions of avian tuberculosis were observed in young pigeons, which were kept in the infected lofts.

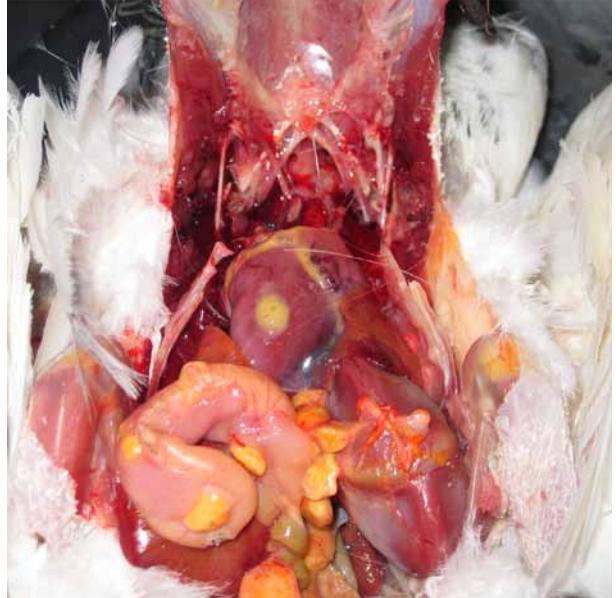
Mycobacterial isolation and identification

Fifty one MAA isolated from pigeons and 1 from eggs. Appearances of the most isolated colonies were small, smooth and transparent and their colors were grayish white. These AFB isolated were examined by PCR method for molecular identification. In 16S rRNA, this figure is not shown, IS1245 and IS901 PCR tests all of them were positive (Fig 6 and 7). Clinical signs, necropsy findings, ZN staining and molecular identification confirmed that the pigeons were infected with MAA.

DISCUSSION

Diagnosis of avian tuberculosis can be difficult because of wide variation in non-pathognomic disease symptoms, long incubation period, inadequate serological tests and difficulty in culturing the organism.² Also such study has never seemed to have

Figure 5: Nodular granulomatous lesions in the intestines of infected pigeons with *Mycobacterium avium* subsp. *avium*.



been documented so far in the Iran. In this study clinical signs, necropsy findings, differential susceptibility of male and female pigeon to infection and disease and vertical transmission of MAA in naturally infected lofts of domestic pigeons were described. Isolation via culturing and/or molecular methods remains the most definitive means of diagnosing mycobacterial infection in birds.¹⁶ In present study all of the 52 isolates carried IS901 insertion sequence, a determinant of pathogenicity, and also IS1245 locus. Such isolates belong to serotypes 1, 2 and 3 of *Mycobacterium avium* are considered as the most pathogenic strains of *Mycobacterium avium* in birds.^{1,17} Beside, morphology of colonies indicated that they belong to the virulent strains.⁴ Factors that enhanced the development of disease on the pigeon lofts included stress, due to keeping in a small area, and poor health conditions.³ The remarkable clinical sign of the disease was swollen joints, which along with other clinical signs or external lesions could help diagnose of the disease though these signs are not pathognomic.⁸ Clinical signs, necropsy findings and the isolation of acid

fast bacteria did not reveal any significant difference in susceptibility to infection and disease between male and female pigeons ($p < 0.05$); and this finding was consistent with other studies and show that susceptibility to infection and disease was unrelated to the sex of the pigeons.¹⁸ In the all avian species, the infection is acquired by ingestion. However, an occasional occurrence of aerogenic pulmonary infection and transmission of *Mycobacterium avium* infection through eggs has also been described,¹⁹ which correlated with our findings, because lung lesions were observed only in one pigeon, while liver and intestines were the most affected organs in the rest of them. It seemed that lung lesion in one pigeon was due to secondary hematogenous spread of infection, because lesions in the liver and abdominal cavity of this pigeon were also seen.^{10,20} Isolation of MAA from the contents of one egg, and observation of lesions in 3 pigeons oviduct, show that vertical transmission can be happen.⁴ In all 80 suspected pigeons no macroscopic granuloma in the gonads, kidneys and central nervous system were seen which indicated that gonads, kidneys and central nervous system are rarely affected.^{6,21} Mycobacteriosis appears to be less prevalent in young birds because in older ones the disease has a greater opportunity to become established through a longer period of exposure.^{22,23} during our investigations no clinical signs or necropsy lesions of avian tuberculosis were observed in young pigeons which were kept in the infected lofts. In the necropsy examinations conducted no macroscopic mineralization was found. This finding was consistent with lesions in mycobacterial infections described in birds by Tell et al.²⁴

During the necropsy examinations of the all pigeons, the largest sizes of tuberculous granuloma in the abdominal cavity were observed. It seems abundance of oxygen and enough space in the abdominal cavity provided suitable environment for metabolism and growth of bacteria.²⁵ In this study four forms of disease manifestations were described, the classic form (a disease with

widespread tubercle formation in most organs); the intestinal form: In which only intestines had the typical macroscopic lesions; the liver form: In which only liver had the typical macroscopic lesions; and the non-TB form: In which the cultures were positive without the presence of any gross TB lesion, but Prunker- Radkovic et al.,²⁶ described three forms of disease manifestations; this study was consistent with the three forms but one more form which it called the liver form was described as well.

Pigeons are extensively kept in urban area for homing and racing purposes. They are also frequent visitors of cattle, pig and poultry farms where they search for food and water and also release their droppings thus infecting the environment and exposing people and these animal farms to pathogenic MAA. It was concluded that on the basis of frequency of clinical signs, necropsy lesions, isolation and identification of MAA from suspected pigeons, frequency of avian tuberculosis in the pigeons was high, but it needs further investigation, especially molecular epidemiology studies.

ACKNOWLEDGMENT

This study was supported by research grant from research deputy of Shahid Chamran University of Ahvaz and Razi Vaccine and Serum Research Institute, Karaj, Iran.

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