DOI: 10.22059/ijvm.2018.243573.1004850 *Iranian Journal of Veterinary Medicine*

Isolation and Molecular Identification of *Mycoplasma* spp. From Pigeons in the North-East of Iran

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Abstract:

BACKGROUND: Mycoplasma is one of the most important pathogens of respiratory system in poultry.

OBJECTIVES: The aim of the present study was to identify *Mycoplasma* spp. isolated from pigeons.

METHODS: Sixteen pooled samples were provided and cultured on PPLO medium and finally the DNA was extracted from the resulting single colonies.

RESULTS: Through 16S rRNA gene amplification Mycoplasma genus has been detected. Overall, 31% (5 out of 16) of pooled samples were positive which were identified as *Mycoplasma cloumborale* and *Mycoplasma gallinaceum*.

CONCLUSIONS: Screening of large numbers of pigeons for known poultry pathogenic mycoplasmas will be required to establish the role of pigeons in the spread and maintenance of these organisms in the environment.

Keywords:

Mycoplasma, *Mycoplasma cloumborale*, *Mycoplasma gallinaceum*, PCR, Pigeon

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Received: 24 December 2017

Accepted: 18 March 2018

Introduction

One of the considerable causes of economic losses to the poultry industry is avian mycoplasmosis, especially in chickens and turkeys, globally (Ley, 2008). Respiratory problems is one of the common problems of racing pigeons which are difficult to diagnose, prevent and treat. However, different kinds of diseases may be involved such as avian chlamydiosis, pigeon pox, Escherichia coli infection, pigeon Herpesvirus I (PHV I) infection and some lentogenic strains of Newcastle disease virus (Keymer et al., 1984), but the role of mycoplasmas in pigeons is debatable. Although mycoplasmosis is regarded as a clinical entity by many pigeon breeders and some veterinarians, there is little evidence for this view. In Japan, many strains of *M. columbinum* and *M. columborale* have been recovered from the trachea and oropharynx of clinically healthy feral pigeons (Shimizu et al., 1978) and from the respiratory tract and esophagus of apparently healthy feral pigeons in Britain (Jordan et al., 1981).

Sinclair (1980) isolated *M. columbinasale* from pigeons showing respiratory disease (Ley, 2008). MacOwan et al. (1981) isolated *M. columborale* from pigeons also affected with a similar syndrome in Britain. Recognized pigeon species of *Mycoplasma* are *M. columborale*, *M. columbinum* and *M. columbinasale*. These have been recovered from pigeons in Britain (Sinclair,1980; Jordan et al., 1981; MacOwan et al. 1981). No previous report of their isolation in Iran exists.

Mycoplasma gallisepticum was isolated from pigeons kept near an infected chicken house (Bencina et al., 1987). *Mycoplasma gallisepticum* could be transmitted both vertically and horizontally by contaminated fomites and workers (Ley, 2008). Few number of studies have reported *M. gallisepticum* infection in pigeons. A serological survey of racing pigeons in Taiwan revealed 1.3% positive results using SPA (Tsai and Lee, 2006). In additional reports, other *Mycoplasma* species were isolated from pigeons but not *M. gallisepticum* (Sinclair, 1980; Keymer et al., 1984; Reece et al., 1986; Loria et al., 2005). Present study was aimed to isolate *Mycoplasma* spp. from pigeons and to recognize *Mycoplasma* spp. at the species level.

Materials and Methods

Sampling and Mycoplasma culture: Sixteen pooled samples were provided from sixteen lofts. Each pooled sample consisted of three to five different pigeons. Swabs were collected from choanal clefts or trachea in live birds or air sacs and trachea in carcasses. Tracheal swabs were placed in peptone water 0.1% and by use of 0.45 µm filters were cultured immediately after sampling for detection of Mycoplasma, using PPLO broth media which contained NAD and cystein. Cultured Broths were incubated at 37 °C. Any turbidity or color change during the first 24 hours was considered as contamination and was removed from incubator. The positive cultures were filtered and sub-cultured on PPLO agar media at 37 °C, moreover, cultured plates were screened for Mycoplasma colonies up to 10 days using a light microscope.

DNA extraction and PCR: DNA extraction of tracheal swabs and single pure colony of the selected isolates were performed by boiling method. The extracted

Table 1. Sequence of primers, Annealing temperatures and sizes of PCR products.

Mycoplasma spp.	Primer Sequence	Annealing	Product (bp)
Mycoplasma (GPF MGSO)	5'-GCT-GGC-TGT-GTG-CCT-3'	30 sec	1013
	5' -TGC-ACC-ATC-TGT-CAC-TCT-GTT-AAC-CTC-3'	56 °C	
M. gallisepticum	MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'	30 sec	185
	MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'	94°C	
M. synoviae	MS–F: 5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'	30 sec	211
	MS–R: 5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'	56°C	
M. melagridis	MMF: 5'-CGA-GCG-AAG-TTT-TTC-GGA-AC-3'	30 sec	422
	MMR: 5'-GGT-ACC-GTC-AGG-ATA-AAT-GC-3'	56°C	
M. iowae	MIF: 5'-AGT-CGA-ACG-GGG-TGC-TTG-CAC-3'	30 sec	237
	MIR:5'-TGC-CTC-TCA-GCA-CAG-CTA-TGC-3'	60°C	

DNA was subjected to 16S rRNA gene PCR by using a genus-specific PCR with 1013 bp PCR product according to Lierz et al (2007).

Amplification of bacterial DNA was performed by TC 3000 Temperature Cycling System. Every reaction contained 25 µl of ready-to-use premix (Ampliqon, Denmark), 2 µl of forward and reverse primers, and 2.5 µl (25 ng) of the DNA template, to a final volume of 50 µl. The PCR condition was carried out as follows: 4 min at 94 °C for initial denaturation, followed by 35 cycles of 94 °C for 30 seconds, different annealing temperature according to primer, and 72 °C for 50 seconds and a final extension of 72 °C for 10 min.

Positive samples in genus specific PCR were further investigated using species-specific PCR tests for the detection of *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, and *M. iowae*. Positive samples which were negative in species-specific PCR, were sent for sequence analysis. Deionized distilled water was used as negative control.

Four microlitres of the PCR products was separated on a 1.5% agarose gel (80 volts, 2 hours) (Merck, Germany) and visualized on a transilluminator after ethidium bromide staining. The primer's sequences, annealing temperatures and sizes of PCR products are shown in Table 1. The PCR products were sequenced by using the same forward and reverse primers by a commercial DNA sequencing service (Macrogen, Korea). Manual editing of the sequenced samples was performed by ChromasPro (version 2.1.4, Technelysium Pty Ltd, Australia) software. Partial sequences were aligned with CLC software (CLC Main Workbench 7, Denmark) and the phylogenetic tree was constructed using different related sequences deposited in GenBank.

Results

In pigeons, 31% (5 out of 16) of pooled samples were positive. Based on the nucleotide sequencing and comparison of sequences with those recorded in Genbank, 25% (four samples) were *M. cloumborale* and 6% (one sample) was *M. gallinaceum* (Fig 1).

Discussion

Keymer et al. (1984) examined live and dead racing pigeons (*Columba livia*) which were clinically healthy and those affected by mild respiratory disease, from five lofts for *Mycoplasma* spp. Oropharynx, the na-

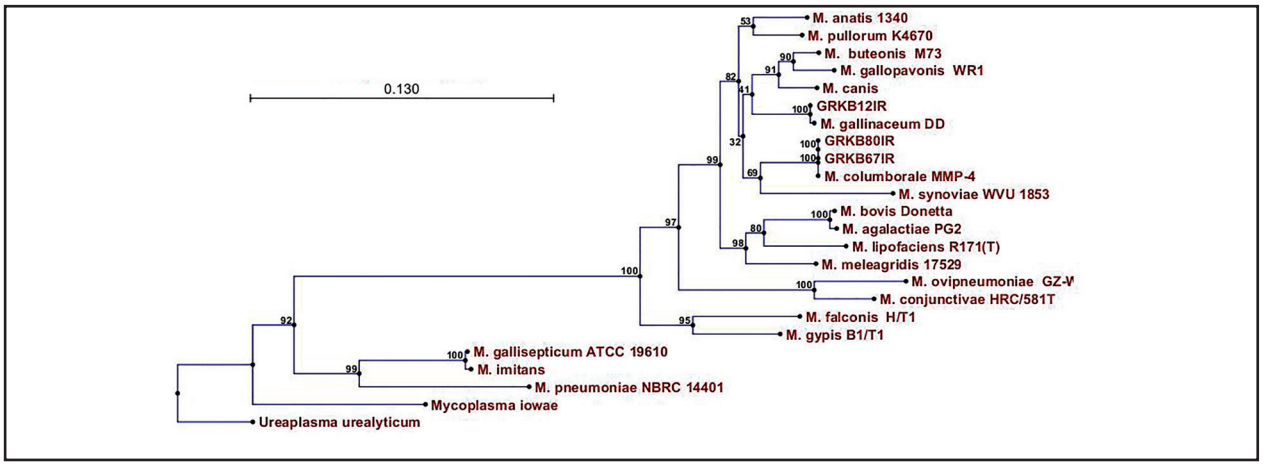

Figure 1. Phylogenic tree of Mycoplasma spp.

sal sinuses and other tissues were sampled and cultured. *Mycoplasma columbinum*, *M. columborale* and *M. columbinasale* were isolated from the oropharynges and nasal sinuses; *M. columbinum* and *M. columbinasale* from the brain and *M. columbinum* and *M. columborale* from lungs and air sacs. One or more of these three *Mycoplasma* spp. were isolated at necropsy from 16 (28%) of 58 pigeons. Only 4 (11%) of 37 pigeons reacted serologically to *M. columbinum* and none to *M. columborale*. Twenty-five birds examined for *M. gallisepticum* antibody were negative. About 10% of pigeons in all five lofts showed clinical signs of the respiratory disease sometimes described as 'mycoplasmosis catarrh', but most of the dead birds from which *Mycoplasma* spp. were isolated also had concomitant infections of various kinds (Keymer et al., 1984). Although the results of these investigations provide no clear evidence that *Mycoplasma* spp. are aetiologically involved in common respiratory disease of pigeons.

In the present study, Mycoplasma was isolated in five (31%) out of sixteen evaluated lofts. Culture and molecular diagnosis were totally in agreement. Nucleotide sequencing and comparison of sequences in GenBank showed 100% similarity with *M. columborale* and *M. gallinaceumin* 4 (25%) and 1 (6%) pooled sample, respectively.

Molokwu and Adegboye, 1988 reported *M. columbinum* and *M. columborale* from pigeons with respiratory symptoms in Nigeria. Samples were taken from oropharynx, trachea, lungs and air sacs. However, poultry species such as *M. gallisepticum* (MG), *M. synoviae* (MS) and *M. meleagridis* were not isolated Molokwu and Adegboye, 1988. Some studies have reported the isolation of *M. columborale* and *M. columbinum* from the trachea and oropharynx of clinically healthy pigeons (Shimizu et al., 1978; Jordan et al., 1981). These have also been detected in respiratory diseases of pigeons Molokwu and Adegboye, 1988. The pathogenic potential of *M. columborale* for chicks was demonstrated by the same authors. Pigeons and other wild birds are regularly found near poultry farms; therefore, pigeons may play a major role in the spread of poultry pathogens from one farm to another. The fact that M.columborale and *M. columbinum* species, thought to be specific for pigeons, have also been isolated from chickens (Molokwu et al., 1987) may be an indicator of contact between pigeons and

chickens. In the present study, isolation of *M. gallinaceum* has shown the importance of pigeons in the transmission of Mycoplasma to poultry.

In another study, 18 and 32 Mycoplasma were recognized as *M. columbinum* and *M. columborale* from tracheas and oropharynxes of pigeons (Shimizu et al., 1978). Gharaibeh and Hailat (2011) reported low rate detection of *M. gallisepticum* by culture from pigeons. Several of the previous reports on *M. gallisepticum* infection in sparrows and pigeons have studied wild bird populations or captive flocks (Sinclair 1980; Stallknecht et al., 1982; Reece et al., 1986; Bencina et al., 1987; Tsai and Lee, 2006). Their findings indicated that pigeons may play a role in *M. gallisepticum* transmission between poultry farms as mechanical vectors or temporary biological carriers.

Ferreira et al. (2016) performed rapid serum agglutination test (RST) for the detection of antibodies against *Mycoplasma synoviae*, *Mycoplasma gallisepticum* in feral pigeons in Brazil. Antibodies were 3.3% positive for *M. synoviae* and 2.5% for *M. gallisepticum* (Ferreira et al., 2016).

Prevalence of mycoplasmosis in wildlife has reached an alarming level. These findings emphasize the urgent need for increased surveillance and control of mycoplasma in wildlife. Also, their findings show that host specificity of Mycoplasma could be reconsidered (Sumithra et al., 2016).

Based on the results of the present study, it would be necessary to screen large numbers of pigeons by using culture method and genetically for known poultry pathogenic mycoplasmas in order to determine their prevalence, and reveal the role of pigeons in the spread and maintenance of these organisms in the environment.

Acknowledgments

This research was funded by a grant (No. 21396) from the Research Council of the Ferdowsi University of Mashhad. We would like to thank Mr. Ali Kargar for his assistance in laboratory works.

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مجله طب دامی ایران، ۱۳۹۷، دوره ۱۲، شماره ۳، ۱۸۶-۱۸۱

جداسازی و شناسایی مولکولی گونه‌های مایکوپلاسما از کبوتر در شمال شرقی ایران

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(دریافت مقاله: ۳ دی ماه ۱۳۹۶، پذیرش نهایی: ۲۷ اسفند ماه ۱۳۹۶)

چکیده

زمینه مطالعه: مایکوپلاسما یکی از مهمترین عوامل پاتوژن تنفسی در طیور می‌باشد.

هدف: هدف مطالعه حاضر شناسایی گونه‌های مایکوپلاسمای جدا شده از کبوتران می‌باشد.

روش کار: شانزده نمونه تجمیعی تهیه و در محیط PPLO کشت داده شدند و نهایتاً DNA از تک کلونی‌های حاصله استخراج گردید.

نتایج: با تکثیر ژن، جنس مایکوپلاسما شناسایی گردید. میزان ۳۱٪(۵ تا از ۱۶) نمونه‌های تجمعی مثبت بودند که مایکوپلاسما کلومبور\له و مایکوپلاسما گالیناسئوم بودند.

نتیجه‌گیری نهایی: پایش تعداد زیادی از کبوتران برای شناسایی مایکوپلاسمای پاتوژن طیور جهت شناسایی نقش کبوتران در انتشار و بقای این ارگانیسم‌ها در محیط مورد نیاز است.

واژه‌های کلیدی: مایکوپلاسما، مایکوپلاسما کلومبور\له، مایکوپلاسما گالیناسئوم، PCR، کبوتر

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