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Short Communication

Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* among Commercial Poultry in Khouzestan Province, Iran

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ABSTRACT

Mycoplasmas are important avian pathogens, which can cause both respiratory disease and synovitis in poultry that result in considerable economic losses to the poultry industry all over the world. The aim of this study was to determine the prevalence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections among commercial poultry flocks in Khouzestan province, Iran, using the polymerase chain reaction (PCR) technique. Totally, 290 tracheal swab samples were collected from 19 broiler flocks and 4 layer-breeder flocks, with or without respiratory signs, in different areas of Khouzestan province within six months. The PCR tests were applied for the specific amplification of *16S rRNA* (185 bp) and *vlhA* (392 bp) genes. Out of 100 swab samples obtained from the layer-breeder flocks, 1 and 72 specimens were positive for *M. gallisepticum* and *M. synoviae*, respectively. In this regard, out of the 4 layer-breeder flocks, 1 (25%) and 4 (100%) flocks were positive for *M. gallisepticum* and *M. synoviae*, respectively. However, none of the studied broiler flocks were *M. gallisepticum*- or *M. synoviae*-positive. According to the results, the PCR technique could be concluded as a rapid method for the accurate identification of *M. gallisepticum* and *M. synoviae* infections in commercial poultry flocks. The results were indicative of the low prevalence of *M. gallisepticum* in the studied flocks in Khouzestan province. On the other hand, *M. synoviae* was widely distributed among layer-breeder flocks in this province.

Keywords: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Poultry, PCR, Khouzestan

Détection de *Mycoplasma gallisepticum* et *Mycoplasma synoviae* chez des volailles commerciales dans la province de Khouzestan, Iran

Résumé: Les mycoplasmes sont des pathogènes aviaires importants, qui peuvent provoquer à la fois des maladies respiratoires et des synovites chez les volailles. Ceci entraîne des pertes économiques considérables pour l'industrie aviairedans le monde entier. Le but de cette étude était de déterminer la prévalence des infections au*Mycoplasma gallisepticum* et *Mycoplasma synoviae* parmi les troupeaux de volailles commerciales dans la province iranienne du Khouzestan, en utilisant la technique de réaction en chaîne de la polymérase (PCR). Untotal de 290 échantillons d'écouvillons trachéaux a été prélevé à partir de 19 troupeaux de poulets de chair et 4 élevages de pondeuses avec ou sans signes respiratoires, dans différentes zones de la province de Khouzestan durant six mois. Les tests PCR ont été appliqués pour l'amplification spécifique des gènes de l'ARNr 16S (185 pb) et vlhA (392 pb). Sur 100 échantillons d'écouvillons obtenus à partir des troupeaux de pondeuses, 1 et 72

Spécimens étaient respectivement positifs pour *M. gallisepticum* et *M. synoviae*. De plus, sur les quatre troupeaux reproducteurs, 1 (25%) et 4 (100%) étaient respectivement positifs pour *M. gallisepticum* et M. *synoviae*. Cependant, aucun des troupeaux de poulets de chair étudiés n'était positif à M. gallisepticum ou à *M. synoviae. Selon les résultats, la technique PCR peutêtre considérée comme une méthode rapide pour l'identification précise des infections à M. gallisepticum et M. synoviae dans les élevages de volailles commerciales. Les résultats ont révéléla faible prévalence de M. gallisepticum parmiles troupeaux étudiés dans* la province de Khouzestan. D'autre part, M. synoviae infectait largement les troupeaux de pondeuses de cette province.

Mots-clés: *Mycoplasma gallisepticum, Mycoplasma synoviae,* Volaille, PCR, Khouzestan

INTRODUCTION

Mycoplasmas are bacterial agents that belong to the class *Mollicutes* and differ from other bacteria in their genetic inability to form a cell wall. This feature accounts for the pleomorphic and plastic shape of this genus and its resistance to antimicrobials that affect cell wall synthesis. *Mycoplasmas* characteristically form fried-egg-shaped microcolonies that grow into agar media. Although they have been considered as extracellular organism, researchers admit nowadays that some of them are obligatory intracellular microorganism, while all other *Mycoplasmas* are considered facultative intracellular agents (Markey et al., 2013). These organisms tend to be host-specific; accordingly, avian *Mycoplasmas* are not generally known to infect mammalian or other species. The most important *Mycoplasmas* for different avian species, especially chickens and turkeys, are *M. synoviae*, *Mycoplasma gallisepticum*, *M. meleagridis*, and *M. iowae*. Avian mycoplasmosis imposes considerable economic losses to the poultry industry around the world (Lierz et al., 2008). *M. gallisepticum* infection is usually determined as a chronic respiratory disease in chickens and infectious sinusitis in turkeys (Kempf et al., 1994). It is characterized by sneezing, coughing, snicks, rales, as well as nasal and ocular discharge. Although *M. gallisepticum* infections mainly affect the respiratory tract, they may occasionally cause arthritis, salpingitis, keratoconjunctivitis, and fatal encephalopathy (Haque et al., 2015). In breeders and layers, the disease causes a decline in egg production and an increase in embryonic mortality. In broilers, it leads to the reduction of feed conversion efficiency and weight gain, as well as the enhancement of mortality rate and condemnations in abattoirs. *M. gallisepticum* also causes some respiratory diseases, including sinusitis and conjunctivitis, in turkeys, ducks, geese, quails, partridges, pheasants, and other avian species (Avakian and Kleven, 1990). *M. synoviae* infection most frequently occurs as a subclinical upper respiratory tract infection. It may cause air sac disease described as a severe air sacculitis. The co-infection of *M. gallisepticum* or *M. synoviae* with respiratory virus infections, such as Newcastle disease, infectious bronchitis, or both can exacerbate the respiratory condition. It is usually known as infectious synovitis, an acute-to-chronic infectious disease of chickens and turkeys, which primarily involves the synovial membranes of the joints and tendon sheaths (Lockaby et al., 1998; Dufour-Gesbert et al., 2006). In chickens, turkeys, and other birds, a milder form of some of *M. gallisepticum* symptoms can be seen in *M. synoviae* infections, besides pale comb and head, lameness, as well as swollen hocks and foot pad. *M. synoviae* has a broader host range than other *Mycoplasmas*; in this regard, chickens, turkeys, and guineafowl are the natural hosts of the organism. Both *M. gallisepticum*- and *M. synoviae*-induced diseases are economically important and may be transmitted horizontally and vertically. Moreover, they can remain in the flock constantly as subclinical forms (Nascimento et al.,

2005). The success of control programs depends on the accurate and timely diagnosis of infected flocks. The diagnosis of *M. gallisepticum* and *M. synoviae* infections can be accomplished by means of various methods, such as the isolation and identification of the organism, serological assays to detect antibody production, and molecular tests. The gold standard test for the confirmation of diagnosis is the isolation and recognition of the organism. However, these techniques are expensive, difficult, and time-consuming and require sterile conditions. Serological tests for *M. gallisepticum* and *M. synoviae* include the haemagglutination inhibition test, rapid slide agglutination test, and enzyme-linked immunosorbent assays (ELISAs) (Kempf, 1998; Feberwee et al., 2005).

Polymerase chain reaction (PCR) techniques are interesting alternatives for the detection and analysis of DNA with a high specificity, sensitivity, and capability for a large scale of data, and economic viability (Nascimento et al., 1991). These tests have been used for the rapid detection and identification of *M. gallisepticum* and *M. synoviae* from cultures or directly from clinical samples without the need for isolation (Hess et al., 2007; Grodio et al., 2008). With this background in mind, the present study aimed to determine the prevalence of *M. gallisepticum* and *M. synoviae* among the commercial poultry flocks in Khouzestan province, southwest of Iran, using the PCR technique.

MATERIALS AND METHODS

Sampling. A total of 290 tracheal swab samples were randomly collected from 23 commercial poultry flocks, including 19 broiler flocks and 4 layer-breeder flocks (i.e., 10 samples from each broiler flock and 25 samples from each layer-breeder flock), with or without respiratory signs, in different areas of Khouzestan province during June to December 2015 (Table 1). The information recorded for the flocks in each farm included geographical area of flock, farm capacity, flock age, administration of any vaccination against *M. Gallisepticum* and *M. synoviae* in flock and their parent flocks, egg production rate, mortality rate, and respiratory symptoms, such as sneezing, coughing, rales, and nasal discharge. The samples were transferred to the Department of Microbiology, Shahid Chamran University of Ahwaz, in test tubes with PPLO broth medium and in special sterile ice-filled containers to reserve the organism and prevent swabs from drying out after sampling.

DNA extraction. DNA extraction was performed according to the previously described procedure (Liu et al., 2001). Briefly, the swabs were submerged into 3 mL of PPLO broth, then agitated on a vortex mixer for 30 sec. The swabs were discarded. After an over-night incubation of mediums at 37 ^{o}C, 1 mL of the culture was centrifuged at 13,000 rpm for 10 min, then the sediment was washed twice in 1 mL of phosphate buffered saline (pH=7.2) and re-suspended into 25 µL of the same buffer. Subsequently, the cell suspension was heated at 100 ^{o}C for 10 min, and then ice-cooled for 10 min. After cooling, the lysate was centrifuged at 13,000 rpm for 4 min. The supernatant containing DNA was collected and stored at -20^{o}C until further use.

Molecular assays. Following the protocol of Khalda et al. (2008), we used two forward and reverse *M. gallisepticum*-specific primers, amplifying a 185 base pair (bp) region of *16S rRNA* gene. *M. synoviae*-specific primers (i.e., *M. synoviaecons*-F and *M. synoviaecons*-R) had been already designed by Jeffery et al. (2007), amplifying a 392 bp region of *vlhA* gene. The primer sequences with their corresponding genes are presented in Table 2. Amplifications were carried out in a total volume of 25 µL containing 6 µL DNA template, 1.5 µL of each primer (10 picomol), 4 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM deoxynucleotide, 1 U of Taq DNA polymerase, 2.5 µL of 10x PCR buffer (all reagents were purchased from SinaClon Bioscience Co., Iran), and sterile distilled water. The reaction mixture was thermocycled (Eppendorf, Mastercycler Gradient, Germany) for 35 cycles, beginning with an

initial denaturation step at 95 °C for 5 min. The temperature and time profile of each cycle included 95°C for 45 sec(denaturation), 53ºC for 30 sec (annealing), and 72 °C for 30 sec (extension). The PCRs were finished with a final extension step at 72°C for 5 min. The separation of 10 µL aliquot of each PCR products was accomplished through electrophoresis (90 volts for 30 min) in an 1.5% agarose gel stained with 0.032 µL/mL safe stain. DNA fragments were visualized by UV transilluminator (UVitec, United Kingdom). The molecular size of the PCR products were compared with a 100 bp DNA ladder. We used the reference strains of *M. Gallisepticum* and *M. synoviae* as positive controls, which were obtained from *Mycoplasma* Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran, and sterile distilled water as negative control.

Statistical analysis. The data were statistically analyzed in SPSS software, version 19.0 (SPSS Inc., Chicago, IL). Descriptive statistics were computed to characterize the proportions of the isolation of different bacteria species in various areas and from different flocks. Furthermore, the Chi-square test was employed to compare the proportions.

RESULTS AND DISCUSSION

According to the results, none of the 19 (0.0%) broiler flocks were positive for *M. Gallisepticum.* However, regarding the layer-breeder flocks, one isolate (i.e., 25% of 4 layer-breeder flocks, 1% of 100 tracheal swabs from these flocks, and 0.34% of 290 swab samples) was *M. gallisepticum*-positive in PCR assay and produced the predicted 185 bp amplification product (Table 1, Figure 1).

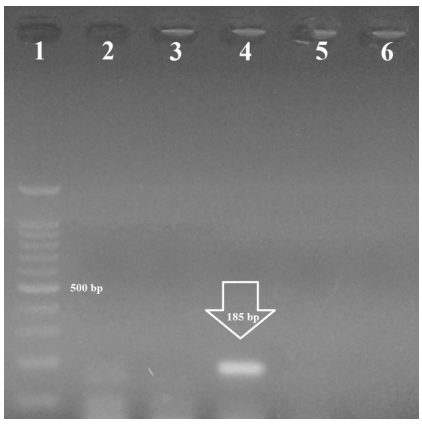

Figure 1. Electrophoresis of polymerase chain reaction products on 1.5% agarose gel stained with safe stain; lane 1) 100 bp molecular weight marker, lanes 2, 3, 5) negative samples, lane 4) *Mycoplasma gallisepticum*-specific185 bp band, lane 6) negative control.

In terms of *M. Synoviae*, none of the broiler flocks were positive. Nonetheless, 72 isolates from tracheal swabs obtained from four layer-breeder flocks (100% of 4 layer-breeder flocks, 72% of 100 tracheal swab samples from the layer-breeder flocks, and 24.8% of 290 swab samples) were positive for *M. synoviaepcr* and gave 392 bp products, similar to the positive control when visualized through electrophoresis (Table 1, Figure 2). These findings were indicative of the wide distribution of *M. Synoviae* among the layer-breeder flocks of Khouzestan province. In addition, the results revealed a significant difference between the prevalence of *M. Synoviae* and *M. Gallisepticum* in the layer-breeder flocks of the province (P<0.001). Statistical analysis showed no significant difference between age groups regarding the prevalence of *M. Synoviae* in the layer-breeder flocks. The current study was performed to determine the prevalence of *M. Gallisepticum* and *M. synoviae* infections in commercial poultry flocks in Khouzestan province using the PCR technique. As the findings indicated, *M. gallisepticum* had a low prevalence in the studied flocks, and only one positive case was detected in the layer-breeder flocks.

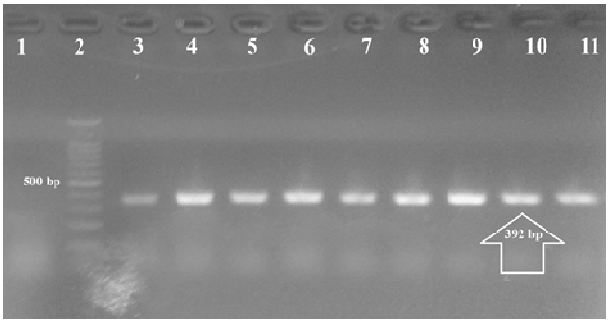

Figure 2. Electrophoresis of polymerase chain reaction products on 1.5% agarose gel stained with safe stain; lane 1) negative

control, lane 2) 100 bp molecular weight marker, lane 3) positive control, lanes 4-11) *Mycoplasma synovia*-specific 392 bp band.

On the other hand, the prevalence of *M. Synoviae* was high among the layer-breeder flocks. The low prevalence of *M. gallisepticum* in layer-breeder flocks could be due to the intense control of these flocks against this agent, which has been conducted for several years. Our findings showed that *M. Synoviae* was widely distributed among the layer-breeder flocks. There was a significant difference between the rate of *M. synoviae* isolation from understudy commercial poultry flocks of the province (P<0.001). The high prevalence of *M. synoviae* in these birds is probably due to the fact that the vaccine against this species is uncommonly used, and none of the studied farms made use of it. There were no significant difference between the age groups and respiratory problems regarding the prevalence of *M. Synoviae* in the layer-breeder flocks. Considering the already acknowledged vertical transition characteristics of mycoplasmosis (Nascimento et al., 2005), the *Mycoplasma* prevalence rate of 73% in the layer-breeder flock can imply the continuous dissemination of this infectious agent in the subsequent chicks, which amplifies the disease incidence and the following economic losses. There are studies reporting different prevalence rates from the rates obtained in the present study. In this regard, in a study conducted by Buim et al. in 2009, *Mycoplasmas* was isolated from 72.7% of poultry flocks in Brazil by multiplex PCR with the predominance of *M. Synoviae*. Moreover, in another research performed by Köhn et al. in 2009, 84% of commercial layer flocks during laying period were positive for *Mycoplasmas* in PCR test; in this regard, 75% of them turned out positive for *M. Synoviae*, and all were negative for *M. Gallisepticum*. On the other hand, in 2013, Rachida et al. (2013) detected the prevalence rates of 35.5% and 18.5% for *M. Gallisepticum* and *M. Synoviae* in laying hens in Algeria, respectively. In a study carried out in Germany, Lierz et al. (2008) reported that none of the tracheal swabs obtained from healthy captive and free-ranging birds of prey were positive for *M. Gallisepticum* and *M. Synoviae* by PCR assay. In line with the present study, Feberwee et al. (2008) showed that *M. synoviae* had the seroprevalence rate of 73% in commercial poultry flocks in Dutch based on serum plate agglutination test. Similarly, in a study conducted by Seifi and Shirzad () 2012in Iran, the results of serum plate agglutination and ELISA tests revealed that the seroprevalence of *M. Synoviae* in broiler breeder flocks was 47.8%. Furthermore, Saâdia et al. (2014) demonstrated that *M. synoviae* had the highest frequency (16th week: 64%, 32th week: 82%, and 56th week: 100%) in the tracheal swabs collected from broiler breeder flocks in Morocco by PCR assay. Additionally, in 2009, Suzuki et al. reported the *M. synoviae* seroprevalence rate of 53% based on ELISA test. Inconsistent with our findings, in a study carried out in Turkey, Yilmaz and Timurkaan (2011) showed that the presence of *M. synoviae* antigens in pneumonic broiler chicken lungs was 4% by immunohistochemical method. Similarly, Sarkar et al (2005) and Barua et al. (2006) reported that the seroprevalence rates of *M. Gallisepticum* in different

Table 1. Results of identification of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in 290 tracheal swab samples collected from 23 commercial poultry flocks in Khouzestan province, Iran during June to December 2015 by polymerase chain reaction technique

Area (city)	Number of flocks	Flock size	Age (days)	Condition		Number of sampled	Number of positives (%)	
				Normal	With respiratory signs		*M. gallisepticum*	*M. synoviae*
Broiler flocks								
Dezfoul	1	20000	42	■	□	10	0	0
Ahvaz	2	25000	48	□	■	10	0	0
	3	36000	45	■	□	10	0	0
	4	20000	42	■	□	10	0	0
	5	20000	50	■	□	10	0	0
Andimeshk	6	25000	42	■	□	10	0	0
	7	36000	45	□	■	10	0	0
Shadeghan	8	20000	48	■	□	10	0	0
	9	10000	42	■	□	10	0	0
Masjed-soleyman	10	10000	49	■	□	10	0	0
Laaly	11	20000	44	□	■	10	0	0
	12	10000	45	■	□	10	0	0
Baghmalek	13	20000	45	□	■	10	0	0
Behbahan	14	20000	42	■	□	10	0	0
	15	32000	48	■	□	10	0	0
Hendyjan	16	30000	46	■	□	10	0	0
RaM. synoviaehyr	17	25000	50	□	■	10	0	0
Dasht-azadeghan	18	20000	45	■	□	10	0	0
Maahshahr	19	10000	46	■	□	10	0	0
Total						**190**	**0 (0.0%)**	**0 (0.0%)**
Layer-breeder flocks								
Ahvaz	20	5000	420	■	□	25	1 (4%)	16 (64%)
Ahvaz	21	5000	420	□	■	25	0	24 (96%)
Ahvaz	22	5000	84	■	□	25	0	10 (40%)
Ahvaz	23	5000	84	□	■	25	0	22 (88%)
Total						**100**	**1 (1%)**	**72 (72%)**
						290	**1 (0.34%)**	**72 (24.8%)**

areas of Bangladesh were 58.9%, 49.5%, and 66.5% in breeder, broiler, and layer poultry farms, respectively. Additionally, Haghighi-Khoshkhoo et al. (2011) showed that 6.2% and 18.5% of serum samples collected from commercial layer flocks in the north of Iran were positive for *M. Gallisepticum* and *M. Synoviae*, respectively. Based on a study performed by Hosseini et al. (2012) in Mazandaran province, Iran, 10 (31.2%) out of 32 trachea and air sac samples of poultry flocks were found to be infected with *M. Synoviae* by PCR test. In addition, in a research carried out by Tebyanian et al. (2014), 24.5% of lung and trachea samples collected from the slaughtered ostriches in Kerman province were positive for *M. Synoviae.*

According to the results of the current study, *M. gallisepticum* had a low prevalence in the studied flocks in Khouzestan province. On the other hand, *M. synoviae* was found to be widely distributed among layer-breeder flocks in the district under investigation. These findings indicated that the PCR technique could be a rapid and accurate method for the identification of *M. Gallisepticum* and *M. Synoviae* infections, especially in commercial poultry flocks.

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Table 2. Sequences of primers and sizes of polymerase chain reaction products of the oligonucleotide primers used for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synovia*

Organism	Primer	Sequence	Amplicon size (bp)	Reference
Mycoplasma gallisepticum	*16S rRNA*	5' AGCTAATCTGTAAAGTTGGTC 3' 5' GCTTCCTTGCGGTTAGCAAC 3'	185	15
Mycoplasma synovia	*vlhA*	5'TACTATTAGCAGCTAGTGC 3' 5'AGTAACCGATCCGCTTAAT 3'	392	12

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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