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Prevalence study of *Mycoplasma synoviae* in broiler flocks with lameness in Shahrekord and Isfahan, Iran

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ABSTRACT

Identification of *Mycoplasma synoviae* (*M. synoviae*), an economically important pathogen in poultry industries worldwide, in clinical samples has critical importance. This study was conducted to determine the prevalence of *M. synoviae* in broiler flocks with clinical lameness using serological and polymerase chain reaction (PCR) methods. Blood serum samples (200) and joint fluid samples (200) were collected from 20 broiler flocks. All serum samples were studied using the serum plate agglutination (SPA) test while all joint fluid samples were studied using the PCR technique for detection of vIhA gene of *M. synoviae*. Results showed that 61 serum samples from 11 broiler flocks were positive for SPA test while 42 out of 61 samples were positive using SPA with 1/2 dilution. The PCR method indicated that 102 joint fluid samples of 16 broiler flocks were positive for presence of the *vIhA* gene of *M. synoviae*. Our results showed that the SPA and PCR techniques can be used for determination of the prevalence of *M. synoviae* in clinical samples of poultry.

Key words: Chicken, Iran, *Mycoplasma synoviae*, PCR

Mycoplasmas are Gram-positive bacteria lacking a cell wall. They belong to the class Mollicutes and have a 580 kb to 1,358 kb genome (Nascimento *et al.* 2007). Avian mycoplasmas occur in a variety of bird species with most important mycoplasmas for chickens being *Mycoplasma gallisepticum* (*M. gallisepticum*) and *Mycoplasma synoviae* (*M. synoviae*). Transmission occurs vertically from parents to their offspring, through contamination of eggs (transovarian transmission) (Nascimento *et al.* 2007, Ley 2008). *Mycoplasma synoviae* infection can cause considerable economic losses in chickens such as subclinical upper respiratory infection, air sac lesions, infectious synovitis, bursitis, lameness, pale combs and heads, swollen hocks and foot pads, drop in egg production and increase in embryo mortality (Stipkovits and Szatmari 2009). Acutely affected birds may show green feces, but respiratory infections caused by *M. synoviae* are usually asymptomatic (Kleven and Noel 2013). Therefore, accurate diagnosis of *M. synoviae* cannot be made clinically.

There are various methods for diagnosis of mycoplasma species. Culture methods were developed for detection of *Mycoplasma* species, but they are time-consuming and expensive. Tentative diagnosis and monitoring of *M. synoviae* infection is usually made using serological assays, while definitive diagnosis is by isolation and identification of the organism. Diagnosis of bacterium using serological tests is time-consuming and occurrence of cross contamination is inevitable, but some studies report the high sensitivity and specificity of some serological methods like the serum plate agglutination (SPA) test (Ley 2008). Several studies showed that molecular technique like polymerase chain reaction (PCR) is a rapid and accurate technique for definitive identification of *Mycoplasma* species in clinical samples (Stanley and Kleven 2008). The present study determined the prevalence rate of *M. synoviae* in broiler poultry farms using PCR and SPA methods in central cities of Iran.

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MATERIALS AND METHODS

Sample collection: In the winter of 2011, broiler flocks (20) in the Shahrekord and Isfahan (central cities of Iran) were studied for prevalence of *M. synoviae*. The population of each flock ranged from 10,000 to 20,000 chickens. All chickens were 30 or more days of age until 60 days old. All the chickens sampled were lame, which is often associated with *M. synoviae* infection. There were no signs trauma in selected chickens. Also, there was no nutritional factor which could be causing lameness in selected chickens. Blood samples (200) were collected from these birds and sera prepared by centrifugation. After euthanization, 200 joint fluid samples were collected using sterile needle with gauge 22. All samples were transferred immediately to the laboratory in a cooler with ice pack within a day of collection.

Serological method: The serum plate agglutination (SPA) test was done with crystal violet stain *M. synoviae* antigen. For this test, 30 µl of antigen and 30 µl of sera samples were placed side by side with a sampler on a glass plate and mixed by stirring with a small tooth pick followed by gentle rocking. The agglutination end point was determined by preparing 2-fold (1/2) dilutions of sera in saline. Results were read within 2 minutes. In positive cases, agglutination formed slowly, which was seen during rocking, but in negative case no such agglutination formed within 2 minutes. Sera that reach to 1/8 or higher levels are considered positive, which may also be useful in differentiating between specific and non-specific reactions (Awatef *et al.* 2010).

DNA extraction and PCR method: Purification of DNA from joint fluid samples was achieved using a Genomic DNA purification kit according to the manufacturer's instructions. The primers for amplification of *vIhA* gene were *MS cons-F* (5'-TACTATTAGCAGCTAGTGC-3') and *MS cons-R* (5'-AGTAACCGATCCGCTTAAT-3') (van Eck *et al.* 1980). The PCR was performed using DNA engine thermo cycler in 25 µl PCR reaction mix consisting of 2.5 µl of 10× PCR buffer, 4 µl of 1.5 mM $MgCl_2$, 0.5 µl of 200 mM dNTPs, 0.1 µl of each of *MS cons-F* and *MS cons-R* primers (20 µM each), 0.25 µl Taq DNA polymerase (2.5 U/µl), 16.55 µl of deionized distilled water and 1 µg/µl of template DNA in a 25 µl PCR reaction. All amplification reaction were performed in a gradient mastercycler termocycler using the following temperatures and time cycles. After denaturation at 94°C for 4 min, the first reaction was performed in 32 cycles with denaturation (94°C for 1 min), annealing (58°C for 1 min), and primary extension (72°C for 1 min), and a final extension at 72°C for 7 min. The PCR products were detected by electrophoresis in 2% agarose gel in TAE buffer. Gels were run using 15 µl of final PCR products for 90 min at 60V, stained with ethidium bromide, along with 100 bp DNA ladder.

In this study, MS vaccine was used as positive control and sterile water was used as negative control.

Table 1. Results of SPA and PCR for investigating the distribution of *M. synoviae* in blood serum joint fluids and of broiler poultry farms in central cities of Iran

Flock number	Number of sample	Positive SPA (%)	Positive SPA (1/2 Dilution) (%)	Positive PCR (%)
1	10	5(50)	4(40)	8(80)
2	10	6(60)	5(50)	8(80)
3	10	6(60)	5(50)	8(80)
4	10	0(0)	0	0
5	10	5(50)	4(40)	7(70)
6	10	0(0)	0	4(40)
7	10	0(0)	0	0
8	10	0(0)	0	4(40)
9	10	7(70)	6(60)	8(80)
10	10	0(0)	0	4(40)
11	10	3(30)	0	6(60)
12	10	0(0)	0	0
13	10	0(0)	0	4(40)
14	10	5(50)	4(40)	8(80)
15	10	7(70)	5(50)	8(80)
16	10	6(60)	5(50)	8(80)
17	10	0(0)	0	0
18	10	0(0)	0	2(20)
19	10	5(50)	4(40)	6(60)
20	10	6(60)	0	9(90)
Total flocks	Total sample	Total positive sample	Total positive sample	Total positive sample
20	200	61 (30.5%)	42 (21%)	102 (51%)

RESULTS AND DISCUSSION

vIhA gene was amplified in positive samples (Fig. 1). In this study, a sample in 1 flock was positive, that flock was identified as positive. Sera samples (61) in 11 flocks were positive using the SPA method. Besides, 40 sera of 61 samples were positive using the SPA with 1/2 dilution. After the PCR technique, it was recognized that 102 samples in 16 flocks had a bandwidth 400-bp size, which were considered as positive for presence of *M. synoviae* (Table 1).

This study illustrated that *M. synoviae* infection had a high prevalence among broiler flocks with clinical lameness. Our results showed that from 20 flocks which had lameness, 11 flocks were diagnosed positive using the SPA method while 16 flocks were diagnosed positive using the PCR technique. Therefore, our results indicated that the PCR method was more accurate than the SPA test for detection of *M. synoviae* in clinical samples. Although respiratory infections caused by *M. synoviae* are generally considered being subclinical (Morrow *et al.* 1990), many reports have documented economic losses attributable to respiratory infections (Lockaby *et al.* 1998). Our study showed that the lameness can be economically devastating because results suggest that the majority numbers of lameness in flocks may be due to *M. synoviae*. Sixteen flocks with lameness background were positive for *M. synoviae* and only 4 flocks with the history of lameness were free of *M. synoviae* according to SPA and PCR testing. Previous study on commercial layer flocks in the north in Iran showed that 17 of 40 (42.5%) flocks were positive and 23 of 40 (57.5%) were negative for presence of *M. synoviae* (Haghighi-Khoshkhoo *et al.* 2011).

Another study showed that the prevalence of *Mycoplasma* species in poultry is different in various seasons (the highest prevalence in winter and the lowest in summer) (Barua *et al.* 2006). Another study in India confirmed the seasonal pattern for prevalence of mycoplasmosis in poultry farms (Chakraborty *et al.* 2001). A study showed that the *M. synoviae* had the higher prevalence than *M. gallisepticum* in poultry farms (Kleven and Noel 2013).

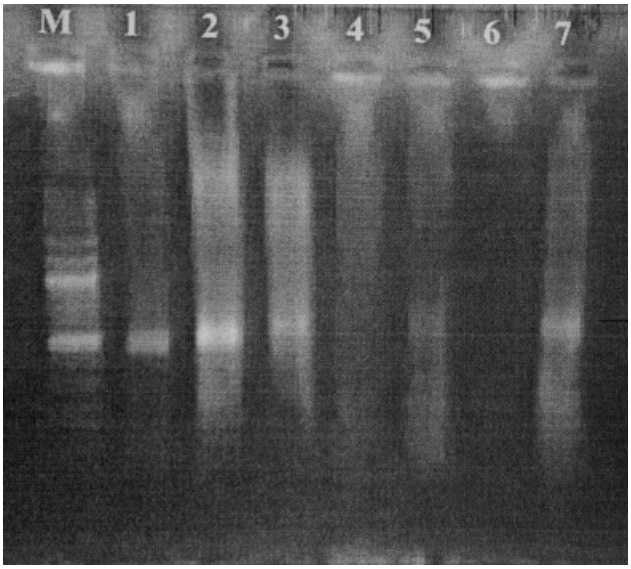

Fig. 1. Gel electrophoresis of PCR product. Column, Marker 100 bp; column 1, positive control; column 2, 3 and 7, positive samples; column 4 and 5, negative samples; column 6, negative control.

One study in Paraguay showed that 55% seropositivity of *M. synoviae* in chickens using the ELISA test (Suzuki *et al*. 2009). The ELISA technique showed that 84% of commercial layer flocks during laying period were positive for mycoplasmas in Germany, while 75% turned out positive for *M. synoviae* and all were negative for *M. gallisepticum* (Kohn *et al.* 2009). Results from a Dutch study showed that the seroprevalence of *M. synoviae* in commercial poultry was 73% using SPA test (Feberwee *et al.* 2008). Another study in Sebria indicated that overall seroprevalence of the *M. gallisepticum* and *M. synoviae* in the poultry flocks was 9.01 and 47.49% in 2000 and was 11.59 and 22.17% in 2009 by SPA and ELISA tests, respectively (Kapetanov *et al.* 2010). The age of flocks which we chose was 30 days or more. A study showed that the age of the poultry flocks has a dominant role on prevalence of mycoplasmosis (Botus *et al.* 2008).

In this study, we used both SPA and PCR tests for detection of *M. synoviae* in broiler poultry farms. The SPA test is quick, relatively inexpensive and sensitive. Moreover, it can be widely used as an initial screening test for flock monitoring and serodiagnosis. The SPA is more sensitive than ELISA and HI, but less specific than them (Ley 2008). Serological screening is relatively inexpensive; nevertheless, it is not always sufficiently specific or sensitive, while cultures can be insensitive and time consuming (Awatef *et al.* 2010).

Earlier *M. synoviae* specific PCR*s* were based on the 16S rRNA gene (Awatef *et al.* 2010), and more recently some have been based on haemagglutinin genes (Kleven and Noel 2008). The PCR and DNA sequence analysis of the N-terminal end of the hemagglutinin encoding *vlhA* gene were used as an alternative method for detection and initial typing of field strains of *M. synoviae* in commercial poultry (Kleven and Noel 2008).

From this study, it can be concluded that *M. synoviae* infection had a high prevalence among broiler flocks with clinical lameness. Also, our study showed that the lameness can be economically devastating, because results suggest that the majority numbers of lameness in flocks may be due to *M. synoviae*. The most effective method of control can be regular flock monitoring and elimination of positive breeder flocks and this can be achieved by using accurate and reliable diagnostic assays. Overall, it is suggested that the PCR could be an alternative method for accurate identification of the *M. synoviae* infection in broiler chicken flocks.

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