Isolation and Identification of *Mycoplasma synoviae* From Suspected Ostriches by Polymerase Chain Reaction, in Kerman Province, Iran

Hamid Tebyanian [1]; Seyed Hanif Mirhosseiny [1]; Babak Kheirkhah [1,*]; Mehdi Hassanshahian [2]; Hamze Farhadian [1]

[1]Department of Microbiology, Kerman Science and Research Branch, Islamic Azad University, Kerman, IR Iran
[2]Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, IR Iran

Corresponding author: Babak Kheirkhah, Department of Microbiology, Kerman Science and Research Branch, Islamic Azad University, Kerman, IR Iran. Tel: +98-9133454787, Fax: +98-3412818815, E-mail: babakkheirkhah@yahoo.com

Background: *Mycoplasma synoviae* is an important avian pathogen which can cause both respiratory disease and synovial joint inflammation (*synovitis*) in poultry. *Mycoplasmas* spp. may cause the respiratory system infection in ostriches with symptoms such as inflammation of the nose, trachea and also damages of lungs.

Objectives: The current study aimed to use the *M. synoviae* specific Polymerase Chain Reaction (PCR) and microbiological methods in order to isolate and identify *M. synoviae* from suspected ostriches in Kerman Province, Iran, and compare the two methods (microbiological and PCR) employed to confirm Mycoplasmal contamination of ostrich lungs.

Materials and Methods: Fifty three samples of different parts of lung and trachea were immediately collected after slaughtering the ostriches in Kerman Province six months. Samples were cultured in the same conditions in pleuropneumonia-like organism (PPLO) broth and to isolate and identify *M. synoviae*, PCR and microbiological methods were conducted. The identified isolates were confirmed by specific amplification of 16S rRNA gene (163 and 207 base pair).

Results: In the current study, 25 and 17 out of 53 ostrich samples were identified as *Mycoplasma*-positive in the PCR and microbiological methods, respectively; and 13 out of 25 the mentioned *Mycoplasma*-positive samples were also confirmed by PCR method.

Conclusions: The current study showed that PCR method is time consuming, effective, and efficient method to detect *M. synoviae* infection in ostriches. PCR method could be recommended as an alternative for culturing; *M. synoviae* was isolated from ostriches for first time in Kerman Province, Iran.

Keywords: Mycoplasma synoviae; PCR; Microbiological methods

1. Background

Mycoplasmas are very small prokaryotes, devoid of cell walls and bounded only by a plasma membrane. *Mycoplasma synoviae* is one of the most important pathogenic chickens *Mycoplasmas*. *M. synoviae* infection occurs as a subclinical upper respiratory infection. It may cause air sac lesions when combined with Newcastle Disease (ND), infectious bronchitis (IB), or both. Less frequently, *M. synoviae* becomes systemic and results in infectious *synovitis*, acute to chronic infectious disease of chickens and turkeys, involving primarily the synovial membranes of joints and tendon sheaths, and bursitis. Lameness and respiratory disorder caused by this infection results in decreased growth rate and loss of egg production. As the vertical transmission plays a major role in the spread of *M. synoviae* in chickens, the most effective control method is to monitor the flocks regularly and also eliminate the positive breeder flocks. Reliable and rapid diagnoses are needed to prevent infection dissemination (1). *Mycoplasmas* are consisted of wall-less prokaryotes which are the smallest fastidious bacteria and unusually have small genomes (2).

M. synoviae belongs to the *Mycoplasmataceae* family, and is a facultative anaerobic bacterium (3). Other important *Mycoplasmas*, namely *M. mycoides*, with large colonies and *M. capricolum,* are claimed to cause similar syndromes (4, 5). The disease occurs mainly in the Mediterranean region, but is also reported in many other areas of the world (6) including Iran. The Polymerase Chain Reaction (PCR) with *Mycoplasma* 16S ribosomal RNA has been applied to detect a variety of *Mycoplasma* species (6, 7). Computer alignment studies of these rRNA sequences which have revealed the existence of regions with highly conserved sequences and zones which display sequence variability at the genus and species levels, allow the selection of genus and species specific primers for PCR (8). The rRNA is naturally present in high copy numbers (up to 10'000 molecules per cell) (9). The PCR method has been applied to detect a variety of *Mycoplasma* species that cause other *Mycoplasma* diseases in some research works (6, 7, 10, 11).

M. synoviae has a broader host range than the other *Mycoplasmas*. Chickens, turkeys, and guinea fowl are the natural hosts. Several other species have been naturally infected, and others have been infected by artificial inoculation. *Mycoplasmas* may be transmitted horizontally, through infectious aerosols coughed and sneezed by infected birds and through contaminated feed, water, personal contact and communication with animals, mainly birds. Transmission occurs vertically from parents to their offspring, through contamination of laid eggs (transovarian transmission). *Mycoplasma* is commonly transmitted within species and/or between closely related species, that is, they are host-specific, with rare exceptions (12, 13).

2. Objectives

The current study aimed to isolate and identify *M. synoviae* from suspected Iranian ostrich samples by PCR and microbiological methods in Kerman Province, and the obtained results to confirm *Mycoplasmal* contamination of ostrich lung.

3. Materials and Methods

3.1. Mycoplasmas, Isolation and Enrichment

Fifty three suspected samples were collected by multiple random sampling of different parts of ostrich lungs within six months, stored at 4°C and transferred to the Reference *Mycoplasma* Laboratory in Razi Vaccine and Serum Research Institute, Karaj, Iran for examination. Overall, 53 samples were placed into the PPLO broth as transport medium on ice packs and were transferred to laboratory within 12 hours (14).

3.2. Bacteriology

For primary isolation, the specimens were diluted and filtered by 0.45μL syringe filter (Nunk, Denmark) in fresh PPLO broth and inoculated onto PPLO agar medium (BBL, Becton Dickinson and company Cockeyville, Sparks, MD, USA). The media was supplemented with 12% of equal volumes of inactivated horse or swine sera. Thallium acetate (1:4000) and penicillin (Roche company, Germany) (1000 IU/mL) as fungal and bacterial inhibitors, nicotinamide adenine dinucleotide (NAD) (Roche company, Germany) (1:10000 w/v) as a necessary requirement of *M. synoviae*, and cysteine hydrochloride (1:10000 w/v) (Roche company, Germany) as a reducing agent for NAD were also added to the media. Inoculated broth and agar media were incubated under microaerophilic condition (7% CO_2) at 37°C with 98% relative humidity and checked for color change of broth and typical *Mycoplasma* colonies on agar. As soon as the phenol red indicator changed to yellow, the subculture onto the fresh broth and agar were carried out (7, 15). Several passages until 21 to 28 days were subcultured. Identification of the *M. synoviae* samples was carried out by serological methods, after appearing the specific colonies of *Mycoplasma*.

3.3. Agglutination Test

Suspension of the suspected colony was made in drops of saline on slide. The slide was discarded if auto-agglutination occurred or the suspension was rough in saline. A drop (approximately 50 μL) of *M. synoviae* specific antiserum (Material No. 10100761) (SPAFAS Canada) was added and mixed to one drop (approximately 50 μL) of smooth suspension. If agglutination was observed within one or two minutes, the isolate was considered as *M. synoviae* (14).

3.4. DNA Extraction

DNA was extracted from the enriched samples, according to Kojima et al. protocol (7). As per European Pharmacopoeia 2005, negative and positive controls were PPLO broth media and a standard strain of *M. synoviae* (MS-NCTC 10124-05), respectively. About 500 μL of the samples were placed in a 1 × 5 μL Eppendorf tube (Roche Company, Germany) and micro centrifuged at 13000 rpm for 15 minutes. Then, 100 μL of lyses buffer was added to 100 μL of precipitate, which the tube was placed in a 56°C bath for four hours. Then, 200 μL saturated phenol was added and the tube was centrifuged at 13000 rpm for 20 minutes.

The supernatant was transferred to another tube and an equal volume of mixed phenol/chloroform (1:1) was added. After centrifuging at 13000 rpm for 20 minutes, the supernatant was transferred to another tube and an equal volume of pure chloroform was added, and then, centrifuged at 13000 rpm for five minutes. The supernatant was transferred to a new tube, mixed with 1/10 volume of 3 M acetate sodium and was precipitated in -20°C with two-fold cool and pure ethanol for 20 minutes. Next, the tube was centrifuged at 13000 rpm for 15 minutes, then 200 μL of 70% ethanol was added and the tube was centrifuged at 13000 rpm for five minutes, and finally the DNA was dried and re-suspended at 4°C to be used for PCR (4, 7).

3.5. Performing Polymerase Chain Reaction

According to the protocol of Kojima et al., two forward and reverse *Mycoplasma specific* primers (7), which amplify a 163 base pair region of 16S rRNA gene, were used. *M. synoviae specific* primers (MS-1 and MS-2) had been already designed by Lauerman et al. (5) who amplified a 207bp region of lipoprotein gene. The primers sequences with their corresponding genes are shown in Table 1.

Amplifications were carried out in a total volume of 25 μL containing 17.5 μL DNA, 0.1 μL of each primer, 0.5 μL dNTP mix (10 mM) (Cinna Gen Inc.), 4 μL Mgcl2 (25 mM) (Cinna Gen Inc.), 2.5 μL PCR buffer (10X) (Cinna Gen Inc.) and 0.25 μL Tag DNA polymerase (5 units/μL) (Cina Gen Inc.). The reaction mixture was thermocycled (Eppendorf, Germany) for 34 cycles, beginning with an initial denaturation step of minimum 95°C. The temperature

and time profile of each cycle was as follows: 94°C for one minute (Denaturation), 55°C for one minute (Annealing) and 72°C for one minute (Extension), while PCRs were finished with a final extension step at 72°C for five minutes. PCR products were stored at 4°C. PCRs were carried out using two programmable thermocycler apparatuses (Primus and Master gradient). Positive (MS-H vaccine strain) and negative (PPLO broth media) controls were included in all tests (4, 7, 16).

3.6. Amplicon Electrophoresis

An 8 μL aliquot of each PCR products was mixed with 2 μL loading buffer (6X). The PCR products and 100 bp DNA ladder were separated by electrophoresis (Apelex, France) in a 1% agarose gel (Agarose MP, Roche) with 0.5 μL/mL ethidium bromide (100 volts for one hour) following illustration under UV transilluminator and then photographed (Visi-Doc-It system, UVP, UK) (7).

3.7. Specificity and Sensitivity of the PCR Test

The sensitivity of the PCR procedure was carried out by testing the DNA extractions of serial log dilutions of bacterial suspension of MS. The specificity of the PCR was confirmed by testing the extracted DNA of *M. gallisepticum*, *Salmonella entritidis*, and water (14).

4. Results

4.1. Bacteriology

In the current study, 17 out of 53 ostrich samples were found *Mycoplasma*-positive. Figure 1 shows the colonies of MS, after confirmation by PCR.

4.2. Polymerase Chain Reaction

The growth of *Mycoplasmas* in PPLO broth media was demonstrated by changes in color or turbidity due to biochemical activity and metabolism of the bacteria. Fifty-three suspected samples were evaluated by PCR test. *Mycoplasma*-positive isolates were detected in 25 out of 53

Table 1. Nucleotide sequences and Primers Used for Identification of *Mycoplasma synoviae*

Target Gene	Sequence	Length (bp)	Reference
16S rRNA		163	(7)
M1-F	F: 5/-GCTGCGGT-GAATACGTTCT-3/		
M2-R	R: 5/-TCCCCACGTTCTC-GTAGGG-3/		
16S rRNA		207	(5)
MS-1	F: 5/-GAAGCAAAATAGT-GATATCA-3/		
MS-2	R:5/-GTCGTCTCCGAAGT-TAACAA-3/		

ostrich samples in the PCR method, and also all isolations showed a specific amplicon at 163bp to the *Mycoplasma* genus (Figure 2). All *Mycoplasma*-positive samples were analyzed for *M. synoviae* by PCR method; out of 25 *Mycoplasma*-positive samples, 52% were identified as *M. synoviae* (Figure 3). Thirteen out of 25 *Mycoplasma*-positive samples were also positive for *M. synoviae* species. On the other side, 17 out of 53 ostrich samples were identified positive in the microbiological method. The results of the PCR and microbiological tests are presented in Table 2.
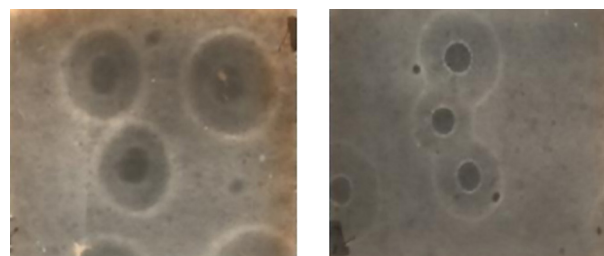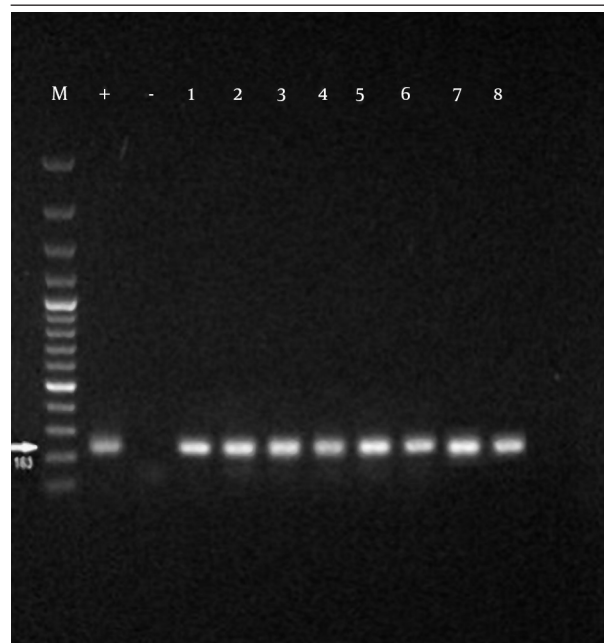

Figure 1. Colonies of *Mycoplasma synoviae* on the PPLO Agar (X 40)

Table 2. Distribution of Samples for *Mycoplasma*-PCR, *M. Synoviae*-PCR and Microbiological Results

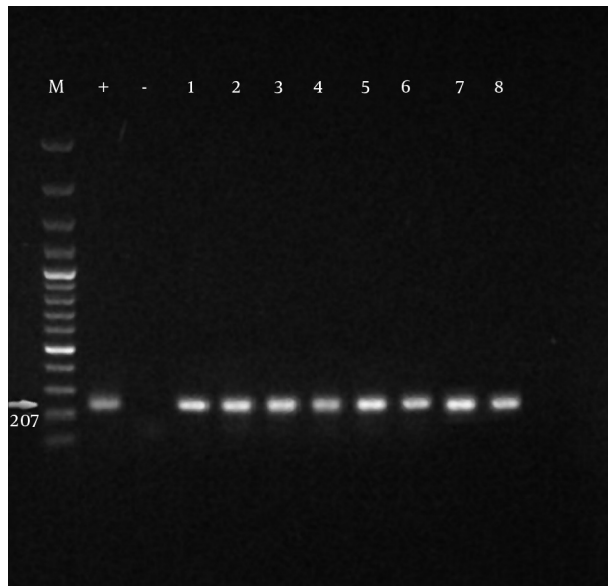| Control	Mycoplasma-PCR, No. (%)	M .synoviae-PCR, No. (%)	Microbiologic, No. (%)
+	25 (47)	13 (52)	17 (32)
-	28 (53)	12 (48)	36 (68)
Total	53 (100)	25 (100)	25 (100)

Figure 2. Specificity of the PCR Assay Using the Specific Primers

M, marker 100 bp; PC (+), positive control [*M. synoviae* (MS-NCTC 10124-05)]; NC (-), negative control; 1 to 8 suspected samples;163bp bands were formed in positive samples.

Figure 3. Specificity of the PCR Assay Using the Primers

MS-1 and MS-2; M: marker 100 bp; PC (+): positive control (*M. synoviae* (MS-NCTC 10124-05)); NC (-): negative control. Positive *Mycoplasma* sp. samples; 207 bp bands were formed in positive species.

5. Discussion

In the current study, *M. synoviae* species was isolated and identified from ostrich samples by PCR and microbiological methods in Kerman Province, Iran. *M. synoviae* infection among slaughtered ostriches was truly confirmed. Efficient *M. synoviae* microbiological and molecular methods are necessary for diagnosis, eradication and research goals of this important poultry pathogen. Although respiratory infections with *M. synoviae* are generally considered sub clinical, an increasing number of reports have documented economic losses related to respiratory infections and arthropathic strains (14, 17). The eggshell pathology and the concomitant egg production losses that result from *M. synoviae* infection, further highlight the economic significance of these bacteria in commercial poultry (18). In the current study, the most specific microbiological and PCR methods, without false negative, were set up and established. The *M. synoviae* infection in ostrich samples was confidently confirmed. Although serological screening is still widely used, it may not detect subclinical *M. synoviae* infections, and monitoring programs that depend solely on detecting seroconversion may be inadequate and nonspecific (14).

Traditionally, *M. synoviae* has been diagnosed by serological screening or microbiological method followed by identification of the *Mycoplasma*. Serological screening is relatively inexpensive but not always sufficiently specific or sensitive, while microbiological tests can be insensitive and time consuming (17). Detection of *M. synoviae* by microbiological methods was not reported from Kerman Province slaughtered ostriches before the current report. The current study successfully isolated 17 *M. synoviae* species by microbiological method. *M. synoviae* culture and isolation are not easy and almost are not accurate in all the Iranian poultry laboratories. Sample collection is important because *M. synoviae* tend to disappear from the lesions after a few weeks. Since *M. synoviae* die rapidly at room temperature, chilling the specimens during transportation should be considered. To control the bacterial and fungal contamination of samples, resistant antibiotic resistance as well as filtration should be considered. Microbiological method is needed for some research projects and even for diagnosis; to prepare the PCR samples, many false negative PCR results might occur without enrichment (19); therefore, culturing should not be ignored. But culturing can be costly and time-consuming, and can also be inconclusive because of low sensitivity (20). In the current study, microbiological tests had some limitations such as: ostrich slaughter was not widely performed, suspected samples were hardly collected, and *M. synoviae* isolation was not so easy.

There are few works on isolation and molecular identification of *Mycoplasmas*. In the Fars Province of Iran, Ghaleh Golab et al. isolated and detected *M. smagallicepticum* in chicken farms using PCR and RFLP assays and microbiological methods (21). Hosseini et al. analyzed the *M. gallisepticum* isolates of different geographical areas of Iran by random amplification of polymorphic DNA (RAPD) (22). Ghaleh Golab et al. identified four *M. synoviae* species from Fars Province using PCR and RFLP assays. The primers in Ghaleh Golab et al. study selectively amplified a 780 bp DNA fragment within the 16S rRNA gene of *M. gallisepticum*, *M. synoviae* and *M. iowae* (23).

Comparing the PCR and culture methods, the current research showed that 17 and 25 out of 53 samples were positive by microbiological and PCR methods, respectively. Furthermore, the specific PCR can be used to identify the *Mycoplasma* sp. After obtaining positive results from the culture tests. To eradicate *M. synoviae* infection, it is important to identify *M. synoviae* rapidly and accurately and molecular methods such as PCR are helpful. Previously, *M. synoviae* specific PCRs were performed based on the 16S rRNA gene (5, 14) and recently they are based on haemagglutinin genes (19, 24).

The current study specific primers of Lauerman et al. were used (5). They indicated a sensitivity of 82% for their *M. synoviae* PCR and a specificity of 100% compared with those of microbiological and serological tests, and epizootiology, and history. To increase the PCR sensitivity, they suggested extraction of RNA and performing reverse transcription of the rRNA. These *M. synoviae* primers were selected from the 16S rRNA gene, and each cell is reported to have 104 ribosomes (5). However, it is suggested to apply other specific species PCR based on haemagglutinin genes. To obtain better *M. synoviae* PCRs results, it is also recommended to enrich the isolates, which is consisted as specimen's incubation for 24 hours prior to detergent treatment in the DNA extraction stage. This is certainly

due to an increase of the DNA starting material and the reduction of PCR inhibitors present in the original samples (19). Because of its sensitivity and specificity, PCR should circumvent the overgrowth problems encountered with culturing the field samples (20). These results strongly support the use of this PCR assay as an efficient alternative or supplement to culture and serological identification, which are labor-intensive, extremely time-consuming, and often provide confusing results. Overall, it is suggested that the PCR could be an alternative method for accurate identification of the *M. synoviae* infection especially in slaughter ostriches.

Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

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