

Full Article

Application of culture and polymerase chain reaction (PCR) methods for isolation and identification of *Mycoplasma synoviae* on broiler chicken farms

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ABSTRACT

Mycoplasma synoviae (*M. synoviae*) is a major worldwide poultry pathogen that causes serious economic losses in the poultry industry. This study was designed to detect *M. synoviae* through culture isolation and polymerase chain reaction (PCR) assay to demonstrated the involvement of *M. synoviae* infection in trachea and the lung/air sac samples taken from commercial broiler chicken farms in 3 main provinces of Iran (Tehran, Markazi and Qazvin), with clinical signs of the disease. Total of 43 samples were cultured in PPLO broth media supplemented for *M. synoviae* isolation. The bacteria DNAs were extracted by phenol/chloroform method and the PCR assay amplifying the conserved region of *16S rRNA* gene was applied for the detection of Mycoplasma genus in 163bp fragment and *M. synoviae* in 207bp fragment from culture as same as in clinical samples. Of the 43 swabs 28(65.1%) yielded one of the potentially pathogenic mycoplasmas evaluated for using PPLO agar culture diagnostic method, and 33(76.8%) yielded one of the potentially pathogenic *Mycoplasmas* evaluated for using *Mycoplasma* genus PCR as diagnostic method, and 24(55.9%) of the swabs yielded *M. synoviae* for using *M. synoviae* PCR as diagnostic method. In this study we had observed the highest quantity of *M. synoviae* infections in broiler chicken with PCR test. In conclusion, PCR is a more rapid, effective, sensitive and inexpensive method than the standard culture technique, that could be used as an alternative method for traditional culture and showed the real number of the *M. synoviae* contaminated broiler chicken farms.

Keywords: *Mycoplasma synoviae*, Broiler chicken, PCR, 16S rRNA, Culture

INTRODUCTION*

Mycoplasma synoviae (*M. synoviae*) is a major worldwide poultry pathogen that causes serious

economic losses in the worldwide poultry industry by decreased in egg production, growth retardation, and condemnation at slaughterhouse (Kleven *et al* 2008, Jordan *et al* 1979). *M. synoviae* infection is most frequently occurs as a subclinical upper respiratory infection. It may cause "Air sac disease" that have been

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described as a severe airsacculitis and it could be a result of coinfectious of *M. gallisepticum* or *M. synoviae* with respiratory virus infection (e.g. infectious bronchitis or Newcastle disease) or *Escherichia coli*. It may become systemic and results in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys, involving primarily the synovial membranes of joints and tendon sheaths producing an exudative synovitis, tenovaginitis, or bursitis (Kleven et al 2008, Kleven et al 1991, Lokaby et al 1998). The contaminated material with *M. synoviae* (dust, feathers and food) could infect chicks, sometimes after remarkably long silent periods (Corinne et al 2005). The success of control programmes depends on accurate and timely diagnosis of infected flocks. Therefore, a diagnostic assay with high sensitivity, high specificity, and a fast detection time is required for monitoring *M. synoviae* in poultry flocks (Buntz et al 1986, Bencina et al 1988, Cookson et al 1994), rapid diagnosis is needed to prevent dissemination of infection and has traditionally been achieved by serological screening for antibodies or by culture of the mycoplasma. Serological screening is still in widespread use but may not detect subclinical *M. synoviae* infections. Monitoring programmes may be inadequate because it depends solely on detecting seroconversion (Ewing et al 1998, Kleven et al 2001). *M. synoviae* strains can vary in antigenic make up and have the ability to alter the expression of major surface antigenic proteins affects the sensitivity and specificity of serologic monitoring systems (Adair et al 1990, Avakian et al 1990, Bradly et al 1988, Noormohammadi et al 1997). Culture can be costly and time-consuming, and can also be inconclusive (Ewing et al 1998), Culture of Mycoplasma is laborious and can take 3–4 wk, and even then, the result can be negative or be hampered by mixed infections (Zain et al 1995, Bradbury et al 1982). For these reasons, use of rapid and sensitive detection methods, like polymerase chain reaction (PCR) method, can be advantageous (Fernandez et al 1993, Hyman et al 1989, Kempf et al 1993), so PCR-based tests are now routinely used for

detecting pathogenic avian mycoplasmas. There are few works about isolation and molecular identification of *M. synoviae* in Iran. Ghaleh Golab et al (2008) used 16S rRNA gene of *M. synoviae* for PCR and RFLP assays to identify *M. synoviae* from chicken farms in Fars province. Pourbakhsh et al (2010) analyzed the *M. synoviae* infection of broiler breeder farms of Tehran province, they had shown the *M. synoviae* infection by using culture methods, serology and specific PCR assay.

The present study was designed to evaluate the technical performance of culture and PCR assays for detection of *M. synoviae* in trachea and the lung/air sac samples taken from commercial broiler chicken farms in 3 main provinces of Iran (Tehran, Markazi and Qazvin), with clinical signs of the disease.

MATERIALS AND METHODS

Sampling and cultures. Samples were collected from 3 main provinces of Commercial broiler chicken farms (Tehran, Markazi and Qazvin). Most of the samples were obtained from flocks with clinical signs of a probable infection by Mycoplasma spp. (*M. gallisepticum*, *M. synoviae*). 43 field samples obtained from trachea and the lung/air sac. Samples were collected on cotton swabs by opening the trachea and vigorously rubbing the mucosa with the tip. After this sampling, swabs placed in to 3ml PPLO broth (modify Frey media) and immediately refrigerated (4 °C) and transported to the laboratory, then agitated on a vortex mixer for 30 sec and the swab discarded. An aliquot of 0.5 ml was removed to a 1.5 ml tube for the DNA extraction. After filtering by 0.45 filters, 0.3 ml sample was taken for broth culture. PPLO broth and PPLO agar medium (BBL, Becton Dickinson and company, Cockeysville, Sparks, MD, USA) that contained 15% of swine serum, thallium acetate, penicillin (1000IU/ml) as mycotic and bacterial inhibitors, 0.0125% β -Nicotinamide adenine dinucleotide (NAD) as a necessary requirement of *M. synoviae* and 0.0125% cysteine hydrochloride as a reducing agent for

NAD were also added to the media. Broth and agar media were incubated under microaerophilic condition with 98% relative humidity and checked for color changes of broth and after tow passage, we had transfered 0/1ml of sample to agar and checked for typical mycoplasma colonies on agar. As soon as the phenol red indicator would change to yellow sub culture onto the fresh broth and agar were carried out. Mycoplasma routine cultures were incubated at 37°C under 5% CO₂ and checked for mycoplasma growth from 10 days up to 15 days even up to 28 days of incubation. *M. synoviae* reference strain (MS-NCTC 10124-05) have been used in this study as a positive control, and used uncultured PPLO broth as a negative control.

DNA Extraction. DNA was extracted from samples using a previously described method by Pourbakhsh et al (2010) with some modifications. 0/5ml of each sample was transferred to Eppendorf tube and centrifuged for 15 min at 13000 rpm. The supernatant fluid was discarded and add lysis buffer (Tris-HCL 50 mM pH=8, SDS 1%, Nacl 100mM, EDTA 50 mM, proteinase K 20 µl to 200µl) to the tube equal volume of the pellet in it and incubate for at least 4 hrs at 56 °C. Equal volume of the material in the tube added phenol and mixed well by vortex. Centrifuged at 13000 rpm for 15 min. Removed all aqueous layer (top layer) and transfered in a new tube. Added phenol: chloroform (1:1) in tube equal volume of the tube containing. Centrifuged at 13000 rpm for 15 min and transfered all aqueous layer in a new tube. Added chloroform in the tube, equal volume of the tube containing mix them well by vortex and centrifuged at 13000 rpm for 15 min. Transfered all aqueous layer in a new tube and sodium acetate was added 1:10 volume of the tube containing and mixed well. Added to them ethanol (ETOH) two fold of material in tube. This solution was placed on -20 for 20 min and centrifuged for 15 min at 13000 rpm. Discarded liquid containing of tube softly and 200 µl of 70% ETOH, centrifuged for 5 min at 13000 rpm. Pour off ETOH and drying tubes then add 50 µl distilled water to them.

Amplification with specific primers (PCR). In this study tow published primer sets were used for the specific detection of genus and species of *M. synoviae*. For genus Mycoplasma as follow: MYF: 5'-GCTGCGGTGAATACGTTCT-3', MYR: 5'-TCCCCACGTTCTCGTAGGG -3'. The 163 bp fragments were amplified (Kojima *et al* 1997). In *M. synoviae* strain as follow: MSF: 5'-GAAGCAAATAGTGATATCA-3', MSR: 5'-GTCGTCTCCGAAGTTAACAA-3'. The 207 bp fragments of *M. synoviae* 16SrRNA gene were amplified (Lauerman *et al* 1993). The PCR mix was performed in a total volume of 25µL per sample, containing 2.5 µl of 10 X PCR buffer(Roch Diagnostics-corporation, Indianapolis, USA), 4 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTPs, 0.1+0.1 µl each primer, 0.5 U Taq DNA polymerase(Roch Diagnostics-Corporation,Indianapolis, USA). Consequently 15.3 µl of deionized distilled water and 2 µl of extracted DNA as template was carried out.The PCR reaction was conducted in a Gradient Mastercycler (Eppendorff, Germany) as follows: In genus: 7.5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C, with a final extension cycle of 5 min at 72 °C. In species: 5 min at 95 °C, followed by 34 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension cycle of 5 min at 72°C. Visualization of amplified products was done by UV illumination after electrophoresis (1% agarose gel in 1× Tris-acetic acid-EDTA (TAE) buffer) and ethidium bromide staining.

RESULTS

43 swabs from 43 broiler chicken poultry farms analyzed simultaneously by culture, *Mycoplasma*PCR (MPCR) and *M. synoviae* PCR (MSPCR). Of the 43 swabs 76.8%(33) yielded one of the potentially pathogenic mycoplasmas evaluated, and only 10 samples (23.2%) scored negative for using PCR as diagnostic method, interestingly, the prevalence of mycoplasmas in the trachea and the lung/air sac was

high in all the flock. Of the 43 swabs, 55.9 %(24) yielded Infectious with *M. synoviae* evaluated, and 44.1%(19) yielded negative for using PCR as diagnostic method for *M. synoviae*. Of the 43 swabs from 3 broiler chicken poultry farms analyzed with culture, 65.1%(28) yielded one of the potentially pathogenic mycoplasmas evaluated, and 34.8%(15) scored negative for using culture diagnostic method. A positive results for mycoplasma genus was obtained in 27(62.7%) samples scored Positive for both diagnostic methods, so the agreement coefficient of the both diagnostic methods (Culture and PCR) is 83.72%. Whereas *M. synoviae* was detected in 24(55.9%) of the swabs tested with both diagnostic methods (Table 1), therefore several mycoplasma strains could be concomitantly carried in the same trachea and the lung/air sac.

Table 1. The culture and PCR methods results.

No. Sample	No. Culture		No. MPCR		No. MSPCR	
43	+	-	+	-	+	-
	28	15	33	10	24	19

Six samples were MPCR positive - culture negative, and only 1 sample was MPCR negative - culture positive (Table 2), also 10 of the samples scored negative for using MPCR and MSPCR and no one of samples are MPCR positive - MSPCR negative (Table 3).

Table 2. Distribution of samples for culture and Mycoplasma- PCR methods results.

No. Sample	Culture	MPCR
27	+	+
9	-	-
1	+	-
6	-	+
43		Total

Table 3. Distribution of samples for *M. synoviae* – PCR and Mycoplasma- PCR results

No. Sample	MSPCR	MPCR
24	+	+
10	-	-
9	-	+
0	+	-
43		Total

The MPCR exhibited positive results to the 2.5 particle/reaction and the MSPCR exhibited a sensitivity to approximately 100CCU (color changing unit). The results that have been obtained from this study indicated that the sensitivity of PCR for detection of *Mycoplasma* is more than 92.85% and the specificity of PCR assay is 66.67%.

$$\text{Agreement coefficient: } \frac{[\text{MPCR positive, culture positive} + \text{MPCR negative, culture negative}]}{\text{total samples}}$$

$$\times 100 = \frac{36}{43} \times 100 = 83.72\%$$

$$\text{Sensitivity: } \frac{[\text{MPCR positive, culture positive} - \text{MPCR negative, culture positive}]}{[\text{MPCR positive, culture positive} + \text{MPCR negative, culture positive}]} \times 100$$

$$= \frac{26}{28} \times 100 = 92.85\%$$

$$\text{Specificity: } \frac{[\text{MPCR negative, culture negative} + \text{MPCR negative, culture positive}]}{[\text{MPCR negative, culture negative} + \text{MPCR positive, culture negative}]} \times 100$$

$$= \frac{10}{15} \times 100 = 66.67\%$$

DISCUSSION

In this study, PCR method was applied to document the involvement of *M. synoviae* infection in trachea and the lung/air sac samples taken from commercial broiler chicken farms in 3 main provinces of Iran (Tehran, Markazi and Qazvin), and the obtained results were compared with culture isolation. PCR as a molecular detection method and traditional culture also successfully eliminated the need of biochemical tests and specific antiserum for the detection of *M. synoviae* colonies.

Lauerman et al (1993), developed PCR assay with high specificity and sensitivity for the detection of *M. synoviae* among the various avian mycoplasmas.

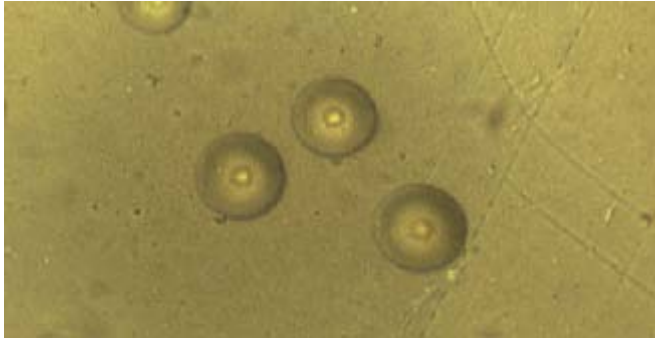


Figure 1. Colonies of *Mycoplasma* on the PPLO agar.

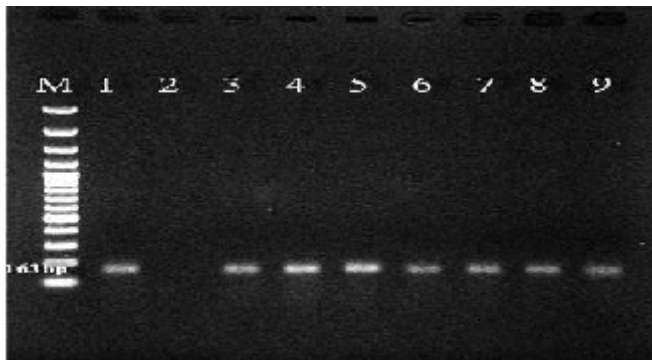


Figure 2. PCR electrophoresis analysis in 1% gel agarose. M: Marker (100bp DNA ladder). Lane 1: Positive control (163bp band, *Mycoplasma* genus, MS-NCTC 10124-05). Lane 2: Negative control (uncultured PPLO broth) and Lane 3 to 9 are positive samples.

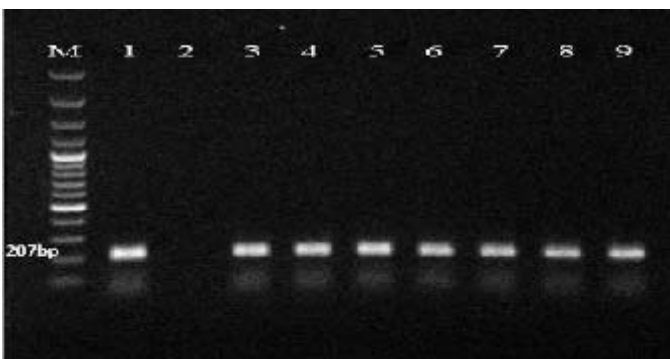


Figure 3. PCR electrophoresis analysis in 1% gel agarose. M: Marker (100bp DNA ladder). Lane 1: Positive control (207bp band, *Mycoplasma synoviae*, MS-NCTC 10124-05). Lane 2: Negative control (uncultured PPLO broth). and Lane 3 to 9 are positive samples.

A PCR assay was developed and evaluated as a diagnostic test, because confirmation of *M. synoviae* infection by culture presents several practical challenges (Kleven *et al* 2008, Jordan *et al* 1979, Garcia *et al* 1996, Lauerman *et al* 1993, Lauerman 1998, Feberwee *et al* 2005). *M. synoviae* is fastidious organism with unique medium requirements; it requires 4 days up to 5 days for growth (Kleven *et al* 2008), even up to 28 days of incubation (Feberwee *et al* 2005), and initial cultures commonly contain other mycoplasmas, such as *M. gallinarum* and *M. gallinaceum* (Kleven 1998). The particular aspect of *M. synoviae* is that, so clinically healthy and serologically negative chickens can carry the causative mycoplasmas at the most common infection sites, like the synovial membranes of joints and upper respiratory system (Kleven *et al* 2008, Ewing *et al* 1998, Kleven 1998). A PCR assay has several features that simplify the diagnosis of *M. synoviae* infection. As a diagnosis test, PCR assay can be completed in one day by using an mucosal swabs without special transport requirements other than a container to prevent cross-contamination between flock specimens, swabs submitted in this manner are not suitable for culture, and thus isolate are not available for pathogenicity testing (Kleven *et al* 2008, Lauerman *et al* 1993, Feberwee *et al* 2005, Kojima *et al* 1997, Salisch *et al* 1998, Kleven 1998). In this study 76.8% of the samples taken proved positive for *Mycoplasma* genus and 55.9% of the samples taken proved positive for *M. synoviae*, these are high number of carriers, it is warning to tent rapid detection *M. synoviae* in poultry flocks. In the present study, PCR provided a rapid diagnosis and identification of *M. synoviae* when it was performed on broth or colonies from agar media inoculated in the traditional manner. Evaluation of sensitivity and specificity of a new diagnostic test requires comparison with a proven standard (Lauerman *et al* 1993, Feberwee *et al* 2005), thus the *M. synoviae* PCR was compared with another test, traditional culture. As for culture and PCR tests, despite the comparable sensitivity, fast results and relatively low costs of the PCR compared with culture

(Lauerma *et al* 1993, Feberwee *et al* 2005, Sakhaei *et al* 2009, Pourbakhsh *et al* 2010, Joaquin *et al* 2010). The cause of the failure of the *M. synoviae* PCR test to detect the *M. synoviae* in the infected group may be due to one or more mutations in the genome of the highly passaged strain at the target sequence of the PCR primer (Feberwee *et al* 2005). One of our samples was PCR negative but yielded a positive culture. Several factors such as the presence of Taq DNA polymerase inhibitors or gene sequence variations lead to false negative PCR results (Joaquin *et al* 2010). Six of our samples were PCR positive but yielded negative culture. Several factors such as deficiency in culture requirement (serum, NAD, CO₂ and other materials), during transportation chilling of specimens and dying *M. synoviae* rapidly at room temperature lead to false negative culture results (Pourbakhsh *et al* 2010), so the PCR can detect and identified the dead particles of organisms in the culture and original samples from chickens swabs. The PCR is currently under consideration as a confirmation test in the OIE Terrestrial Manual. The *M. synoviae* PCR has value as a confirmation test when the flock history, clinical signs, and pattern of serological reactors suggest *M. synoviae* infection (Lauerma *et al* 1993, Lauerma 1998). Although generally regarded as the 'gold standard' for definitive diagnosis, mycoplasma culture has the disadvantage of being very laborious, particularly in cases of mixed infections, and it also depends on the presence of viable organisms (Lauerma *et al* 1993, Feberwee *et al* 2005, Sakhaei *et al* 2009, Kojima *et al* 1997, Salisch *et al* 1998, Stellrecht *et al* 2004, Pourbakhsh *et al* 2010, Joaquin *et al* 2010). Lauerma *et al* (1993), Salisch *et al* (1998), Feberwee *et al* (2005) and Pourbakhsh *et al* (2010) have been reported that, PCR is a more rapid, sensitive and useful method than the standard culture technique for the detection of *M. synoviae*, which is in agreement with our experience. Whereas in this study, positive culture results obtained within 10 to 15 days, up to 30 days and may be indicated negative results, and on the other hand PCR take only 1 day for the detection of

mycoplasmas and are not dependent upon viability of the organisms. It is suggested that the PCR could be an alternative method for accurate identification of the *M. synoviae* infection especially in breeder chicken flocks (Pourbakhsh *et al* 2010). Recently a PCR assay for detection of variable hemagglutinin gene (*vlhA* gene) of *M. synoviae* (Hong *et al* 2004, Harada *et al* 2009) proved useful tool in detection and typing of different *M. synoviae* strains. Comparing *vlhA*-PCR with *16S rRNA*-PCR (Hong *et al* 2004) also projected that in very early stage of infection the *16S rRNA* procedure was more sensitive than the *vlhA* method. Hosseini *et al* (2010) in DNA sequence analysis of some *M. synoviae* isolates from Mazandran province poultry flocks and compares them with *M. synoviae* from other countries showed that the different molecular structure and heterogeneity among *M. synoviae* isolated from Mazandran province, so it is suggested to apply the *16S rRNA* gene sequence for phylogenetic analysis of *M. synoviae* in field samples by comparison of the sequence with another *M. synoviae 16S rRNA* gene sequence in GeneBank. In this study PCR and culture applied altogether and the agreement coefficient of these methods is 83.72%, and the sensitivity of PCR assay is 92.85%, and 6 samples were PCR-positive culture-negative, and only one sample was PCR-negative culture-positive. The prevalence of *M. synoviae* in broiler chicken was observed highest quantity as measured through PCR, therefore, this study showed that the real number of the *M. synoviae* contaminated broiler chicken farms characterized by the PCR assays, and it is the most reliable than culture method.

In conclusion, PCR is a more rapid, effective, sensitive and inexpensive method than the standard culture technique, therefore, PCR can be an alternative method for traditional culture toward the detection of *M. synoviae* in broiler chicken farms.

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