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Full Article

Detection of *Mycoplasma synoviae* infection in broiler breeder farms of Tehran province using PCR and culture methods

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ABSTRACT

Mycoplasma synoviae (MS) is an important avian pathogen that can cause both respiratory disease and joint inflammation synovitis in poultry, inducing economic losses to the Iranian chicken industry especially breeder farms. The aim of this study was to use the MS specific PCR and culture methods in order to detect of *M. synoviae* from breeder farms where located in Tehran province. A total of 475 samples including choanal cleft, trachea, ovary and /or joint cavities from 23 broiler breeder farms of Tehran area were collected. Samples were cultured in PPLO broth media supplemented for MS isolation. The bacteria DNAs were extracted by phenol/chloroform method. Specific published primers amplify a 207 bp region of the 16S rRNA gene of MS were used for PCR method. Out of 475 samples, 146 cultures were shown positive and typical *Mycoplasma* colonies, 85 samples were also identified MS based on agglutination test with specific MS antiserum and the PCR method. A total of 122 samples, a band with 207 bp was shown as MS specific PCR product in electrophoresis. In addition to these 85 samples that were positives in both culture and PCR, 37 samples that had not grown in *Mycoplasma* media were positive in MS specific PCR. A total of 292 samples were negatives in both culture and PCR methods. 122 positive samples out of 475 samples (25.7%) were belonged to 7 breeder farms (30.4%). On conclusions, the MS infection of broiler breeder farms of Tehran area was confirmed truly. From the results, as the PCR method reduces the time consuming, an effectiveness and efficient for detection of *M. synoviae* infection of chicken breeder. It is then suggested that the PCR method could be an alternative method for culturing.

Keywords: *Mycoplasma synoviae,* broiler breeder, PCR, culture, Tehran

INTRODUCTION

Mycoplasma synoviae(MS) is one of the most important pathogenic chickens *Mycoplasmas*. MS 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, MS becomes 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, and bursitis (Landman & Feberwee 2001, 2004; Kleven

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2003). Lameness and respiratory disorder caused by this infection results in decreased growth rate and loss of egg production and causing economic loss in intensive production. As the vertical transmission plays a major role in spread of MS in chickens the most effective method of control is regular flocks monitoring and eliminating of positive breeder flocks. Reliable and rapid diagnosis is needed to prevent dissemination of infection (Lockaby *et al* 1998, Kleven 2003). In Iran, rapid serum agglutination (RSA) is used for flocks monitoring as screening test. Various serologic, bacteriologic and molecular methods are used to confirm the RSA results. Serological screening is still in widespread use but may not be detected subclinical *M. synoviae* infections and monitoring programmes that depend solely on detecting seroconversion may be inadequate (Ewing *et al* 1998, Kleven *et al* 2001). However, culturing of MS could be costly, time-consuming and inconclusive (Ewing *et al* 1998). PCR-based tests are now routinely used for detecting pathogenic avian mycoplasmas. Some PCRs are based on the 16S rRNA gene, some are in-house tests and others are produced commercially kits (Garcia *et al* 1995, Lauerman *et al* 1993, Lauerman *et al* 1998). The attempts to eradicate the *Mycoplasma gallicepticum* (MG) infection in Iranian poultry farms have been commenced earlier than MS. Furthermore the culture of MS is more difficult than MG, so the reports of isolation and molecular detection of MG are more than MS. MS is a fastidious organism that requires medium enriched with many factors and some factors especially yeast extract and serum may vary in their ability to support growth. MS is not stable at pH 6.8 or lower and it is sensitive to temperatures greater than 39º C and dehydration. Cultures incubated for more than a few hours after the phenol red indicator has changed to yellow (pH<6.8) may no longer be viable. Incubation of agar should be placed in a closed container to prevent dehydration of agars (Kleven *et al* 1998, 2003). There are few works about isolation and molecular identification of MG in Iran (Ghaleh Golab *et al* 2005, Hosseini *et al* 2006, Ghaleh Golab *et al* 2008) and there is only one report about isolation and molecular identification of MS (Ghaleh Golab *et al* 2005). In Fars province of Iran, Ghaleh Golab *et al* (2005) isolated and detected *Mycoplasma gallicepticum* in chicken farms using PCR and RFLP assay and culture method. Hosseini *et al* (2006) analyzed the isolates of MG from different geographical areas of Iran by random amplification of polymorphic DNA (RAPD). Ghaleh Golab *et al* (2008) identified 4 MS isolates from Fars province using PCR and RFLP assay. The primers of Ghaleh Golab *et al* study selectively amplify a 780 base pair DNA fragment within the 16S rRNA gene of MG, MS and *M. iowae* (MI). They had to use another method such as RFLP for specific detection of MS and differentiation from MG and MI. Our aim was to use the MS specific PCR assay and culture method in order to detect *M. synoviae* infection from some broiler breeder farms located in Tehran province.

MATERIALS AND METHODS

Samples. During 2004 and 2007, a total of 23 broiler breeder chicken farms were sampled. Specimen for *M. synoviae* isolation were taken from live birds with clinical signs suspected to MS infection. Swabs from the choanal cleft and trachea collected. After human euthanizing the live birds they were also sampled via the ovaries and /or joint cavities. Overall 475 swab samples were placed into the PPLO broth as transport medium on ice packs and were shipped to laboratory within 12 hours.

Bacteriology. For primary isolation, the specimens were diluted and filtered in fresh PPLO broth and inoculated onto PPLO agar medium (BBL, Becton Dickinson and company Cockeyville, Sparks, MD, USA). The media supplemented with 12% of equal volumes of

inactivated horse or swine sera. Thallium acetate (1:4000) and penicillin (1000IU/ml) as mycotic and bacterial inhibitors and Nicotinamide adenine dinucleotide (NAD) (1:10000w/v) as a necessary requirement of MS and cysteine hydrochloride (1:10000w/v) 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 ºC with 98% relative humidity and checked for color change of broth and 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 (Kleven *et al* 1998). Several passages until 21-28 days were subcultured. After appearing the specific colonies of mycoplasma identification of the MS samples were carried out by serology.

Agglutination test. Suspension of the suspected colony was made in drops of saline on slide. If auto-agglutination occurred or the suspension was rough in saline the slide was discarded. A drop (approximately 50 µl) of MS specific antiserum (SPAFAS Canada) was added and mixed to one drop (approximately 50 µl) of smooth suspension. If agglutination was observed within 1-2 minutes the isolate was considered as MS.

DNA Extraction. The DNA Extraction was processed from an 8 hours culture as an enrichment step only for direct clinical samples. The MS isolates that were obtained from culture method were also extracted and tested in PCR for more confirmation. Bacterial DNA was extracted by Phenol/Chlorophorm method. One ml of each PPLO broth culture was centrifuged in a microcentrifuge at 14000 rpm for 5 minutes. Cell pellets were re-suspended in phosphate buffered saline (PBS). 100 µl of harvested bacterial suspension was added to 100 µl lysis buffer and tube was placed in a 56 ºC bath for 4 hours. Then 200 µl saturated phenol was added and tube was centrifuged (13000 rpm or 15700 g) for 20 min. Upper phase was transferred to the next tube and equal volume of mixed phenol / chlorophorm (1:1) was added. After centrifugation at 13000 rpm for 20 min the aqueous phase was transferred and added to equal volume of pure chloroform and was centrifuged (13000 rpm) for 5 min. Upper phase mixed with 1/10 volume of acetate sodium and were precipitated with 2 fold volume of cool and pure ethanol. After final precipitation using by 70% ethanol the DNA was dried and re-suspended in 50 µl TE buffer at 4 ºC and used for PCR.

Primers. In this study two primers MS-1: 5′-GAA GCA AAA TAG TGA TAT CA-3′ and MS-2: 5′-GTC GTC TCC GAA GTT AAC AA-3′ which have been designed by Lauerman *et al* previously (1993) were used. They flank and amplify a 207 bp region of the 16S rRNA gene of MS.

PCR Parameters and Optimization. DNA amplifications were carried out in a total volume of 25 µl containing 17 µl DNA, 0.1 µl of each primer, 0.5 µl dNTP mix (10mM) [CinnaGen Inc.], 4 µl $MgCl_2$ (25mM) [CinnaGen Inc.], 2.5 µl PCR buffer (10X) [CinnaGen Inc.], and 0.1 µl Taq DNA polymeras (5 unit / µl) [Cinna Gen Inc.]. Reaction mixtures were thermocycled 34 times beginning with an initial denaturation step of 5 min at 95 ºC. The temperature and time profile of each cycle was as follows: 94 ºC for 1min (Denaturation), 50 ºC for 1min (Annealing), and 72 ºC for 1min. PCRs were finished with a final extension step of 5 min at 72 ºC. PCR products were stored at 4 ºC. PCRs were carried out using two programmable thermal cyclers (Primus and Mastercycler gradient). The MS-H vaccine strain as positive and water as negative controls were included in all tests.

Amplicon Electrophoresis. A 8 µl aliquot of each PCR products was mixed with 2 µl loading buffer (6X) and separated by electrophoresis in an 1% agarose gel with 0.5 µl /ml ethidium bromide (100 volts for 1 hour) following U.V. transillumination.

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 DNA extracted of *Mycoplasma gallisepticum, Salmonella entritidis* and water.

RESULTS

Bacteriology. Of 475 cultures, 146 (30.74%) were positive and showed typical mycoplasma colonies and among them 85 samples were MS based on serology test. Figure 1 shows the colonies of MS after confirmation by PCR.

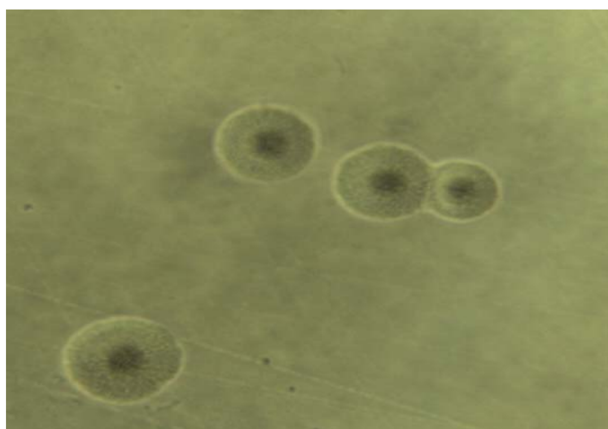

Figure1. Colonies of *Mycoplasma synoviae* on the PPLO agar specific for MS isolation(x 40)**.**

PCR. The PCR showed amplification only when the DNA was processed from an 8 hours culture (enrichment step). A total of 122 samples turned out to be PCR positive. This PCR test successfully amplified the DNA of both propagated MS in culture method and the samples which were negative in culture. The amplified PCR product of about 207 bp was separated by electrophoresis in 1% agarose gel using 100bp DNA ladder and this PCR product was not observed for negative controls (Figure 2). 85 out of 146 mycoplasma isolates were positive in MS specific PCR. In addition to those 85 samples that were positives in both culture and PCR, 37 samples that had not grown in mycoplasma media were also positive in MS specific PCR. A total of 122 samples were positive in PCR method and 292 samples were negatives in both culture and PCR methods. The results of culture method based on serology confirmation using MS specific antiserum were considered as golden standard test. The sensitivity and specificity were 100% and 90.5% respectively, for PCR method against the golden standard test. Also, the correlation rate between two methods was 92.2 %. 122 positive samples out of 475 samples (25.7%) were belonged to 7 out of 23 breeder farms (30.4%).

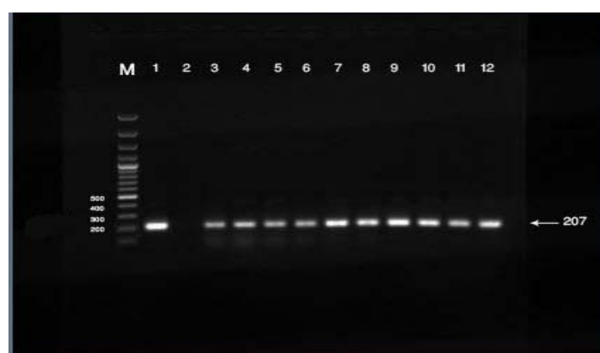

Figure 2. Electrophoresis of PCR products. M: Marker (100bp DNA ladder) *Lane 1*: Positive control, *Lane* 2: Negative control, *Lane* 3: MS-H strain, *Lane* 4-12: MS isolates from broiler breeder flocks of Tehran.

Specificity and Sensitivity of the PCR test. The MS PCR exhibited a sensitivity equivalent to approximately 100 color changing unit (CCU) and this PCR did not react with DNA template of *Mycoplasma gallisepticum* and *Salmonella entritidis* (Figure 3).

DISCUSSION

In this study, *Mycoplasma synoviae* infection was shown in broiler breeder farms of Tehran province using PCR and culture methods. MS infection of broiler breeder farms of Tehran area was confirmed truly. Efficient *M. synoviae* culture and molecular methods are necessary in order to diagnosis, eradication and research goals of this important poultry pathogen. Although respiratory infections

with *M. synoviae* are generally considered to be subclinical (Van Eck *et al* 1980), an increasing number of reports have documented economic losses attributable to respiratory infections (Morrow *et al* 1990, Lockaby *et al* 1998, Kang *et al* 2002) and arthropathic strains (Landman & Feberwee, 2001, van Beek *et al* 2002, Kleven *et al* 2003).

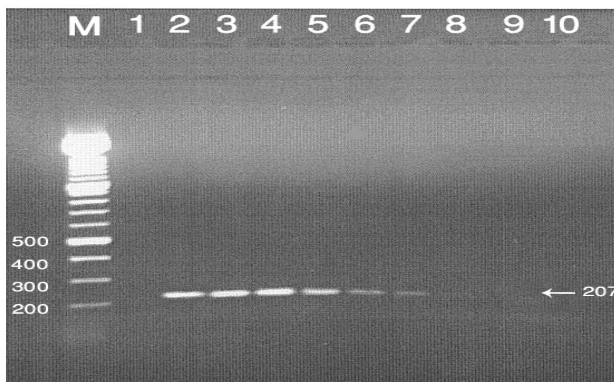

Figure 3. Specificity and Sensitivity of the PCR test. M: Marker (100bp DNA ladder) ***Lane 1:*** Negative control, ***Lane 2:*** MS-H strain, ***Lane3-8:*** DNA extractions of serial log $_{10}$ dilutions of bacterial suspension of MS-H. ***Lane 9:*** DNA template of *Mycoplasma gallisepticum* ***Lane 10:*** *Salmonella entritidis*.

The eggshell pathology and the concomitant egg production losses that result from it further highlight the economic significance of *M. synoviae* in commercial poultry. (Feberwee *et al* 2009). As the vertical transmission plays a major role in spread of MS in chickens the most effective method of control is the regular flocks monitoring and eliminating of positive breeder flocks (Kleven *et al* 2003). In this study, the most specific culture and PCR methods without false negative were set up and established. The MS infection of chicken breeder flocks of Tehran area was confirmed confidently. Serologic monitoring of MS infection in chicken breeder farms of Iran has been carried out routinely and regularly since 1986. Although serological screening is still in widespread use but may not detect subclinical *M. synoviae* infections, and monitoring programmes that depend solely on detecting seroconversion may be inadequate and nonspecific (Ewing *et al* 1998, Kleven *et al* 2001). Traditionally, MS has been diagnosed by serological screening or by culture method followed by identification of the *Mycoplasma*. Serological screening is relatively inexpensive but is not always sufficiently specific or sensitive while culture can be insensitive and time consuming (Kleven *et al* 2003). Detection of MS by culture has not been reported from Tehran breeder chicken farms before this and has been reported from other areas of Iran only from Fars province by Ghaleh Golab *et al* (2008). They identified 100 Mycoplasma isolates by PCR and RFLP and among them they pointed to 4 isolates of MS. The media that they used for MS culture were described previously by Frey *et al* (1968). They did not explain the details of MS culture more.

In our study modifications of Jordan's medium were applied (Kleven *et al* 1998) and we successfully isolated 85 MS bacteria by culture method and identified using PCR assay. MS Culture and isolation is not easy and almost was not successful in all the Iranian poultry laboratories. Some of the reasons of this are discussed here. Sample collection is of importance because MS tend to disappear from lesions after a few weeks. During transportation chilling of specimens should be considered because MS die rapidly at room temperature. Additions of more antibiotics have been ineffective in controlling bacterial contamination as well as filtrations are needed. Culture method is needed for some research projects and even for diagnosis aims, without enrichment culture in order to prepare the PCR samples, many false negative PCR results might occur (Ben Abdelmoumen Mardassi *et al* 2005), so the culture should not be ignored. But culture can be costly and time-consuming, and can also be inconclusive because of low sensitivity (Ewing *et al* 1998). Marois *et al* (2000) in comparison the PCR and culture methods showed that in the experimental infection, 10/96 and 46/96 samples of food, drinking water, feathers, droppings or dust were positive by culture and Mycoplasma-PCR. In field conditions,

the number of positive results for environmental samples were respectively 7/28 and 17/28. PCR-based tests are now routinely used for detecting pathogenic avian mycoplasmas. Furthermore the specific PCR can be used to identify the mycoplasma isolate after appear positive result in culture. For eradication of MS infection, rapid and accurate identification of Ms is of great importance and molecular methods such as the PCR have been developed to improve this. Earlier MS specific PCRs were based on the 16S rRNA gene (Lauerman *et al* 1993 & Garcia *et al* 1995) and more recently, some have been based on haemagglutinin genes (Hong *et al* 2004, Ben Abdelmoumen Mardassi *et al* 2005). In this study, we used species specific primers of Lauerman et al. (1993). They indicated sensitivity for their MS PCR of 82% and a specificity of 100% as determined by comparison with culture, serology, epizootiology, and history. For increasing the sensitivity of the PCR they suggested extraction of the sample RNA and performing reverse transcription of the rRNA. These MS primers were selected from the 16S rRNA gene, and each cell is reported to have 10^4 ribosomes (Lauerman *et al* 1993). The PCR method of Garcia *et al* (1995) used by Galeh Golab et al (2005, 2008) is also based on the 16S rRNA gene and requires additional step of RFLP, but the PCR used in this study (Lauerman *et al* 1993) is species specific and has been used in recent years by other workers successfully (Buim *et al* 2010). However it is suggested to apply other specific species PCR based on haemagglutinin genes. It is also recommended to use the enrichment step for better results of MS PCRs, which consists of the incubation of specimens for 24 h 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 (Ben Abdelmoumen Mardassi *et al* 2005). Because of its sensitivity and specificity, PCR should circumvent the overgrowth problems encountered with culturing of field samples (Ewing *et al* 1998). 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 MS infection especially in breeder chicken flocks.

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