

Original Research Article

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## Molecular Detection of Avian Mycoplasmas in Poultry Affected with Respiratory Infections in Haryana (India)

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### ABSTRACT

*Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are the most economically significant *Mycoplasma* pathogen of poultry. The objective of this study was molecular detection of avian *Mycoplasmas* in poultry affected with respiratory infections in Haryana (India). In this study 92 pooled tissue samples including trachea, lungs and air sacs were collected from 92 different poultry flocks affected with respiratory infections and were undergone mycoplasmaological examination by Polymerase chain reaction (PCR). Four sets of primers including a universal primer (16S rRNA) for genus *Mycoplasma*, two primers for *M. gallisepticum* (16S rRNA gene and 16S-23S rRNA IGSR) and one for *M. synoviae* (16S rRNA gene) were employed for amplification of genes of all the 92 DNA samples extracted from tissue samples. Direct detection of avian *Mycoplasma* in tissue specimen by PCR using 16S rRNA universal primers for genus *Mycoplasma* followed by MG and MS specific PCR revealed, 34/92(36.9%) *Mycoplasma*, 25/92(27%) MG and 2/92 (2.1%) MS. This study demonstrated the high prevalence of MG infection in commercial Broilers farms of Haryana. Therefore, the high prevalence and wide distribution of MG infection warrants immediate attention and preventive strategies to minimize economic impact of MG infection. The present study suggests that the PCR assays performed for MG and MS provide a simple, quick and precise tool to specifically detect these organisms from the field samples which are always found to be complicated by other pathogens.

#### Keywords

*Mycoplasma gallisepticum*,  
*Mycoplasma synoviae*, PCR.

#### Article Info

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### Introduction

Haryana is a small state of India but it is the 5<sup>th</sup> largest in poultry population (Livestock census, 2012). There are many poultry pathogens which are responsible for huge economic losses to poultry industry through respiratory infections such as *Mycoplasma*, *E. coli*, Newcastle disease virus, Infectious bronchitis virus. The mycoplasmas do not have cell wall and are smallest self-replicating

prokaryotes. There are mainly two *Mycoplasma* species which are pathogenic in nature viz. *M. gallisepticum* (MG) and *M. synoviae* (MS). In chickens *M. gallisepticum* causes Chronic Respiratory Disease and usually show loss of appetite, dullness, depression, tendency to huddle together, poor growth, emaciation, respiratory rales, tracheitis, air sacculitis, coughing, sneezing,

open mouth breathing, nasal discharges and dyspnea (Gupta *et al.*, 2009). Conjunctivitis with frothy ocular exudate is common in turkeys and occurs occasionally in chickens. In broilers due to MG infection, there is a reduction in weight gain of up to 20% to 30%, a 10% to 20% decrease in food conversion efficiency, a 5% to 10% mortality rate and 10% to 20% of carcasses are condemned at the processing plant (Kleven, 1990). In breeders and layers, the disease causes a 10% to 20% decrease in egg production (nearly 16 fewer eggs per hen) and a 5% to 10% increase in embryo mortality (Kleven, 1990). Another serologically distinct *Mycoplasma* associated with infectious synovitis of poultry was first reported by (Chalquest and Fabricant, 1960) and had been named *M. synoviae* by (Olson *et al.*, 1964). Generally the preliminary detection of flock for MG and MS is done by serological tests namely slide/plate agglutination test and/or ELISA and confirmation can be done by culture and/ or molecular identification (Ramadass *et al.*, 2006; Zhang *et al.*, 2011). Several workers have described the use of conventional PCR in detecting avian mycoplasmas directly from clinical samples such as nasal swab, trachea and air sac (Ramadass *et al.*, 2006; Bibank *et al.*, 2013). PCR has some advantage over cultural detection as it can detect the pathogens in dead organism also and also rapid, inexpensive and sensitive method. Therefore, the present study was carried out to detect the *Mycoplasma* infection in poultry affected with respiratory infection by PCR.

## Materials and Methods

### Samples

Pooled tissue samples were collected from 92 poultry flocks which were brought from different parts of Haryana for disease investigation during July 2015 to January 2016 in the Department. Ninety two pooled tissue samples including trachea, lungs and air

sacs were collected post necropsy from chicken affected with respiratory infections and immediately processed for PCR examination. These 92 pooled samples represented 92 different poultry flock.

### Genus and species specific PCR

DNA was extracted directly from tissue samples collected from various poultry flocks using the DNA extraction mini kit (Qiagen, Germany) following the manufacturer's recommendations. Polymerase chain reaction assay was carried out on extracted DNA samples from 92 pooled tissue samples to amplify the 16S rRNA gene fragment specific to Genus *Mycoplasma*, 16S rRNA and 16S-23S rRNA fragments specific to *M. gallisepticum* and 16S rRNA specific to *M. synoviae* species as per protocols described by (Zhi *et al.*, 2010; OIE, 2008; Raviv *et al.*, 2007) respectively. The primer specific to Genus *Mycoplasma* was; F-5'GGCGAATGGGTGAGTAACACG3' and R-5'CGGATAACGCTTGCGACCTATG3' and size of amplified product was 461bp (Zhi *et al.*, 2010). Two sets of primers were used to amplify MG DNA. First is 16S rRNA, F-5'GAGCTAATCTGTAAAGTTGGTC3' R-5'GCTTCCTTGCGGTTAGCAAC3' and yielded 185 bp product (OIE, 2008). Second primer was IGS (16S to 23S rRNA) with sequence, F-5'GTAGGGCCGGTGATTGGA GTTA3' and R-5'CCCGTAGCATTTCGCA GGTTC3' and yielded a product size of 812 bp (Raviv *et al.*, 2007). Species specific primer for MS, 16S rRNA F-5'GAGAAG CAAAATAGTGATATCA3' and R-5'CAGT CGTCTCCGAAGTTAAC AA3' with 207 bp product size OIE (2008).

S-6 serotype antigen (Salsbury laboratories, U.K.) of MG and *M. synoviae* antigen (Soleil, Biovac Animal Health, France) were used as positive control to carry out PCR specific to genus *Mycoplasma*, MG and MS. At the time of each run, DNA extracted from standard

strains of MG and MS were used as positive controls. Beside, 100 bp ladder was used as marker DNA in each run. Amplification conditions were optimised for each of the primers separately. The PCR reaction mix of 25 µl was optimum included 0.5 µl of each primer (10 µM), 12.5 µl of Master Mix (HotstarTaq master mix Qiagen, 2X), 10.5 µl of Nuclease free water (NFW) and 1 µl of template DNA. Amplification of 16S rRNA specific to Genus *Mycoplasma*, species MG and MS were done using protocol as described by Zhi *et al.*, (2010) and OIE (2008) respectively with initial denaturation of DNA at 94°C for 15 min. It was followed by 40 cycles of denaturation, annealing and extension at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min respectively and final extension was carried out at 72°C for 5 min. For the second primer of MG (16S-23S rRNA) amplification of species specific DNA for *M. gallisepticum* was done as per method described by (Raviv *et al.*, 2007). The PCR amplification was achieved with initial denaturation of DNA at 94°C for 15 min. It was followed by 30 cycles of denaturation, annealing and extension at 94°C for 20 sec, 55°C for 30 sec and 72°C for 1 min respectively, and final extension was carried out at 72°C for 5 min. The amplified PCR products were analysed by agarose gel electrophoresis using 2% agarose containing 0.5 µg/ml ethidium bromide in tris-acetate EDTA (TAE) buffer and visualized by gel documentation system (Alphaimager, HP), as per the method of (Sambrooke *et al.*, 1989). The size of the amplified product was ascertained by comparison with standard DNA marker (100 bp DNA ladder, Thermo scientific, USA).

## Results and Discussion

Our findings show, amongst ninety two DNA samples, 34 (36.9%) to be positive for genus *Mycoplasma* and the product size of amplicon

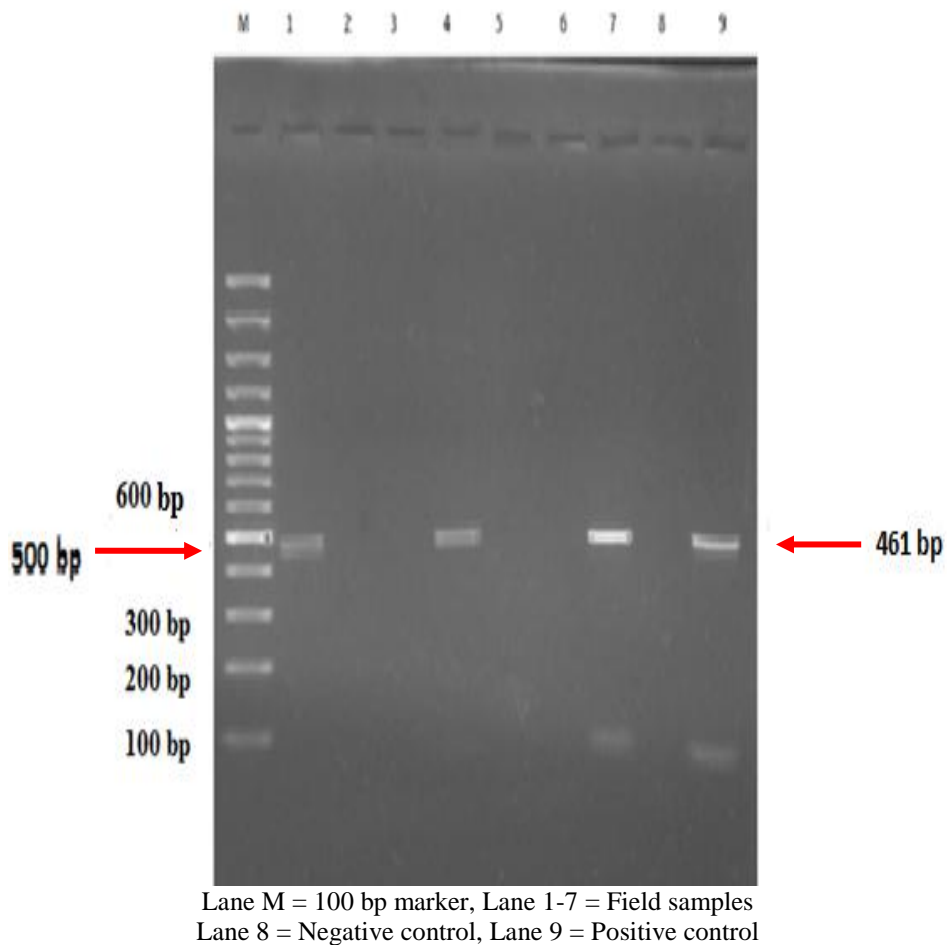
was recorded 461 bp (Figure 1). The DNA from these 34 *Mycoplasma* on further amplification with MG and MS specific primer revealed, 25 (27%) *M. gallisepticum* and 2 (2.1%) *M. synoviae*. The product size of amplicon was recorded 185 bp for MG and 207 bp for MS (Figures 2 and 3). Thirty four DNA of mycoplasmas amplified using IGSR primer for MG also revealed 25 (27%) MG. However the product size of amplicon was recorded 812 bp (Figure 4).

In this study we only sampled one flock per farm which could result in an underestimation of the prevalence if biosecurity measures are well applied in farms. However, we assumed, based on a study by Feberwee *et al.*, (2005) which demonstrated that the reproduction ratio R0 of *M. gallisepticum* is higher than 1, that once there is an infection in a flock it will spread quickly within the whole flock. As evident from above, samples subjected to genus specific PCR, 34 (36.9%) were found positive for *Mycoplasma* genus specific PCR. In concurrence with present study, (Qasem *et al.*, 2015) reported the higher prevalence (48%) of Mollicutes by PCR from poultry flock affected with respiratory infection. However in India, (Sankar, 2012) reported 24% genus *Mycoplasma* using genus specific PCR from poultry tissue affected with avian mycoplasmosis. This high prevalence of genus *Mycoplasma* was due to detection of both pathogenic and non-pathogenic commensal mycoplasmas which could not be differentiated using genus specific PCR. Tissue specimens subjected to genus specific PCR using were followed by MG and MS specific PCRs. We used two sets of primers for amplification of MG DNA targeting 16S rRNA gene and IGSR (16S-23S rRNA). It is known that two phylogenetically related avian mycoplasmas, MG and *Mycoplasma imitans*, have very similar 16S rRNA genes and that the primers targeted this gene give similar products. Due to the fact 16S rRNA gene has

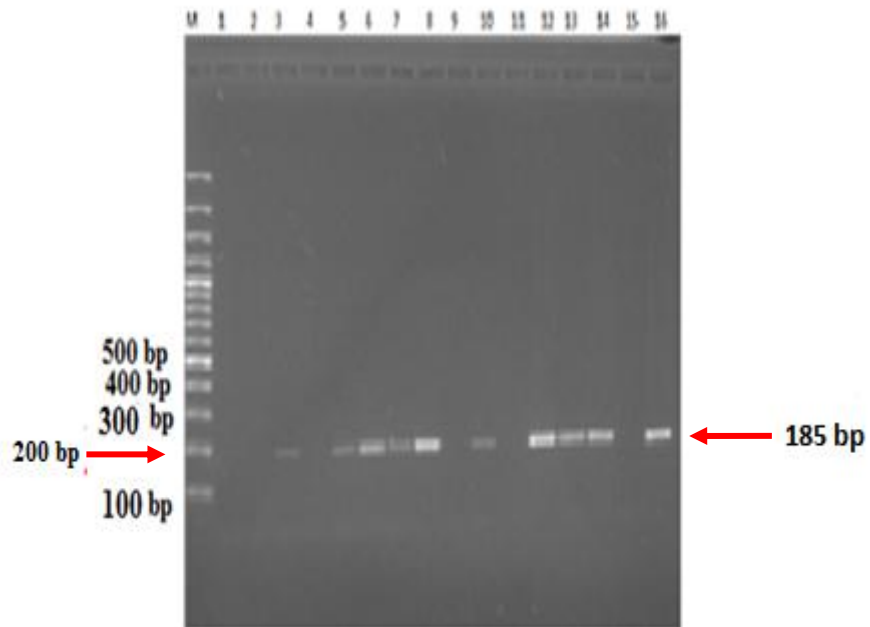
two copies on the MG and MS genomes, the PCR targeted 16S rRNA have higher sensitivity. The genome sequencing analysis of different avian mycoplasmas revealed that there is a region, the IGSR located between 16S and 23S rRNA, which has greater variation within different species and strains than in the 16S rRNA gene. Due to the above facts about we have selected both these type of primer for the amplification of MG DNA. Higher prevalence of MG (27%) was recorded by PCR using the 16S rRNA and IGSR primers showing 25 samples positive.

Our findings appear to correspond with other studies that found 32% prevalence of MG (Buim *et al.*, 2009) and (Singh, 2013). Likewise, (Rauf *et al.*, 2013) also reported higher prevalence of MG (68%) by PCR from poultry affected with respiratory syndrome. This high prevalence may be explained by the increase of vertical transmission due to the lack of surveillance programme in breeder poultry flocks of India which results in spread of MG infection from breeder hens to their offspring.

**Fig.1** Agarose gel electrophoresis of PCR products of DNA extracted from tissue samples depicting the presence of *Mycoplasma* species, as evident by a band of 461 bp using genus specific universal primer (16S rRNA)

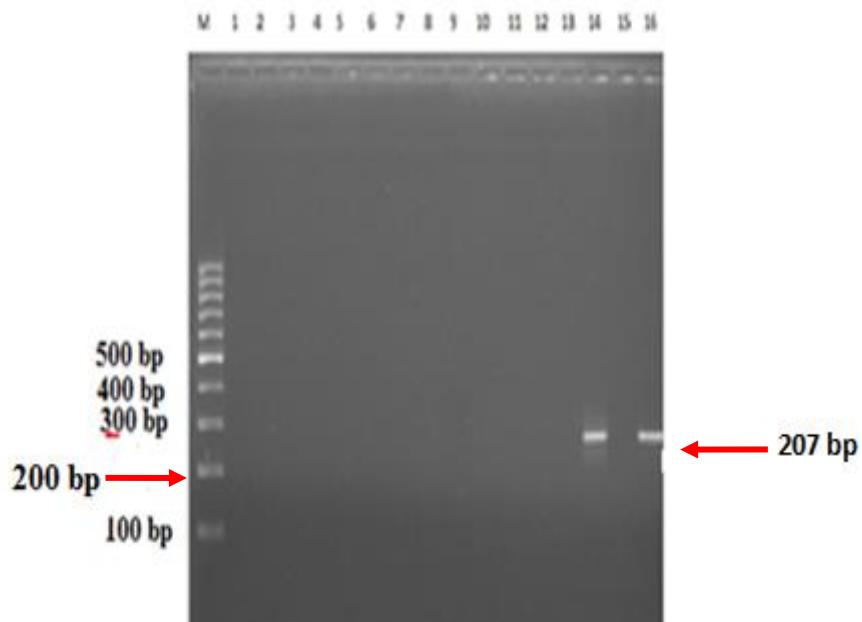


**Fig.2** Agarose gel electrophoresis of PCR products of DNA extracted from tissue samples depicting the presence of *M. gallisepticum* (MG), as evident by a band of 185 bp using species specific primer for MG (16S rRNA)



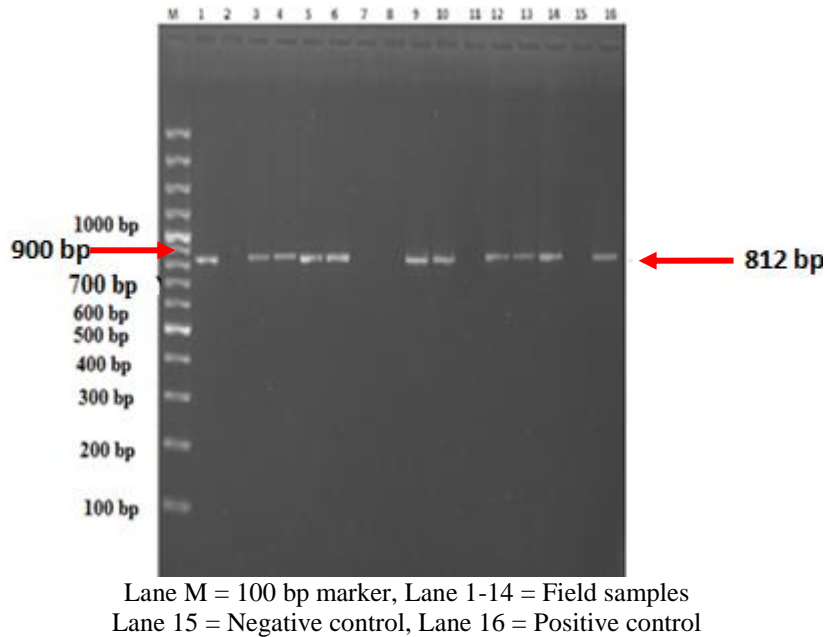
Lane M = 100 bp marker, Lane 1-14 = Field samples  
Lane 15 = Negative control, Lane 16 = Positive control

**Fig.3** Agarose gel electrophoresis of PCR products of DNA extracted from tissue depicting the presence of *M. synoviae* (MS), as evident by a band of 207 bp using species specific primer for MS (16S rRNA)



Lane M = 100 bp marker, Lane 1-14 = Field samples  
Lane 15 = Negative control, Lane 16 = Positive control

**Fig.4** Agarose gel electrophoresis of PCR products of DNA extracted from tissue samples depicting the presence of *M. gallisepticum*, as evident by a band of 812 bp using species specific primer for MG (IGSR)



MG prevalence in chickens has been reported to be high in many countries with no control strategy against MG (Gharaibeh and Al Roussan, 2008). Whereas in India (Ramdass *et al.*, 2006) reported lower prevalence (3.4%) of MG on direct detection by PCR from tissue of poultry affected with respiratory infections and 4% by (Michiels *et al.*, 2016) from poultry flocks affected with respiratory infection in Belgium. This low prevalence can be due to difference in sampling strategy, surveillance programme, and season of sample collection. In Belgium there is reduction of vertical transmission due to the mandatory surveillance programme in breeder poultry flocks that aims to prevent and control spread from breeder hens to their offspring and protect national and international trade markets (Michiels *et al.*, 2016). Lower prevalence of MS (2%) was recorded by PCR using the 16S rRNA primer showing 2 samples positive. The results of present study are comparable with 2% prevalence of MS as reported by (Buim *et al.*, 2009) and 0.8% by

(Saritha and Veeregowda, 2013). In contrary (Ramdass *et al.*, 2006) reported somewhat higher prevalence of MS (6.4%) by PCR from poultry affected with respiratory disease at Chennai.

This study demonstrated the high prevalence of MG infection in Commercial broilers farms of Haryana. Therefore, the high prevalence and wide distribution of MG infection warrants immediate attention and preventive strategies to minimize economic impact of MG infection. The present study suggests that the PCR assays performed for MG and MS provide a simple, quick and precise tool to specifically detect these organisms from the field samples which are always found to be complicated by other pathogens. One of the benefits of PCR over cultural isolation of avian *Mycoplasma* is that PCR is not dependent on viable or structurally intact cells and the presence of DNA in the tissues is sufficient to yield a positive result. The PCR

being more sensitive, specific and reliable test can be applied for the early diagnosis of avian mycoplasmosis in poultry flocks.

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