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Isolation and Phylogenetic Analysis of Avian *Mycoplasmas* from Poultry Affected with Respiratory Infections in India

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ABSTRACT

Mycoplasma gallisepticum (MG) *and Mycoplasma synoviae* (MS) *are the two most pathogenic avian mycoplasmas.* In the present study, examination of 92 pooled tissue samples from broiler chicken of 92 different poultry flocks of Haryana (India) exhibiting respiratory infections resulted in isolation of 13 (14%) *Mollicutes*. Based on biochemical reactions, growth inhibition test, PCR and/or sequencing, 8 (8.6%) isolates could be characterized as MG, 1 (1.08%) as MS, 3 (3.24%) as *M. gallinarum* and 1 (1.08%) as *Acholeplasma laidlawii*. The phylogenetic analysis using Intergenic spacer region (IGSR) of these MG isolates revealed that they clustered with USA strain whereas the vaccine strains were in different clade. Single locus sequence typing (SLST) revealed considerable nucleotides variation between 8 MG isolates and vaccine strains. Conclusively, Sequencing of IGSR region of MG can be used as a valuable epidemiological investigation tool for the differentiation of wild-type MG strains from vaccine strains.

Key words: Avian mycoplasmosis, Isolation, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Nucleotide sequencing.

INTRODUCTION

The poultry industry in India has emerged as the most dynamic and rapidly expanding segment of livestock sector as evident from the production statistics reaching about 69 billion eggs and 791 million broilers per year (Livestock Census, 2012). Haryana ranks 6th in poultry population in India among all states and union territories and is the 5th largest egg producer in the country (Livestock Census, 2012). Poultry industry faces economic losses due to increased mortality resulting from respiratory complications prominently due to *Mycoplasma* spp. infections. Avian mycoplasmas infection in poultry industry has gained worldwide importance due to significant economic losses in chickens (Bibak *et al*., 2013). *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) are the most important pathogenic avian mycoplasmas (Bradbury, 2001; Bibak *et al*., 2013). *Mycoplasma synoviae* causes infectious synovitis whereas *M. gallisepticum* infection causes chronic respiratory disease (CRD) in chickens (Osman *et al*., 2009). *M. gallisepticum* infections are transmitted both horizontally as well as vertically and persist in the flock in subclinical form. The accurate and rapid detection of *M. gallisepticum* is the first and important step for controlling the infection. Serological assays namely plate agglutination and ELISA are routine screening tests and confirmation can be done either by culture or molecular techniques (Ramadass *et al*., 2006; Zhang *et al*., 2011). Despite being laborious, expensive and time-consuming cultural isolation is still gold standard for detecting avian mycoplasmas. However further identification of cultured isolates of mycoplasmas up to species level by PCR and nucleotide sequencing is a better option over the biochemical characterization for confirmation.

Additionally, there has been increasing trend of vaccination against MG to control this disease, thus there is need to differentiate wild-type (field strain) of *M. gallisepticum* from the vaccine strains. In earlier studies sequencing of IGSR region of *M. gallisepticum* has been proved to facilitate strain differentiation, allowing the better discriminatory power of MG strains (Armour *et al*., 2013). Keeping in view the above facts, the present study was conducted to culture the avian mycoplasmas followed by their confirmation through PCR and gene sequence analysis.

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MATERIALS AND METHODS

Tissue specimens

Pooled tissue samples were collected at necropsy from 92 broiler chicken flocks which were suspected for avian mycoplasmosis and were belonged to 92 different poultry farms which were brought to the Disease Investigation Laboratory, LUVAS, Hisar from different parts of Haryana and adjoining area of Rajasthan (India) for disease diagnosis (Table 1). From each flock, pieces of trachea, lungs and air sacs (1-2 gm each) from at least 4-5 birds were collected and pooled to make one sample. From one flock, only one pooled sample was prepared. Thus, a total of 92 pooled samples were collected from 92 broiler chicken flocks. Pooled samples will hereafter be referred as 'samples'.

Isolation of avian mycoplasmas/*Mollicutes*

In the present study, two media consisting of Frey's medium with and without nicotinamide adenine dinucleotide (NAD) and cysteine hydrochloride were used for isolation of *M. synoviae* and *M. gallisepticum* respectively following standard culture method of (OIE, 2008) with some modifications. The slight modification in this process was before making serial dilution, triturated tissue suspension kept for 4h incubation at 37°C to increase the concentration of mycoplasma organisms. The biochemical and serological characterization of *Mollicutes* was done as per Clark *et al*., 1960. Diene's Staining of *Mollicutes* was done as described by Singh (1983). Further classification of *Mollicutes* to their respective genus of *Mycoplasma* and *Acholeplasma* was done on the basis of their sensitivity to digitonin and sterol requirement for their growth (Tully, 1983). These isolates were further characterized up to species level on the basis of different biochemical reactions and growth inhibition test as described by (Aluotto *et al*., 1970).

Molecular characterization of avian mycoplasmas

The extraction of genomic DNA from the broth culture of above field isolates was carried out by boiling, as per (Rauf *et al*., 2013). The *M. gallisepticum* and *M. synoviae* antigen (Soleil, Biovac, France) were used as positive controls to carry out PCR specific to genus *Mycoplasma* as well as MG and MS species respectively. The PCR was carried out on extracted DNA samples from field isolates of *Mollicutes* to amplify the 16S rRNA gene fragment specific to Genus *Mycoplasma*, as well as *M. gallisepticum and M. synoviae* species using respective sets of published primers (Zhi *et al*., 2010; Raviv *et al.,* 2007; OIE, 2008). Details of the primers used are given in Table 2. The PCR reaction mix (25 µl) contained 0.5 µl of each primer (10 µM), 12.5 µl of Master mix (HotstarTaq master mix 2X, Qiagen), 10.5 µl of nuclease free water (NFW) and 1 µl of template DNA. Amplification of 16S rRNA specific to Genus *Mycoplasma* was done using the protocol as described by Zhi *et al.* (2010). For amplification of DNA of MG two sets of primers were used. For the first primer (16S rRNA) amplification of *M. gallisepticum* DNA was done as per the method of (OIE, 2008) and for the second primer (16S-23S rRNA) protocol of (Raviv *et al*., 2007) was followed. Amplification for *M. synoviae* DNA was done as per OIE, (2008).

Nucleotide sequencing

PCR product of each isolate was purified from the gel directly using the Gel Extraction Kit (QIAquick®, Qiagen, Germany)

Table 1: Prevalence of *Mollicutes*, *M. gallisepticum* (MG) and *M. synoviae* (MS) in poultry flocks of Haryana and adjoining area of Rajasthan.

Location of poultry farms	No. of flocks tested	Mollicutes culture (%) confirmed by genus specific PCR	MG positive culture (%) confirmed by MG specific PCR	MS positive culture (%) confirmed by MS specific PCR
Hisar	18	6 (33.33)	5 (27.77)	-
Hansi	2	-	-	-
Jind	10	2 (20)	-	1 (10)
Fatehabad	5	-	-	-
Meham	2	1 (50)	-	-
Bhiwani	10	3 (30)	2 (20)	-
Behal	6	1 (16.66)	1 (16.66)	-
Rohtak	8	-	-	-
Kaithal	7	-	-	-
Karnal	10	-	-	-
Mahendragarh	4	-	-	-
Jhunjhunu, Pilani and Ganganagar (Rajasthan)	10	-	-	-
Total	92	13 (14%)	8 (8.6%)	1 (1.08%)

Table 2: Detailed description of primers.

Primers used	Nature of primers	Polarity	Primers sequence	Amplicon size (base pairs)	Reference
Universal primer specific	16S	Forward	5'- GGCGAATGGGTGAGTAACACG- 3'	461	Zhi *et al.*
to Genus *Mycoplasma*	rRNA	Reverse	5'- CGGATAACGCTTGCGACCTATG- 3'		(2010)
	16S	Forward	5'- GAGCTAATCTGTAAAGTTGGTC- 3'	185	OIE
Species- specific	rRNA	Reverse	5'- GCTTCCTTGCGGTTAGCAAC- 3'		(2008)
primer for MG	IGSR (16S to	Forward	5'- GTAGGGCCGGTGATTGGAGTTA- 3'	812	Raviv *et al.*
	23S rRNA)	Reverse	5'- CCCGTAGCATTTCGCAGGTTTG- 3'		(2007)
Species- specific	16S	Forward	5'- GAGAAGCAAAATAGTGATATCA- 3'	207	OIE
primer for MS	rRNA	Reverse	5'- CAGTCGTCTCCGAAGTTAACAA- 3'		(2008)

following the manufacturer's recommendations. All the purified PCR products from 13 field isolates of mycoplasmas were sequenced on an automated DNA sequencer (3130 XL Genetic Analyzer, Applied Biosystem™, USA). The forward and reverse nucleotide sequences obtained after sequencing were aligned using MEGA 6 (molecular evolutionary genetic analysis) software by ClustalW method. The aligned sequences were analyzed on NCBI website http://www.ncbi.nlm.nih.gov using BLAST to confirm their identity. The isolates of present study were got sequenced and were submitted to NCBI for accession numbers through BankIt submission option. The accession numbers provided by NCBI for 8 *M. gallisepticum* isolates were KX759101, KX759102, KX759103, KX759104, KX759105, KX759106, KX759107 and KX759108, for one *A. laidlawii* isolate it was KY486502, for 3 *M. gallinarum* isolates KY486503, KY486504 and KY486505 and for *M. synoviae* it was KY486506. Twenty-four unrelated strains/isolates of MG including live MG vaccine strains (F-strain, ts-11 and 6/85) and reference strain of MG were downloaded from http://www.ncbi.nlm.nih.gov/genbank for comparison with our isolates sequences and to construct dendrogram using MEGA 7 software by ClustalW method. The evolutionary distances were computed by pairwise distance matrix using the Maximum Composite Likelihood Method. A phylogenetic tree of aligned sequences was constructed by Neighbor-Joining method (1000 replicates for bootstrap). Percentage identity was computed by MegAlign™ program (Lasergene®; DNASTAR, Madison, WI, USA).

RESULTS AND DISCUSSION

Avian mycoplasmosis causes huge economic losses in the poultry industry. Considering the economic importance and increased incidence of avian mycoplasmosis in broiler chickens, the present study was designed to know the cultural prevalence of MG and MS followed by PCR and nucleotide sequencing in broiler chickens of Haryana (India).

In this study, the reported cultural incidence of *Mollicutes* was 14% and were showing typical nipple shaped and small sized colonies with fried egg appearance visualized under the stereomicroscope at 40x magnification which was further confirmed using Diene's staining (Fig 1) and genus-specific PCR (16S rRNA) (Table 1). Similarly, in Kuwait, 14% prevalence of *Mollicutes* was noted from poultry affected with respiratory infections (Qasem *et al.,* 2015). However, earlier studies in India had reported higher isolation rate (27%) of *Mollicutes* (Rauf *et al*., 2013). The reported high incidence of *Mycoplasma*/PPLO in healthy or CRD affected birds by the researchers may be due to the difference in cultivation medium and lack of clear demarcation between pathogenic and non pathogenic mycoplasmas during reporting. High population density in the intensive system and hot and humid climate also increases the infection rate of mycoplasmas. On the contrary, in the recent past, several workers have reported a lower prevalence of *Mollicutes* ranging from 1.63% to 10.66% from poultry flocks affected with the respiratory syndrome (Ramdass *et al*., 2006; Barot 2011; Bibak *et al.,* 2013) as compared to the present study. Lower prevalence can be due to the fastidious nature of mycoplasmas and use of different culture media for isolation as they are likely to die during culture processing.

Based on biochemical reactions, growth inhibition test, PCR and/or sequencing, these 13 isolates could be characterized as 8 (8.6%) *M. gallisepticum*, 1 (1.08%) *M. synoviae*, 3 (3.24%) *M. gallinarum* and 1 (1.08%) *A. Laidlawii* (Fig 2). The 8.6% cultural prevalence of MG in the present investigation is in agreement with the finding of earlier studies of India (Barot, 2011) who reported 6.9% and 7.14% MG incidence from poultry affected with the respiratory disease in Uttar Pradesh (Bareilly). However higher prevalence of MG (14%-21%) from poultry flocks affected with CRD was reported in Pakistan and Kuwait (Rauf *et al*. 2013; Qasem *et al*. 2015). On the other hand other researchers reported lower cultural prevalence of MG (1.05%, 2.3%) from poultry farms in India and Sudan which might be due to difference in the type of samples and season of sample collection.

In the present study although the low prevalence of *M. synoviae* (1.08%) was recorded by cultural isolation yet it seems to be the first report of isolation of *M. synoviae* from chicken affected with respiratory infections in northern region of the country. Similarly, in Jordan and India (Chennai) MS isolation was reported up to 2.3% and 2.4% respectively from tracheal swabs of chicken flocks affected with respiratory infections (Khalifa *et al*., 2013; Ramadass *et al.,* 2006).

Although both *M. gallinarum* and *A. laidlawii* are usually considered as non- pathogenic mycoplasmas but sometimes serve as a cofactor for pathogenic respiratory viral infections in poultry. In this study, their isolation from the chicken affected with respiratory infections appears to be the first

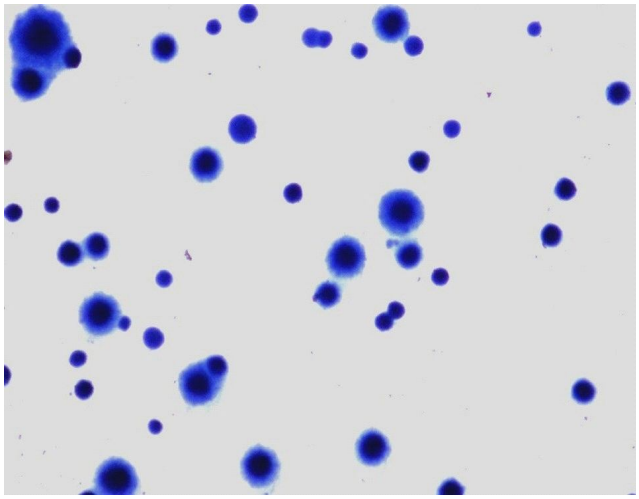

Fig 1: Characteristic colonial appearance of *Mycoplasma gallisepticum* on glass slide stained with Diene's stain showing light blue periphery and dark blue centre having typical nipple shaped small sized colonies (x40).

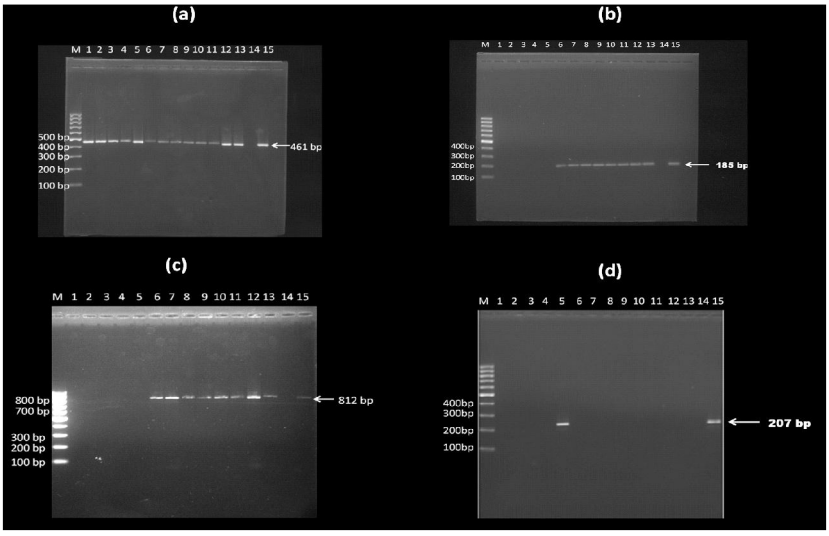

Fig 2: Agarose gel electrophoresis of PCR products of field isolate showed the presence of *Mycoplasma* species as evident by a band of 461 bp using universal primer specific to Genus *Mycoplasma* (16S rRNA) (a), 185 bp using species-specific primer for MG (16S rRNA) (b), 812 bp using species-specific primer for MG (IGSR) (c), 207 bp using species-specific primer for MS (16S rRNA) (d). Lane M = 100 bp marker, Lane 1-13= Field isolates. Lane 14 = Non template control, Lane 15= Positive control.

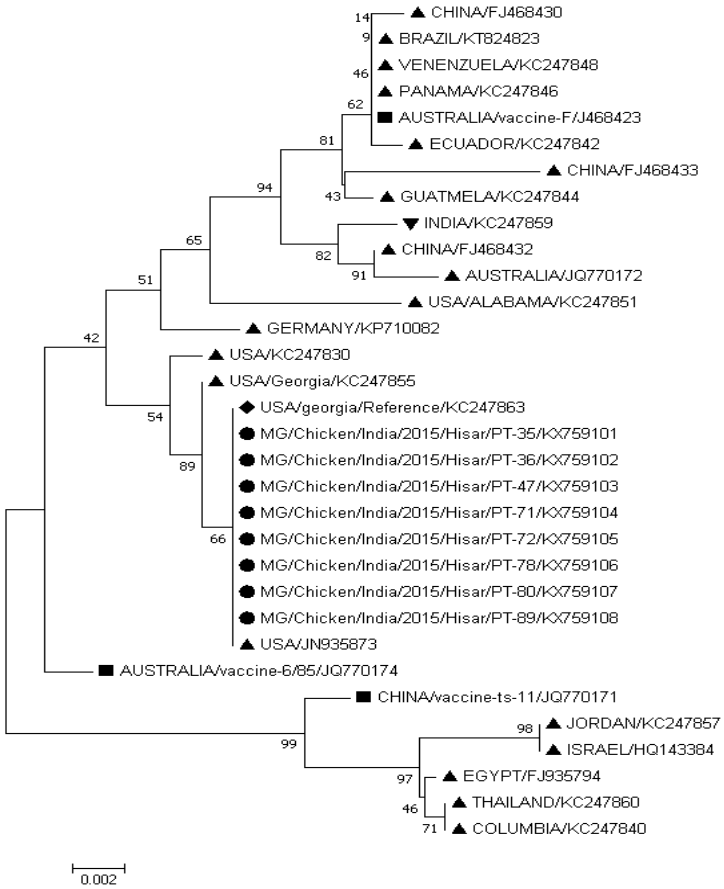

Fig 3: Phylogenetic tree based on partial nucleotide sequences of IGSR (16S-23S rRNA) region of *M. gallisepticum.* Phylogenetic tree constructed by the neighbour joining method using 1000 bootstrap replicates value in Mega7 software. Sequences which are labeled as circles are from the present study, squares for vaccine strains, rhomboid for reference strain; upside down triangles for Indian isolates and upright triangles for other previously published sequences.

report in India. In the present study, low cultural prevalence (3.24%) of *M. gallinarum* was observed. The however, there are reports of higher prevalence of *M. gallinarum* (47%) at poultry farms of Yugoslavia using cultural isolation (Bencina *et al*., 1987). Similarly, higher incidence (20%) of *M. gallinarum* has been reported in chicken flocks of Egypt (Eissa *et al.*, 2009). The current study revealed 1% prevalence of *A. laidlawii* which is in agreement with the findings of the previous study (Sayed *et al.*, 1981) in India (Haryana) which reported the low prevalence of *A. laidlawii* (2.94%) by culture from turkeys affected with sinusitis. However higher incidence of *A. laidlawii* (20%) in healthy chicken flocks of Egypt was also reported which indicates non pathogenic nature of the organism (Eissa *et al*., 2009).

This is the first study in India using single locus sequence typing (SLST) tool for differentiation of Indian field MG isolates from vaccine strains using Intergenic spacer region (16S rRNA - 23S rRNA). Previously, Raviv *et al*. (2007) reported that even one base variation is considerable for isolate differentiation. Based on SLST, it was found that all the MG isolates of current study were wild, suggested by presence of multiple nucleotide base variation from the vaccine strains in IGSR whereas very high similarity with the wild strains. The phylogenetic analysis based on the sequences of MG isolates using IGSR showed that the 8 MG field isolates were closer to the sequences of USA strains than the strains from Tamil Nadu/India and vaccine strains (Fig 3), which is in close agreement with the earlier findings (Singh, 2013) who suggested that U.S strains of MG may be circulating in Haryana region. This closeness of sequences of field isolates from India and those from USA may be due to increasing poultry trade between USA and India. There were very small changes observed in 16S rRNA partial gene sequence of MS isolate. The 16S rRNA sequence is highly conserved among the same species of *Mycoplasma*, therefore, the phylogenetic analysis of these sequence is not of much importance.

CONCLUSION

From the above study, it is concluded that although conventional culture technique is difficult yet it gives a confirmatory diagnosis and it is the gold standard test for diagnosis of animal and poultry mycoplasmosis. Secondly, a high infection rate of MG confirms the endemic nature of the disease in Haryana and it also indicates that majority of isolates are very virulent which is evident from heavy mortality that had been reported in poultry farms. It was also observed that PCR/Sequencing is a better tool in differentiating species and strains of avian mycoplasmas from culture media. Conclusively, the present study provides an epidemiological analysis of field strains of mycoplasmas in poultry in Haryana (India) by isolation and molecular characterization. The sequencing of IGSR region of MG has proved to be a valuable tool in epidemiological investigation and revealed considerable genotypic polymorphism which can aid in the differentiation of wild-type MG strains from vaccine strains.

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