Detection of *Mycoplasma gallinaceum* by PCR amplification of the 16S rRNA gene from respiratory disease in village chickens

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**ABSTRACT**

In the present study, *Mycoplasma gallinaceum* was detected by PCR amplification of 16S rRNA gene from chronic respiratory disease in village chickens of Cauvery delta region of Tamil Nadu. Necropsy was performed to find out the etiological agent in desi birds mortality. At necropsy, airsacculitis with caseous exudate were found in the thoracic and abdominal cavity. Caseous material from airsacs was collected aseptically from dead birds for detection of *Mycoplasma* species. DNA was extracted from caseous material by using tissue DNA extraction kit. PCR was carried out using primers to amplify 16S rRNA gene belonging to *Mycoplasma* species. The amplified product yielded approximately 700-bp length (703 to 713 bp) of the 16S rRNA gene specific for *Mycoplasma* species. Further, it was subjected to sequence analysis and confirmed as *Mycoplasma gallinaceum* by NCBI blast analysis. In the present communication, detection of *M. gallinaceum* by PCR amplification of 16S rRNA gene provides a powerful tool for rapid diagnosis.

**Key words:** *M. Gallinaceum*, 16S rRNA sequence analysis, Native chicken.

**INTRODUCTION**

*Mycoplasmas* are the smallest prokaryotic cell belongs to *Mollicutes* and pleomorphic organism varies spherical to filamentous in nature. *Mycoplasma* infection in chickens was primarily described in 1936 (Charlton et al. 1936). *Mycoplasmas* belongs to the *Mycoplasmataceae*, are broadly disseminated in most of the hosts include mammals, birds, reptiles, fish, humans and other vertebrates as well as plants (Razin et al. 1998). More than 200 species belong to the genus *Mycoplasma* were identified. Out of these, more than 20 species are identified to infect avian hosts. Important pathogens associated with avian Mycoplasmosis are *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) (Raviv and Ley, 2013). *Mycoplasma gallisepticum* cause chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys (Ferguson and Noormohammadi, 2013; Raviv and Ley, 2013), CRD is characterized by respiratory rales, coughing, nasal discharges and in association with other pathogens cause huge economic losses to the poultry farming sector in terms of mortality, decreased production, increased cost of medication and reduced feed conversion. *Mycoplasma synoviae* (Ms) cause infectious synovitis which is associated with synovitis and airsacculitis in chickens.

It has been reported that many techniques such as cultivation, serological assays and molecular tests were employed for diagnosis of MG and MS infections in poultry (OIE, 2008). As described by Kleven (2008a), cultural techniques are laborious, time consuming and expensive and require sterile conditions. Further, rapidly growing *Mycoplasma* species and no growth in subculture are the limitations associated with isolation and identification by cultural methods.

Serological tests include the rapid slide agglutination test, haemagglutination inhibition test and ELISA is usually performed for diagnosis of Mycoplasmal infections. However, the results of serological procedures are hindered by lack of specificity and sensitivity (OIE, 2008).

With the advancement of molecular based techniques, PCR was generally used for rapid detection of the avian *Mycoplasma* and act as alternative diagnostic tool (Nascimento et al., 1991; Lauerman et al., 1993; García et al., 2005). Though species-specific PCRs to determine all *Mycoplasmas* associated with respiratory tract infections would be a complicated undertaking.

It has been reported that amplification of a part of the 16S rRNA gene by PCR, followed by nucleotide sequencing and phylogenetic analysis has become a generalized technique for both the classification and identification of etiological agents. (Yoshida et al. 2002)

In the present communication, detection of *M. gallinaceum* by PCR amplification of 16s rRNA gene and phylogenetic analysis provides a powerful tool for rapid diagnosis and also the basis for etiological studies of chronic respiratory disease in chickens.

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MATERIALS AND METHODS

Sample collection: Around 200 village chicken aged 10 weeks were maintained as backyard farming system in Ambalapattu north village, Orathanadu taluk, Thanjavur district, Tamil Nadu. Mortality with history of dullness, respiratory sound and mucous discharge from the nostrils were observed in the flock. On postmortem examination, Caseous exudate from abdominal and thoracic airsacs were noticed and collected aseptically for rapid detection of Mycoplasmosis.

DNA extraction: DNA was extracted directly from caseous material by using DNeasy blood and tissue extraction kit (Qiagen) as per manufacturer’s instruction (Gondal et al. 2015). DNA was stored at -20°C until for further analysis. Then, Polymerase chain reaction was carried out by using primers to amplify 16S rRNA gene belonging to Mycoplasma species. In this study, a forward primer, GPO-1 (5’-ACTCTTACGGAGGCAGCAGTA-3’) and a reverse primer, MGSO (5’-TGCACATCTGTC ACTCTGTTAA CCTC-3’) previously designed by van Kuppeveld et al. 1992 modified by Lierz et al. 2007 were used to amplify an approximately 700-bp length (703 to 713 bp) of the 16S rRNA gene. The PCR reactions were carried out in 25 µL volume of 12.5 µL of master mix (2x), 1 µL of each primer (10 pmol µL-1), 7.5 µL of deionized water and 3 µL of extracted DNA. Thermal condition of amplification included initial denaturation of 94°C for 30 sec (denaturation), 64°C for 30 sec (annealing) and 72°C for 60 sec (elongation) for 40 cycles (Yoshida et al. 2002). The analysis of PCR product was carried out in 1.5 per cent agarose gel stained with ethidium bromide (0.5µg/ml) and documented under Gel documentation system (Osman et al. 2009). The amplicons were purified by using HiYield plus PCR purification kit (cat#QPP100) following manufacturer’s instruction.

Nucleotide Sequencing and data analysis: The purified PCR amplicons were subjected to sequencing by Sanger dideoxy sequencing method in an automated sequencer. The multiple sequence alignment was carried out using the Mega 5.2 software to generate sequence analysis data. The sequences were aligned by multiple sequence alignment by Clustal W. The phylogenetic tree was constructed to estimate the relationship between sequences by using Neighbor Joining (NJ) algorithm using bootstrap values and distance in Mega 5.2 software (Tamura et al. 2011). Homology searches were performed with the NCBI database and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

PCR amplification of 16S rRNA gene by using forward primer, GPO-1 and a reverse primer, MGSO of Mycoplasma genus specific yielded approximately 700bp length. The PCR results were in agreement with Tabatabaei et al. (2014) shown in the Fig 1.

The partial sequence of 16S rRNA gene of uncultured Mycoplasma species of chicken was submitted to GenBank and the accession number MH035883 was obtained. The phylogenetic tree showed a close cluster with other M. gallinarum isolates available in NCBI database and also with ATCC strains 33550 (Accession No. NR_025913.1) than M. anseris alpingitis, M. pullorum, M. edwardii, M. canis and M. glycophillum strains (Fig 2).

The 16S rRNA gene of uncultured Mycoplasma sp. sequences of the present study showed 99 per cent similarity with the sequence of the duck isolate from USA (Accession No. JN935867.1) and chicken isolate of South Africa (Accession No. MF196167.1). It showed 96 per cent similarity with Mycoplasma pullorum (Accession No. MF196179.1) as demonstrated by blast (NCBI [Internet]. Bethesda (MD) 1988).

Fig 1: M-100bp ladder, 1 and 2 positive samples with a band at 700bp with Mycoplasma genus specific 16S rRNA gene primers

Fig 2: Phylogenetic tree based on 16S rRNA gene sequence of Mycoplasma gallinarum strain OND isolate
In the present study Mycoplasma gallinaceum was detected in the respiratory tract infections of village chicken while Mycoplasma gallisepticum (Mg) and Mycoplasma synoviae (Ms) are thought to be associated with clinical infections in poultry. Many studies have been reported that Mycoplasma gallisepticum is the most pathogenic avian Mycoplasma species which cause chronic respiratory disease in poultry results in significant economic burden to the poultry rearing countries worldwide (Raviv and Ley, 2013). Mycoplasma synoviae is associated with upper respiratory disease, airsacculitis, exudative synovitis, tendovaginitis, or bursitis (Ferguson and Noormohammadi, 2013; Senthilnathan et al. 2015).

In the present study DNA was extracted directly from the clinical samples for detection of Mycoplasma species by PCR (Gondal et al. 2015). Though cultural isolation and identification methods are laborious, time consuming, expensive and also problems encountered during culture include overgrowth by rapidly growing Mycoplasma species or other organisms. The Serological tests are the tests used for the detection specific antibodies against Mycoplasma. Limitations associated with serological assays are low specific and sensitive (Kleven, 2008b; OIE, 2008).

In the present study, Mycoplasma gallinaceum was detected by PCR amplification of 16S rRNA gene of Mycoplasma species. Several studies have been reported that detection of 16s rRNA gene of Mycoplasma species by PCR (Franay et al. 2018, Rasoulinezhad et al. 2017). As been reported by Lecis et al. (2016) Mycoplasma species have been detected in birds of prey by sequence analysis of 16S rRNA, 16S-23S rRNA intergenic spacer and RNA polymerase β subunit (rpoB) gene. Mycoplasma gallinaceum was first illustrated in 1982 as a serovar. It has been reported that Mycoplasma gallinaceum ferments glucose, not tetrazolium chloride, does not utilize arginine or urea and low G+C content of 29% (Jordan et al. 1982). M. gallinaceum thought that rapidly growing Mycoplasma species and not considered as major pathogenic avian mycoplasma species (Ahmed, 2016). Despite, its nonpathogenic nature, some researchers have reported a pathogenic role for this bacterium. Some Research reports have been published linking isolation of M. gallinaceum from the upper respiratory tract of affected chickens including the eyes and sinuses (Abolnik and Beylefeld, 2015). M. gallinaceum has also been isolated from the reproductive tract of laying hens (Wang et al. 1990). M. gallinaceum was formerly concerned as the cause of avian mycoplasmosis in pheasants and partridges with incidence of respiratory disease (Bradbury et al., 2001). Abolnik and Beylefeld, (2015) has isolated M. gallinaceum from 62 week old laying hens with typical Mycoplasmal infection symptoms and the complete genome of 8, 45,307 bp was sequenced, assembled and annotated. Adeyemi et al. (2017) reported that M. gallinaceum alone is non pathogenic in chickens, however it augments the pathogenicity of other respiratory pathogens as co-infections.

Adeyemi et al. (2017) has reported that M.gallinaceum and M.gallisepticum appeared to enhance infectious bronchitis virus replication in tracheas of infected chickens. M.gallinaceum was capable to aggravate the respiratory disease and pathogen proliferation when challenged with M. gallinaceum and QX-like infectious bronchitis virus.

In the present study revealed that detection of M. gallinaceum from respiratory tract infection in village chicken represent the pathogenic potential of this pathogen needs to be further studied.

CONCLUSION

In the present study, M.gallinaceum was detected from respiratory disease in village chicken by phylogeny based PCR amplification of 16S rRNA gene of Mycoplasma species. It provides rapid detection, specific and sensitive compared to conventional methods while cultural isolation and identification requires time consuming, laborious and expensive. It can serve as basis for further pathogenic studies of this pathogen associated with multifactorial avian Mycoplasmosis.

REFERENCES


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