



Molecular Detection of *Mycoplasma synoviae* Infection in Poultry

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Authors' contributions

This work was carried out in collaboration among all authors. Author PLL designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author RSG managed the analyses of the study. Author SBM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Mycoplasma synoviae (MS) is an economically important pathogen of poultry, causing respiratory infection and synovitis in chickens and turkeys. Early detection of MS infection is of critical importance for subsequent prevention and control measures; in this view the present research was undertaken for detection of MS infection in the poultry birds from selected farms of India. A total of 165 choanal cleft swabs from chickens (48 broilers + 117 layers) suspected for Mycoplasma infection were processed for direct detection of MS infection by PCR and cultural isolation. Out of 165 choanal cleft swabs subjected to species specific 16S rRNA PCR, MS was detected in overall 4.2% of specimens with incidence rate of 3.4% and 6.25% in layers and broilers respectively. In *vlhA* gene PCR, a gene fragment ranging from ~360bp to ~380bp was amplified in all seven 16S rRNA positive samples. The amplicon size varied in samples belonging to different geographical regions i.e. samples from Maharashtra region yielded ~360 bp product whereas from Delhi and Kolkata regions the PCR product was of ~380bp. The blast analysis of sequences of representative sample showed the 99.34% identity with *vlhA* gene of various MS strains including MS-H strain. The GenBank accession no. MN602747 obtained from NCBI. In cultural isolation all 165 samples including seven PCR positive specimens were turned out to be negative for recovery of MS. Thus, PCR was found to be a rapid, simple, sensitive and cost effective alternative to cultural isolation.

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1. INTRODUCTION

M. synoviae (MS) is an important pathogen of domestic poultry, causing economic losses to the poultry industry due to decreased egg production, growth and hatchability rates and significant downgrading of carcasses due to air sacculitis and arthritis lesions [1]. As the vertical transmission plays a major role in the spread of MS in chickens, the most effective control method is to monitor the flocks regularly and also eliminate the positive breeder flocks. Reliable and rapid diagnoses are needed to prevent infection dissemination [2]. Positive diagnosis may be made by isolation and identification of MS, but isolation and cultivation of MS is very tedious, difficult because of its fastidious nature and complex nutritional requirement and may not give the accurate prevalence [3].

Polymerase chain reaction is a simple, rapid and highly sensitive method of detection of MS-DNA in tissues or culture medium; moreover PCR procedure is comparable in sensitivity to isolation and identification [4,5]. The Polymerase Chain Reaction (PCR) with *Mycoplasma* 16S ribosomal RNA has been applied to detect a variety of *Mycoplasma* species [6,7]. Rapid diagnosis and identification of infections is very important in the poultry industry. The use of PCR makes it possible to shorten the time for obtaining result from research and the effective detection of genetic material of MS. PCR based techniques are now routinely used for detecting pathogenic avian mycoplasmas directly from swabs [8]. Recently several PCR assays have been used for the amplification of the conserved *vlhA* gene of MS [9]. PCR targeting the *vlhA* gene can be also used to type strains of MS due to sequence variability in the N-terminal region of the gene among strains [10,11,8]. However, in spite of being such an important pathogen; very scarce literature on strains of MS and prevalence data is available in India. Therefore, the current study was planned to screen birds from different farms for the presence of MS infection using PCR.

2. MATERIALS AND METHODS

The media and reagents required for cultural isolation of MS were from M/s Hi Media Laboratories Private Limited, Mumbai (India). The reagents used for PCR assay were from

M/s Sigma Aldrich Chemicals Private Limited (USA).

2.1 Collection of Clinical Specimens

A total of 165 choanal cleft swabs from live birds suspected for mycoplasmosis with respiratory symptoms formed the material for investigation under the present study. The samples were collected during the year 2016 from live broiler and layer poultry birds of WLH and RIR breed housed in the different geographical areas of India. The clinical specimens were collected in duplicate for performing PCR and for isolation of MS. The choanal (palatine cleft) swabs were collected from live birds exhibiting respiratory symptoms by opening the beak of birds, inserting and rubbing the sterile swab in choanal cleft (Fig. 1). Before sampling, the swabs were dipped in Pleuropneumonia Like Organism (PPLO) broth supplemented with Nicotinamide adenine dinucleotide (NAD) and Cysteine in order to create optimal conditions for the organism during transport to the laboratory [12].



Fig 1. Collection of choanal swab from poultry

2.2 Molecular Detection of *M. synoviae* Directly in Clinical Specimens

DNA extracted from all 165 choanal swabs [13] was subjected to PCR using 16S rRNA gene primer [12]. The reaction mixture was prepared in 25 μ l [H₂O Ultra-pure 17.1, 10X PCR Buffer 2.5, dNTPs (10 mM) 0.8, F Primer (20 pmole/ μ l) 0.6, R Primer (20 pmole/ μ l) 0.6, Taq Polymerase (5 U/ μ l) 0.4, MgCl₂ (50 mM) 2.5, DNA 0.5] and the cycling conditions of denaturation at 94°C for 5 minutes, annealing at 52°C for 30 seconds

extension at 72°C for 45 minutes followed by final extension at 72°C for 5 min.

Primer	Oligonucleotide sequence
MS-F	5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'
MS-R	5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'

The *VlhA* gene PCR was carried out using the published primer [14]. The reaction mixture of 25µl [H₂O Ultra-pure 18.0, 10X PCR Buffer 2.5, dNTPs (10 mM) 1.0, F Primer (20 pmole/µl) 0.5, R Primer (20 pmole/µl) 0.5, Taq (5 U/µl) 0.4, MgCl₂ (50 mM) 1.5, DNA 0.6] with cycling conditions of initial denaturation at 94°C for 5 minutes followed by subsequent denaturation at 94°C for 30 sec. Annealing at 46°C for 30 sec, extension at 72°C for 45 sec followed by final extension at 72°C for 5 min.

Primer	Oligonucleotide sequence
<i>vlhA</i> -F	5'-GATGCGTAAAATAAAAGGAT-3'
<i>vlhA</i> -R	5'-GCTTCTGTTGTAGTTGCTTC-3'

The Sequencing of representative PCR products of *vlhA* gene of MS was carried out with ABI Big Dye Terminator Kit version 3.1 using automated genetic analyser at Aavanira Biotech Pvt. Ltd., India. The sequences were subjected to

BLAST analysis using BLASTn algorithm and the sequences were submitted to GenBank for obtaining the accession number.

2.3 Isolation and Identification of *M. synoviae* from Clinical Specimens

A total of 165 choanal swabs collected from live birds suspected for MS were inoculated directly in PPLO broth and incubated at 37°C in presence of 5% CO₂ tension. The inoculated tubes were regularly observed for the change of red colour of the broth to pale yellow or orange as evident for growth. The samples showing no change in colour even after 20 days were considered as negative. The broth cultures which exhibited colour change from red to yellow were transferred on the agar plates and incubated at 37°C at 5% CO₂ for further investigation.

3. RESULTS

Out of 165 samples processed for 16S *rRNA* MS specific PCR, 7 (4.2%) were found to be positive for MS yielding the amplicon of approximately 207 bp (Fig. 2). Out of 117 samples from layers and 48 from broilers, 4 (3.4%) and 3 (6.25%) yielded positive results in 16S *rRNA* PCR

respectively with an overall incidence of 4.2%. Out of 20 samples analysed from Delhi region, 2 (10%) were found to be positive for MS, 2 (5.4%) of 37 were positive from Kolkata region and 3 (2.77%) out of 108 samples tested were positive from Maharashtra region. However, MS could not be recovered in cultural isolation from any of the 165 clinical samples.

To detect variations in the strains of MS belonging to different geographical area, 16S *rRNA* PCR positive DNA of seven samples were subjected to surface variable lipoprotein-haemagglutinin (*vlhA*) gene PCR along with the reference strain. All seven samples positive in 16S *rRNA* PCR were also found to be positive for *vlhA* gene of MS (Fig. 3). The amplicon size of *vlhA* gene varied in the size ranging from ~360bp to ~380bp in the specimens originating from different geographical regions. The strains from Maharashtra region yielded PCR product of ~360bp, whereas Delhi and Kolkata regions produced the same amplicons of ~380bp size (Fig. 4). Thus, indicated the variation in the strains of Maharashtra and the strains of Delhi and Kolkata regions. The Phylogenetic analysis and the blast results of sequence of representative sample showed 99.34% identity with *vlhA* gene of various strains including MS-H strain. The GenBank accession no. MN602747 obtained from NCBI (Fig. 5).

4. DISCUSSION

M. synoviae (MS) is one of the most important and pathogenic poultry Mycoplasmas. MS infection occurs as a subclinical upper respiratory infection and outcome of disease is significantly affected by management factors and other respiratory pathogens. MS, more frequently produces a persistent infection of the upper respiratory tract causing decreased growth rate and loss of egg production [15]. An early and accurate detection of etiological agent and diagnosis of disease help in prevention and control of the infection in order to lower the economic burden on poultry farmers and to increase GDP of the country.

Various researchers have performed the identification of MS directly from clinical samples by targeting the 16S *rRNA* gene [16,17,18,19]. In the present study we also used 16S *rRNA* PCR assay which detected the overall incidence rate of 4.2% of MS, close to the reported incidence rates of 6.44% and 6.7% [20,18]. However, lower i.e. 1.8%, 0% [19,21] and higher

55.9%, 49.1% [3,22] incidence rate than in the present study is also reported. The variations in the incidence rate occur due to number of factors viz. managerial practices, other concurrent infections, parental stock etc.

The considerable variation occurs among isolates in their ability to cause disease and many isolates may cause little or no clinical disease [23]. The *vlhA* (variable lipoprotein and hemagglutinin) gene encodes hemagglutinin, an abundant immunodominant surface lipoprotein of MS [10,11]. *VlhA* protein is encoded by *vlhA* gene of which the 5'-end is present in the genome as a single, conserved copy [10]. In the present study PCR assay targeting *vlhA* gene was performed in total seven *16S rRNA* PCR positive clinical specimens (DNA) to know the

strain variation of MS. Expected gene fragment between ~350 to ~390bp was amplified in all seven samples [14]. The amplicon size of MS originating from different geographical regions varied, indicating strain differences. The strains from Maharashtra region yielded PCR product of ~360bp, whereas from Delhi and Kolkata produced ~380bp size amplicons. In one of the other study, oligonucleotide primers complementary to the single copy conserved 5' end of the *vlhA* could generate amplicons of approximately of 400 bp [11]. *VlhA* gene sequence analysis was applied for typing the MS vaccine MS-H strain and field isolates from diseased chickens in Japan [24]. Many workers have used *vlhA* gene amplification for detection of MS and differentiation among the strains [1,8,14,11,24]. The Phylogenetic analysis and

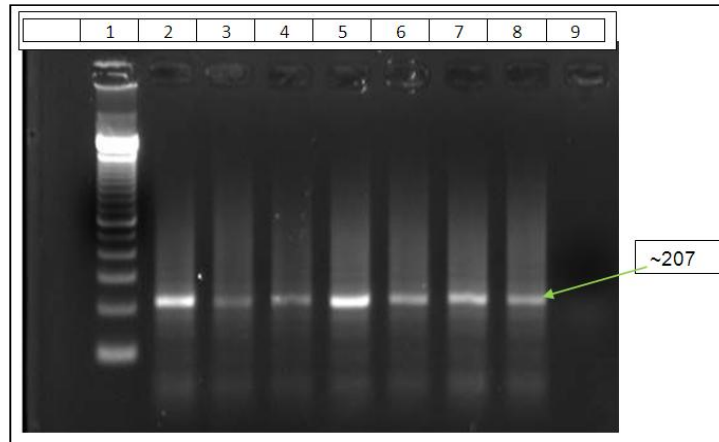


Fig. 2. *M. synoviae* species specific 16S rRNA PCR assay
 Lane 1: 100 bp ladder; 2: Reference strain of MS; 3-8: MS positive clinical specimens showing bands at ~207 bp; 9: Negative

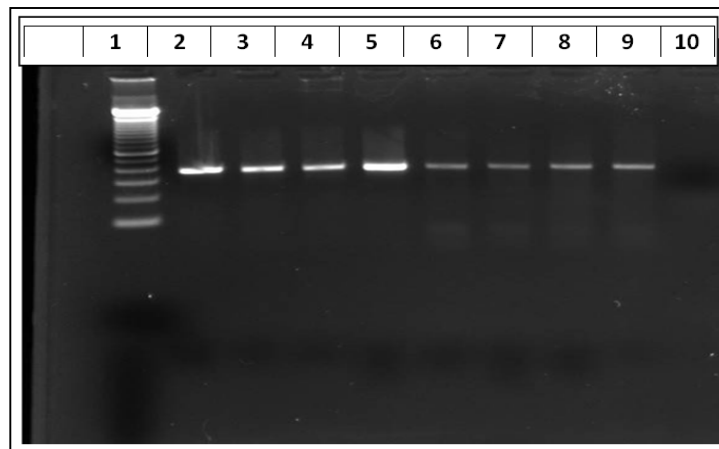


Fig. 3. *M. synoviae* species specific *vlhA* gene PCR assay
 Lane 1: 100 bp DNA ladder; 2-9: Positive clinical specimens for *vlhA* gene; 10: Negative control

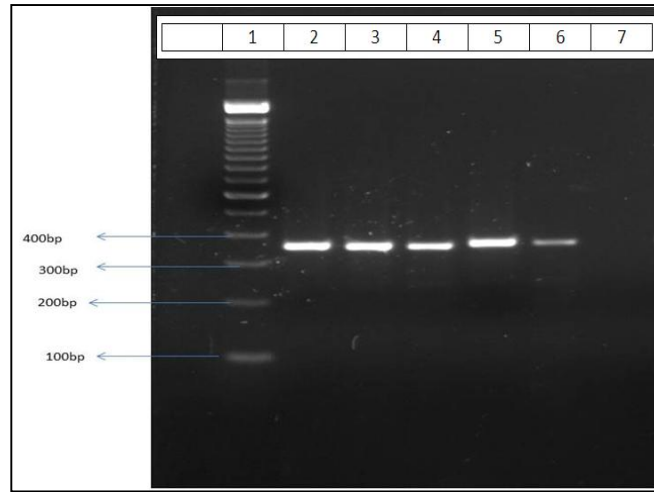


Fig. 4. *M. synoviae* species specific *vIhA* gene PCR assay
 Lane 1- 100bp ladder; 2: *vIhA* amplicons of the reference strain; 3-4: *vIhA* amplicons of MS samples from Maharashtra; 5: *vIhA* amplicons of MS samples from Delhi; 6: *vIhA* amplicons of MS samples from Kolkata; 7: Negative control

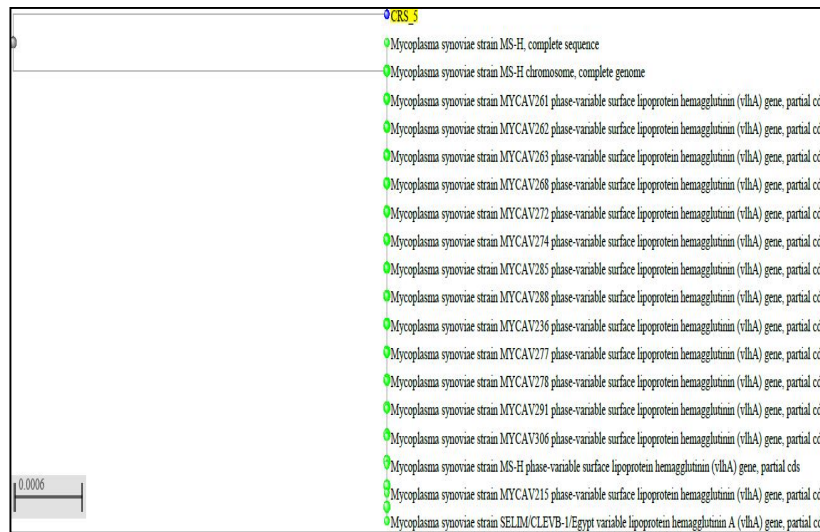


Fig. 5. Phylogenetic tree of *VihA* gene sequence of *M. synoviae*

the blast results of sequence of representative sample showed 99.34% identity with *vIhA* gene of various strains including MS-H strain.

In the present study, MS could not be isolated in *in-vitro* cultivation from any of the clinical samples including all seven 16S rRNA and *vIhA* gene PCR positive samples. The reason for failure in cultural isolation of MS may be due to the fastidious nature and complex nutritional requirement of MS and overgrowth of contaminants which makes cultural isolation very

tedious and difficult. In the present study the failure of isolation of MS may be due to overgrowth of contaminants. Thus cultural isolation may not give the accurate prevalence of MS the organisms in birds thereby increasing the chance of false negative results [3]. Many authors failed to isolate MS from clinical specimens [17,19]; although some authors could succeed in recovering very few MS isolates viz 1.76, 1.9%, 6.3% [18,20,25]. Cultural isolation of MS was found to be less sensitive, time consuming, costly and could not detect the true prevalence of MS [3].

5. CONCLUSION AND RECOMMENDATIONS

The results of the present study indicated the prevalence of MS infection due to different strains pertaining to specific geographical area of India under study. The incidence of MS in respiratory form of disease was higher in broilers than in layers. Polymerase Chain Reaction assay reflects the true picture of epidemiology of poultry mycoplasmosis with strain variability of MS. However, further investigation on large number of samples of broad geographical area is suggested for knowing molecular epidemiology and strains of MS by employing 16SrRNA and *vlhA* gene PCR followed by sequencing. Such study will help to plan the prevention and control strategies for poultry mycoplasmosis.

ETHICAL APPROVAL

As per international standard written ethical permission has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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