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Detection of *Mycoplasma* spp. from Choanal and Tracheal Samples using Genus Specific Polymerase Chain Reaction

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A B S T R A C T

Avian Mycoplasmosis is one of the most substantial global causes of economic losses to the poultry industry, particularly in chickens and turkeys. Present investigation was designed to detect *Mycoplasma species* from suspected broiler breeder/layer using genus specific Polymerase Chain Reaction (PCR). Hundred specimens including 50 each of choanal swabs and tracheal swabs were collected from five layer/breeder poultry farms from different areas of Maharashtra State. The swabs were inoculated in Pleuropneumonia Like Organism (PPLO) broth for isolation of Mycoplasma organisms. DNA extracted from specimens, broth cultures and reference strains (MG and MS) was amplified by 16S rRNA genus specific PCR yielding ~714bp products. Out of 100, 44/50 (88%) choanal swabs, 50/50 (100%) tracheal swabs were found positive for *Mycoplasma spp*., while, from broth cultures, 45/50 (90%) choanal swabs, 45/50 (90%) tracheal swabs were found positive by PCR. The 48 hr. old broth cultures yielded better results than PCR from direct specimens. Current study showed that genus specific 16S rRNA PCR was found to be sensitive for detection of *Mycoplasma* spp. at genus level in direct specimens and broth cultures. Tracheal swabs were better specimens for investigation of *Mycoplasma* infection in poultry.

Introduction

The expansion of poultry sector has led to the establishment of many allied industries. However, the poultry industry is facing multiple challenges due to increased morbidity and mortality of poultry caused by respiratory tract infections (Khatun *et al.*,

2018). Avian mycoplasmosis causes huge losses in broiler breeders due to reduced hatchability and egg production, stunted growth, increased cost of vaccination and medication and destruction of infected flocks under the National Poultry Improvement Plan of MG and MS clean programs (Khalifa *et al.*, 2013).

Among the mycoplasma species that colonize avian hosts, four avian mycoplasmas are commonly recognized as poultry pathogens viz. *M. synoviae* (MS), *M. gallisepticum* (MG), *M. iowae* (MI) and *M. meleagridis* (MM) (Raviv and Kleven, 2009). *Mycoplasma pullorum* was isolated from a seventy two week-old layer hen (Beylefeld and Abolnik, 2017).

Also the various species of genus mycoplasma were recognized from chickens viz. *M. gallinarum, M. gallisepticum, M. pullorum, M. gallinaccium, M. synoviae, M. iners* (3%), as well as one *Acheoplasma laidlawii* strain (Beylefeld *et al.,* 2018). Non-pathogenic Mycoplasma species are generally overlooked, because they have less impact on bird health and production and provide evidence of multidrug resistance traits (Beylefeld *et al.,* 2018).

Mycoplasma gallisepticum is a top ranking Mollicute, isolated from cases of chronic respiratory disease (Khatun *et al.,* 2018), which can get rapidly transmitted both horizontally and vertically (Jarquin *et al.,* 2009). MS causes infectious synovitis in both chicken and turkey and is also responsible for reduced egg production, poor growth and hatchability rate and significant condemnation of carcasses at slaughter (Marois *et al.,* 2000).

Although, transmission of *Mycoplasma synoviae* by vertical transmission has major role in chickens (Lockaby *et al.,* 1998, Kleven, 2003), transmission of MS via direct contact and respiratory aerosols is also reported (Hung-ChihKuo, 2016). The rapid and effective detection of mycoplasma is carried out by PCR based methods which have proven to be excellent for diagnosis of mycoplasmosis (Han Wang *et al.,* 1997; Feberwee *et al.,* 2005).

It is also a sensitive and specific technique which detects smallest amounts of DNA to a level that cannot be easily detected by other methods. Almost all species of the genera Mycoplasma, Spiroplasma, Acholeplasma and Ureaplasma can be detected by genus level PCR (Marois *et al.,* 2002). PCR provides more advantage over conventional methods, as it helps to detect pathogenic agents in samples taken from asymptomatic birds undergoing antibiotic treatment and also in case of immunosuppression (Garcia *et al.,* 1995; Kempf, 1998).

PCR is useful for detection of uncultivable pathogens directly from clinical samples and thus can replace conventional methods. It is considered to be more accurate, rapid and efficient for early identification of etiological agents (Hossam *et al.,* 2016).

Present study was aimed to detect Mycoplasma spp. from breeder/layer at the genus level. Therefore, present study was aimed to test feasibility of using Polymerase Chain Reaction for identification of Mycoplasma spp. directly from the tissues or swabs collected from breeder/layer poultry flocks.

Materials and Methods

Sample collection and preparation:

A total of 100 samples comprising of 50 each of choanal cleft swabs and tracheal swabs from the unvaccinated birds against mycoplasmosis were processed. Investigation

of five (05) layer/breeder poultry farms located in different regions of Maharashtra State was carried out for detection of Mycoplasma spp.

The specimens (choanal and tracheal swabs) were collected aseptically, in duplicates, from birds exhibiting either, history of disease, clinical signs, decreased egg production or reduced hatchability.

The swabs were placed in sterile leak-proof containers, packed in stout polystyrene foam containers and transported to the laboratory on ice (OIE, 2008).

DNA Extraction

The DNA from above clinical specimens were extracted as per the method described by Sambrook and Russell (2001). Briefly, the swabs were suspended in 0.5 ml of TE buffer, with 20 µl of Lysozyme (20 mg/ml) and incubated at 37˚C for 1 hr.

The mixture was added with 200 µl of 10 % SDS and 20 µl of proteinase-k (20 mg/ml) followed by incubation at 37˚C for 2hrs. Subsequently 200 µl NaCl (5M) and 200 µl CTAB (10%) was added and incubated at 65˚C for 20 min.

Equal amount of P:C:I (25:24:1) was added to the suspension and centrifuged at 10,000 rpm/10 min to extract the aqueous. Isopropanol (100%) and 3M sodium acetate (pH 5.2) in 0.6 and 0.1 volumes, respectively, was added. The mixture was left at -20˚C for at least 3 hrs (or overnight) followed by centrifugation at 12000 rpm for 10 min to collect the chromosomal DNA pellet.

Pellet was washed with 70% ethanol allowed to dry by keeping in incubator (37˚C) with lid open. The pellet was dissolved in 30µl of TE buffer/sterile milli-Q containing Rnase-A (20 µg/ml) and kept at 65˚C for 1 hr for proper dissolution and Rnase action. The isolated DNA was stored at -20˚C till further use.

Cultural method

The second set of samples, i. e. 50 specimens each of choanal and tracheal swabs were inoculated in Pleuropneumonia Like Organism (PPLO) MG and MS broth followed by providing the incubation conditions of 37˚C temperature, 5% CO_2 up-to 24-48 hrs.

Extraction of DNA from these broth cultures was performed as per the protocol described by Ley *et al.,* (1997) and Sambrook and Russell (2001) with slight modifications if required. The bacterial ceels were pelleted by high speed centrifugation for 20 mins. DNA was extracted by the method described above.

DNA Amplification

The extracted DNA were subjected to PCR targeting the genus 16S rDNA region of Mycoplasmal genome using the primers mentioned by Kuppeveld *et al.,* (1992). The primer used were GPO-1 (5'-ACT-CCT-ACG-GGA-GGC-AGC-AGT-A -3') and MGSO (5'-TGC-ACC-ATC-TGT-CAC-TCT-GTT-AACCTC -3').

PCR amplification was carried out in a 25 µl reaction comprising of 12.5µl of 2X Mastermix (Takara Bio India Private limited), 0.4 µl each of forward and reverse primers (20 pmol/ µl), 10.7µl nuclease free H_2O and 1.0µl of template DNA (50-100ng/µl).

Cycling conditions used for the amplification include: Initial denaturation at 95˚C for 5 min, 32cCycles of denaturation at 95˚C for 1 min, annealing at 54˚C for 1 min, extension at 72˚C for 1 min and a final extension at 72˚C for 5 min.

The PCR products were loaded on 2% agarose gel containing Ethidium bromide in final concentration of 0.5µg/ml and electrophoresed @ 5V/cm^2 of the gel. A 100bp DNA marker was electrophoresed simultaneously. The ensuing amplified DNA bands were visualised and documented using computerised automatic gel documentation device (Gel document EZ Imager, Bio-Rad).

Results and Discussion

Present study involved the diagnosis of avian mycoplasmosis in poultry directly from the clinical specimens viz. choanal and tracheal swabs. Mycoplasmas are fastidious organisms and difficult to propagate in vitro. Thus, polymerase chain reaction (PCR) can prove to be a better choice for accurate detection of the pathogen from clinical samples. Hundred samples (swabs) of choanal and tracheal regions were collected from live birds (Plates 1 & 2) showing symptoms of respiratory distress and also from apparently healthy birds. The presence of Mycoplasmas in the birds was determined using primers targeting the genus specific region 16S rDNA of Mycoplasma spp. The reference strains and positive samples showed a band of ~714 bp suggesting an amplification of 16S rDNA region of the genome (Plates 3 & 4).

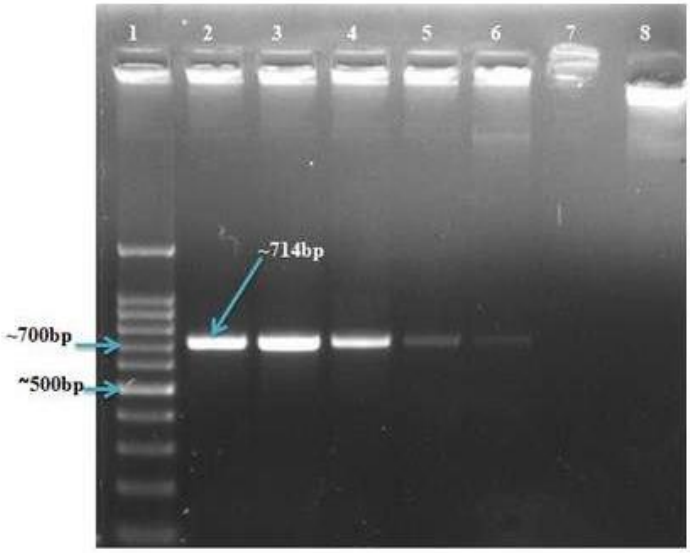
Out of 100 clinical specimens, 94 (94%) were found positive by PCR and remaining 6 (6%) were found negative. Out of these, 88% (44/50) specimens of choanal swabs and 100% (50/50) of the tracheal swabs were found positive for Mycoplasma spp. A second set 100 samples (50 each of choanal and tracheal swabs) from same birds was enriched with PPLO MG and MS broth at 37˚C under 5% CO_2.

The DNA extracted from these broth cultures were also subjected to PCR. Out of 100, 90 (90%) were found positive in the genus specific PCR showing ~714 bp amplicon and remaining 10 (10%) were found to be negative.

Overall, 90% (45/50) broth cultures each of choanal swabs and tracheal swabs were found positive for Mycoplasma spp. by PCR. The farm wise details of the samples collected and results of PCR are depicted in tables 1 and 2.

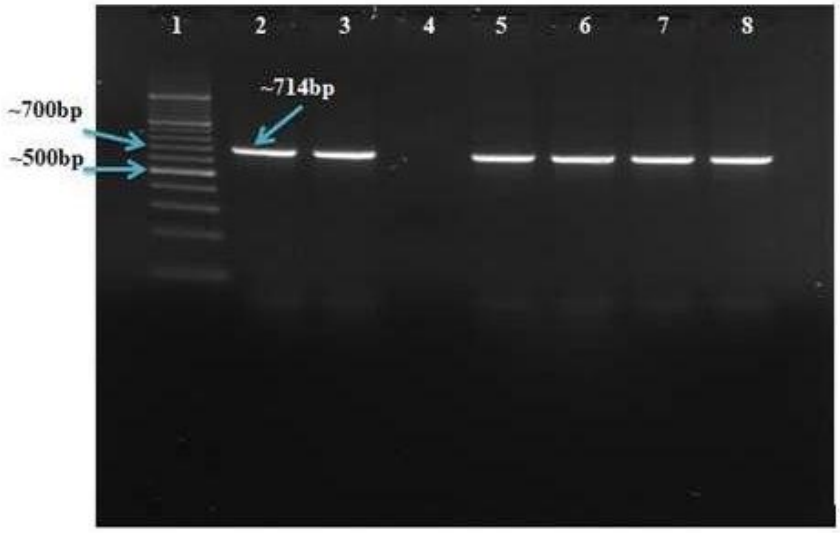
Plate.1 Collection of choanal cleft swab from live bird

Plate.2 Collection of tracheal swab from live bird



Lane 1- Ladder 100bp
Lane 2- Positive control (MG)
Lane 3- Positive control (MS)
Lane 4-6- Positive samples
Lane 7- Negative control
Lane 8- Non-template control

Plate.3 Genus specific 16S rRNA PCR of direct specimens



Lane 1- Ladder 100bp
Lane 2- Positive control (MG)
Lane 3- Positive control (MS)
Lane 4- Negative control
Lane 5-8 - samples

Plate.4 Genus specific 165 rRNA PCR of broth cultures

Table.1 Molecular detection of *Mycoplasma* spp. in direct clinical specimens by PCR

Source	Type of specimens	No. of specimens processed	No. of specimens Positive	% specimens Positive
Farm 1	Choanal swabs	05	04	80%
	Tracheal swabs	05	05	100%
Farm 2	Choanal swabs	10	06	60%
	Tracheal swabs	10	10	100%
Farm 3	Choanal swabs	10	10	100%
	Tracheal swabs	10	10	100%
Farm 4	Choanal swabs	10	09	90%
	Tracheal swabs	10	10	100%
Farm 5	Choanal swabs	15	15	100%
	Tracheal swabs	15	15	100%
	Total	**100**	**94**	**94%**

Table.2 Molecular detection of *Mycoplasma* spp. in broth cultures by PCR

Source	Type of specimens	No. of specimens processed	No. of specimens Positive	% specimens Positive
Farm 1	Choanal swabs	05	05	100%
	Tracheal swabs	05	05	100%
Farm 2	Choanal swabs	10	08	80%
	Tracheal swabs	10	08	80%
Farm 3	Choanal swabs	10	09	90%
	Tracheal swabs	10	07	70%
Farm 4	Choanal swabs	10	08	80%
	Tracheal swabs	10	10	100%
Farm 5	Choanal swabs	15	15	100%
	Tracheal swabs	15	15	100%
	Total	**100**	**90**	**90%**

Indian poultry sector is growing rapidly to provide required amount of food and animal proteins for society. In India commercial poultry is an important sector and with commercial sector, backyard poultry rearing is also increasing day by day.

However, the poultry sector is facing the problem of mycoplasma infection, which is to be addressed and taken care in order to reduce the economic losses in the industry and to poultry farmers.

Vertical transfer of Mycoplasma is also a very critical problem in the layer/breeder stocks. Moreover, transfer of infection in the hatchery is significant problem in commercial farms. Therefore, the present investigation was carried out for detection of Mycoplasma infection in the layer/breeder birds.

In the present study, 100 direct clinical specimens were subjected to the Mycoplasma genus specific PCR, which yielded a total of 94 specimens positive for Mycoplasma spp. with an incidence rate of 94%. Out of total 50 each specimens of choanal swabs, tracheal swabs a total of 44 (88%) and 50 (100%) respectively found positive by PCR.

Genus specific primers for Mycoplasma spp. identification are highly efficient and PCR is a consistent method commonly used for detection of poultry mycoplasmosis (Kupevald *et al.,* 1992; Nehe, 2018). Various authors viz. Garcia *et al.,* (1994), Fan *et al.,* (1995), Kiss *et al.,* (1997), Lienz *et al.,* (2007), Tomar *et al.,* (2017), Ghohestani *et al.,* (2018) also used 16S rRNA gene for detection of Mycoplasma spp. infection in poultry. Fan *et al.,* (1995) detected all Mycoplasma common species by the genus specific PCR by using 16S rRNA gene primer.

In the present study overall incidence of Mycoplasma spp. was found to be 94%. However, lower percentage of Mycoplasma spp. incidence i.e. 27.3%, 36.95%, 31.25% and 50.4% was reported by Gondal *et al.,* (2015), Tomar *et al.,* (2017) Ghohestani *et al.,* (2018) and Nehe (2018), respectively. The higher incidence of Mycoplasma spp. infection in the present study may be due to the improper managemental practices at the farms and the use of Mycoplasma free source of layer/breeder stocks.

The comparative results of choanal and tracheal swabs showed 44/50 (88%) and 50/50 (100%) positive samples in 16S rRNA Mycoplasma genus specific PCR. Thus, the tracheal swabs were found to be better specimen for recovery and detection of Mycoplasma spp. Gondal *et al.,* (2015) also observed similar results with highest positivity from tracheal samples who recovered total 27.3% Mycoplasma spp. from tracheal swabs (39.3%), tracheal tissue (15.9%), lung tissue (27.4%) and air sac (25%). Khatun *et al.,* (2018) investigated 48 samples for detection of MG from tracheal swabs, lung and air sac samples and found 25%, 6.25% and 6.25% samples, respectively, positive by PCR, and indicating highest recovery from tracheal swabs. Moreover, various workers also used tracheal swabs for investigation of mycoplasma infection viz. Kempf (1993), Carli and Eyigor (2003), Garcia *et al.,* (2005), Lysnyansky *et al.,* (2005) and Eissa (2009).

Out of 100 DNAs of 48 h old broth cultures, 90 (90%) were found positive for the genus specific PCR and remaining 10 (10%) were found to be negative. In the present study, the direct clinical specimens and broth cultures showed 94 (94%) and 90 (90%) samples positive, respectively, by 16S rRNA genus specific PCR. The results yielded in direct specimens were higher than those in broth cultures. Similar results from 48 hrs old broth cultures were recorded by Hossam *et al.,* (2016). They used three detection methods of MG and revealed that the highest recovery rate of MG was achieved by PCR on 48 hours incubation in PPLO broth (70.9%), followed by direct PCR on infected tissue (65.45%). Mardassi *et al.,* (2005), Nehe (2018) also used the broth cultures incubated for 24-48 h and achieved better results for detection of mycoplasma infection.

Result from this study indicates that Mycoplasma genus specific 16S rRNA PCR was found to be sensitive for detection of

Mycoplasma spp. at genus level in direct specimens and cultures. Tracheal swabs were found to be better specimens for investigation of Mycoplasma spp. infection in poultry.

It is feasible to directly use the clinical specimens from the birds for detection of mycoplasmosis. It requires less time and is cost effective. Enrichment of the samples, on the other hand, consumes more time and will also increase the cost of the investigation.

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