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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(11): 221-223

www.thepharmajournal.com
Received: 22-09-2021
Accepted: 24-10-2021

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Isolation of *Mycoplasma gallisepticum* from the natural cases of chronic respiratory disease

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DOI: https://doi.org/10.22271/tpi.2021.v10.i11Sd.8708

Abstract
Present work is a postmortem study. Total 428 dead birds, suspected to have died of chronic respiratory disease were considered for sampling. Swabs collected from the respiratory organs of the chickens and subjected for culture of the organism. Collected swabs were inoculated into PPLO broth medium allowed to incubate for 2-3 days. After removal of the contamination, culture from broth medium were inoculated over enriched PPLO agar plate. Typical "fried egg" like colonies appeared after 7 days of incubation. Colonies were confirmed as *Mycoplasma gallisepticum* (MG) by PCR. MG has been proven to cause serious respiratory illness in the chickens under present investigation.

Keywords: *Mycoplasma gallisepticum*, natural cases, chronic respiratory disease

Introduction
Chronic respiratory disease (CRD) is a bacterial illness caused by one of the pleuro pneumonia-like organisms (PPLO). However, the organism directly linked to CRD is *Mycoplasma gallisepticum* (MG), which can also cause secondary problems (Ley, 2008) [1]. Chronic respiratory disease has been recorded all throughout the world, resulting in significant financial losses in huge commercial enterprises. The infection may be undetectable or cause varied degrees of respiratory distress, including rales, trouble breathing, coughing, and sneezing. In uncomplicated instances, morbidity is high and death is low (Bahatti *et al*., 2013) [2]. Typical visual and histological lesions, serological assays to detect antibody generation, and/or isolation and identification of the organism can all be used to diagnose avian mycoplasma infection in chickens (OIE, 2008; Uddin *et al*., 2010) [3, 4]. Present study incorporated the isolation of *Mycoplasma gallisepticum* from the suspected cases of chronic respiratory disease.

Materials and Methods
Total of 428 dead birds included under present investigation. Birds suspected to have died due to chronic respiratory disease were considered for sample collection. All the dead birds were subjected for detailed post mortem examination and gross lesions were recorded carefully. Swabs collected from the birds showed lesions suggestive of CRD i.e., swollen head, sinusitis, air sacculitis, pneumonic lungs and tracheitis (Figure 1). Samples collected from the nostrils, choanal cleft, mouth, trachea, lungs and air sacs from the dead birds with respiratory lesions. All work involving handling of Mycoplasma organism was performed by taking appropriate bio-safety precautions and strict aseptic conditions.

Culture of *Mycoplasma* spp. organism
Collected swabs were pooled area wise and firstly inoculated in the PPLO (Pleuro-pneumonia like organism) broth medium for the isolation of the avian mycoplasmas. Broth was prepared as per the manufacturer guidelines. Briefly, 2.1gm of PPLO broth base (Himedia, M267) was added in 70 ml of distilled water in a conical flask. Media was autoclaved at 15 lbs pressure at 121 °C for 15min. After autoclaving the media was allowed to cool down up to 45 °C. Afterwards, one vial (30 ml) of mycoplasma enrichment supplement (Himedia, FD075) was added in the autoclaved broth media and mixed properly. Prepared enriched broth media than dispensed into the sterile centrifuge tubes aseptically and stored at 4 °C for further use.

Sterilized enriched broth medium were inoculated with the swabs which collected from the suspected cases of the mycoplasma infection and incubated in CO_2 Incubator by maintaining 37 °C temperature and 5% CO_2 concentration for two to three days. The incubated tubes were examined regularly on daily basis for presence mass turbidity due to growth of the organism. All the positive growths were immediately sub cultured in PPLO agar plates after removal of contamination by filtering cultured broth medium with 0.22-micron syringe filter. Inoculated tubes with no turbidity and colour change were kept on maximum 21 days before discarding and considering them as negative (Whitford *et al*., 1994; Yadav *et al.*, 2020) [5, 6].

Positive growths from PPLO growth cultures were streaked into the PPLO agar base after filtration from syringe filter. Manufacturer's guidelines were followed in the process of making enriched PPLO agar plates, suitable for culture of mycoplasmas. In brief, PPLO agar base were suspended in the 70 ml of double distilled water in a conical flask. Medium was autoclaved at 15 lbs pressure at 121 °C for 15 min. Sterile medium was allowed to cool up to 45 °C and added one vial (30 ml) of mycoplasma enrichment supplement (Himedia, FD075). Plates were dried and incubated overnight in order to rule out contamination.

Inoculated enriched PPLO agar plates were kept under CO_2 incubator, maintaining 37 °C temperature and 5% CO_2 concentration. Plates were kept under incubator for 7-15 days for the growth of convincing colonies of mycoplasmas (fried egg). Plates were examined under microscope (40x and 100x) for the growth of characteristic colonies. Plates without visible growth of the organism were kept up to 21 days before discarding and considering them as negative (Yadav *et al*., 2020). Colonies suggestive of avian mycoplasmas (fried egg) subjected for confirmation by doing molecular diagnosis i.e., PCR.

Molecular Diagnosis

DNA was extracted from culture colonies by using DNA purification kit (Himedia). Published primers were used for the PCR (Chandhar *et al.*, 2019) [7]. Details of the primers are showing in Table 1. The PCR amplification of 16S rRNA gene was carried out in a final reaction volume of 10 µl. Components of PCR reaction mixture were nuclease free water (2.2µl), PCR master mix (5 µl), F primer (0.4 µl), R primer (0.4 µl) and genomic DNA (2 µl). The PCR tube was kept in a pre-programmed thermocycler. Standardized reaction programme including denaturation (94 ºC for 30 seconds), annealing (57º C for 30 seconds), initial extension (72 ºC for 60 seconds) and final extension (72 ºC for 5 minutes) was carried out. PCR amplification was confirmed by running 8 µl of PCR product mixed with 2 µl of 6X gel loading dye from each tube on 1.5% agarose gel at a constant voltage, 75 V for 60 minutes. The amplified product was visualized as a single compact fluorescent band of expected size under UV light and documented by gel documentation system.

Table 1: Detail of primer targeting 16S rRNA used in PCR

Primers	Primer Sequence	Product size
MG-14F	GAG-CTA-ATC-TGT-AAA-GTT-GGT-C	185 bp
MG-13R	GCT-TCC-TTG-CGG-TTA-GCA-AC	

Results and Discussion

Cultured colonies were started to appear in enriched PPLO agar plates from 4th days onwards which turned recognizable after 7th days of incubation. Typical "fried egg" like colonies were observed, when examined the colonies under microscope. Morphologically, isolated colonies appeared as smooth, circular, translucent masses with a dense, raised central area and thin and evenly distributed peripheral part. Examination of the colonies was done firstly in 40X followed by 100X. Best resolution for differencing the colonies of our interest was seemed to be 100X. Colonies of *Mycoplasma spp*. were super small and can be examined correctly under discussed focal lens i.e., 100X (Figure 2). Isolated colonies were tested positive for *Mycoplasma gallisepticum*. In positive samples of the PCR amplified product of 185bp of 16S rRNA of *Mycoplasma gallisepticum* gene was clearly visible (Figure 3).

Our findings were in corroboration with Abbas *et al*. (2018) [8]. They isolated the Mycoplasma in modified fray's medium. They observed typical fried egg or nipple like characteristic in the suggestive colonies. Colonies had diameter of 0.1 to 1 mm with a dense raised center in the middle. Logesh *et al*. (2018) [9] collected swab samples from respiratory system of chickens and isolated the organism in PPLO agar plate. At low power of microscope, they detected the colonies of mycoplasma as typical "fried egg" appearance. Equivalent conventional identification criteria for *Mycoplasma spp*. were used by Khalifa *et al.* (2013) and Nouzha *et al*. (2013) [10]. Chandhar *et al.* (2019) [7] confirmed presence of MG in the collected tissue samples collected from the natural cases of MG infection by PCR.

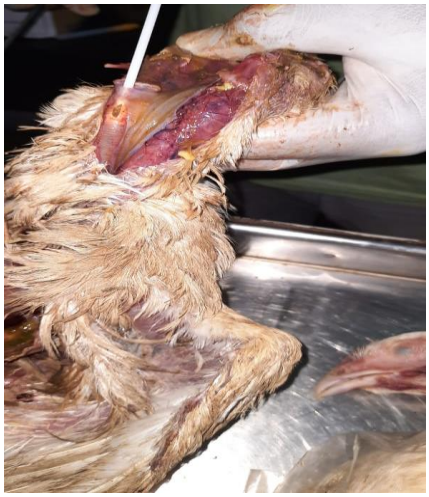

Fig 1: Showing collection of swab samples from the trachea of suspected bird

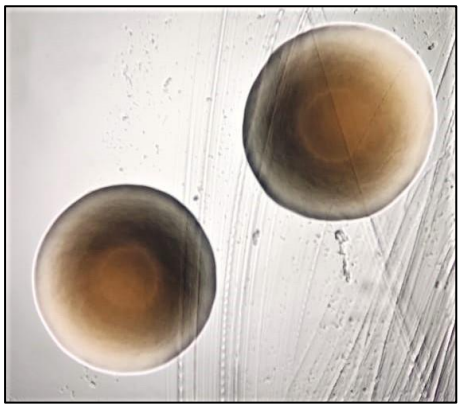

Fig 2: Showing typical colonies of Mycoplasma spp. organism with raised center area

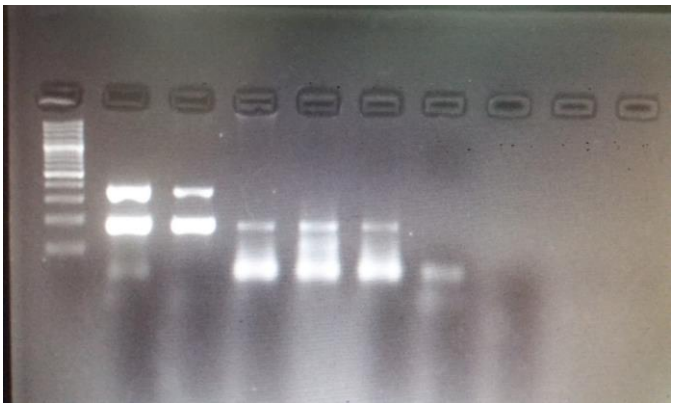

Fig 3: Gel image of PCR done in isolated colonies of *Mycoplasma gallisepticum* Lane 1: ladder, Lane 2 & 3: Positive Control, Lane 4,5,6: positive *M. gallisepticum* colony sample, Lane 7: negative test sample

Conclusion

Culture of the MG is gold standard for diagnosis of disease i.e., CRD. Although it is hard and time taking process, doesn't suit much to diagnose the disease but its contribution in order to make preventive protocols (vaccine making) cannot be overlooked. Other important aspect of the culture is that it provides base to study the particular organism in detail. Culture method helps to investigate physiological and morphological feature of the organism i.e., MG. it also covers mutational aspect of the same so that required preventive and control programme can be designed for removing the infection from selective flock.

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