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Isolation and Molecular Detection of *Mycoplasma gallisepticum* in Commercial Layer Chickens in Sylhet, Bangladesh

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ABSTRACT

Mycoplasma gallisepticum induced poultry diseases are associated with a huge economic crisis and have a considerable impact on the poultry industry worldwide. The aim of the current study was to isolate and perform molecular detection of MG circulating pathogenic strain in the commercial layer farms in the Sylhet district of Bangladesh. The entire study was conducted from January 2018 to January 2019 at three Upazilas of Sylhet district in Bangladesh. A total of 50 dead layer chickens (indicating signs of respiratory distress before death) were collected randomly from 15 different layer farms. The tissue samples, such as air sacs, trachea, and lungs, were taken from suspected dead chickens. Both cultural and PCR-based techniques were applied to identify *Mycoplasma* from tissue samples. The conventional PCR technique was implemented to amplify 185 bp DNA fragments for the MG. Out of 50 samples, 36% (18/50) and 70% (35/50) of MG were identified by cultural method and PCR, respectively. Based on the results of the study, it can be concluded that PCR is an easier, more sensitive, and less time-consuming method for the early diagnosis of MG in chickens, compared to cultural isolation and hence can lower the economic burden to poultry farmers caused by this disease.

Keywords: Culture, Layer chicken, *Mycoplasma gallisepticum*, PCR

INTRODUCTION

Mycoplasma gallisepticum (Nascimento et al., 2005) is one of the significant poultry pathogens responsible for considerable economic losses in the poultry industry worldwide (Ferguson-Noel et al., 2012; Hennigan et al., 2012; Ball et al., 2020). Particularly, in chicken and turkey, it induces chronic respiratory diseases and infectious sinusitis, respectively. The annual economic loss incurred by MG in the poultry industry was reported as over $780 million across the world (Hennigan et al., 2012). *Mycoplsma gallisepticum* is associated with decreased growth and egg production, increased medication costs, reduced feed conversion ratio of chickens, reduced egg production and quality of eggs in layers, and reduced hatchability in breeder flock (Nascimento et al., 2005; Saif et al., 2008; Peebles and Branton, 2012; Felice et al., 2020). The cultural isolation and molecular identification processes are referred to as 'gold standard' techniques for the diagnosis of *Mycoplasma* organisms (Kleven, 2008). Although cultural and biochemical tests are dependable and accurate, it takes a considerably long time to isolate the MG organism due to its slow-growing nature (more than one week). To confront this situation, a molecular tool, namely polymerase chain reaction (PCR), has been started to apply which is a more accurate, sensitive, rapid, and efficient method for early identification and monitoring of MG infection (García et al., 2005; Gharaibeh and Al Roussan, 2008; Rauf et al., 2013). In Bangladesh, the overall prevalence and seroprevalence of *Mycoplasma* range from 46.88% to 64.47% (Sarkar et al., 2005; Sikder et al., 2005; Ali et al., 2015). Therefore, there is a crying need to isolate and identify MG precisely among the commercial layer flocks to know the exact prevalence of the disease and to take immediate necessary steps for the prevention of pathogen transmission from diseased flocks to healthy flocks. The objective of the present study was to molecular detection of MG from naturally infected commercial layer chickens in Sylhet district, Bangladesh.

MATERIALS AND METHODS

Ethical approval

The study design was approved by the ethical committee of Sylhet Agricultural University, Sylhet-3100 Bangladesh before implementation. Mutual (verbal) and written consent was taken from the farmers before data collection.

Sample collection

A total of 50 dead layer chickens (suffered from respiratory illness) were collected from 15 commercial unvaccinated layer farms from 3 Upazilas (Sylhet Sadar, Dakshin Surma, and Bishwanath) of Sylhet district of

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Bangladesh. Tissue samples (air sacs, trachea, and lungs) were then retrieved aseptically from suspected chickens and immediately stored at -20°C in the laboratory, Department of Pathology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University (SAU), Sylhet-3100, Bangladesh.

Isolation and identification of *Mycoplasma gallisepticum* by cultural and biochemical tests

Collected tissue samples were crushed together using mortar and pestle with sterile sand added to simplify tissue crushing. Afterwards, homogenous tissue suspension was passed through a sterile sieve and filter paper, the filtrate was passed into sterile plastic tubes and centrifuged at 5000 rpm for 20 minutes. Then, the supernatant was passed through a sterile bacterial membrane filter of 0.45µm. Finally, the filtrate was dispensed (inoculated) on plates of Hayflick's agar (HiMedia Laboratories Pvt. Ltd) and Frey's *Mycoplasma* broth media (HiMedia Laboratories Pvt. Ltd), the excess fluid was sucked from the plates using a sterile Pasteur pipette and incubated at under micro-aerophilic condition @ 5-10% CO_2 tension. The plates and broth culture were examined daily for any visible colony growth under a stereomicroscope (25×) and change in color as a tentative positive indication. After obtaining pure culture, biochemical tests, such as fermentation of glucose, were performed as preliminary biochemical identification of *Mycoplasma gallisepticum*. The test was performed in the test tubes containing equal volume (5 ml) of pure *Mycoplasma* broth culture and fresh Frey's *Mycoplasma* broth base where 1 to 2 drops of phenol red was added as an indicator. Meanwhile, a test tube containing fresh Frey's *Mycoplasma* broth base was used as a control. Then, 0.1 ml glucose was added to all test tubes and incubated at 37°C with 5-10% CO_2 tension. In the next step, the tubes were examined daily for any color change. In case of the MG positive test tube, it turned reddish-yellow to yellow indicating glucose fermentation. Finally, positive cultures were kept in -20°C until they were used for DNA extraction.

Detection of *Mycoplasma gallisepticum* by PCR

The molecular identification of MG was performed based on the method previously described by Bekele and Assefa (2018). One microtitre broth culture of the respective MG isolates was used for DNA extraction as per manufacturer instructions (Favorgen Biotech Corp, Taiwan). The final volume of the extracted DNA was 50 µl. The MG was detected by PCR amplification of the target gene (mgc2 gene, 185 bp) using specific primer pairs, F primer MG-14F: GAG-CTA-ATC-TGT-AAA-GTT-GGT C, MG-14R: GCT-TCC-TTG-CGG-TTA-GCA-AC R primer as described previously with some modifications (Gedlu et al., 2015). Briefly, the 45 µl reaction mixture comprises DreamTaq Green PCR Master Mix 25 µl, F-primer 2.5 µl (5 pmol/µl), R-primer 2.5 µl (5 pmol/µl) and RNAse free water 15 µl. The tubes were then taken to another clean area where the DNA sample was tested, or positive control (*Mycoplasma* live vaccine), and/or negative control (water, 5 µl) were added to each tube. The PCR was carried out in a thermal cycler (Applied Biosystems, 2720, UK) having the following cycles: initial denaturation (94°C) of 5minutes for 1 cycle; 40 cycles of denaturation (94°C) and annealing (55°C) for 30 seconds each; extension (72°C) for 60 seconds, and rest 1 cycle for the final extension (72°C) for 5 minutes. The PCR product was stored at 4°C in the refrigerator. Finally, 2% Agarose gel was used for electrophoresis and the amplified product was visualized under UV light by incorporating a 1 kb marker (Thermo Scientific, #SM0243).

Statistical analysis

Data were analyzed using Microsoft Excel 2010.

RESULTS

Post mortem examination

After the death of the clinically manifested commercial layer chickens, post mortem examination was conducted on a total of 50 affected chickens from 15 different farms. During the examination, emphasis was mainly given to respiratory organs and lungs, trachea, air sacs, which were collected after post mortem as a sample. The tentative diagnosis was achieved by examination of dead chickens and lesions observed were catarrhal exudates in the nasal passages, infra-orbital sinuses, trachea and bronchi, caseous exudates in the air sacs, and cloudy, congested, and hepatized lungs (Figure 1A). Haemorrhagic and pale-colored liver was observed during heavy infestation (Figure 1B).

Isolation of *Mycoplasma gallisepticum* by cultural and biochemical tests

Out of 50 tissue samples, a total of 18 (36%) positive isolates of MG were detected (Table 1). All positive samples showed the color changes from yellow to orange in Frey's*Mycoplasma* broth (Figure 2A) and typical fried egg-like micro-colonies were visualized under a stereomicroscope (25× magnification) after incubation in Hayflick's agar (Figure 2B). On biochemical tests, such as fermentation of glucose, all positive samples indicated a color change from reddish-yellow to yellow (Figure 2C).

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Table 1. Isolation and Identification of MG by culture in different Upazilas of Sylhet District, Bangladesh

Name of the upazila	Name of farm (5 samples from each farm)	Positive sample for isolation and identification (%)	Overall positive sample (%)
Sylhet Sadar	Farm A	3	18/50 (36%)
	Farm B	2	
	Farm C	2	
	Farm E	2	
	Total	9/20 (45%)	
Dakshin Surma	Farm F	2	
	Farm I	2	
	Farm H	1	
	Total	5/15 (33%)	
Bishwanath	Farm L	2	
	Farm M	1	
	Farm O	1	
	Total	4/15 (26%)	

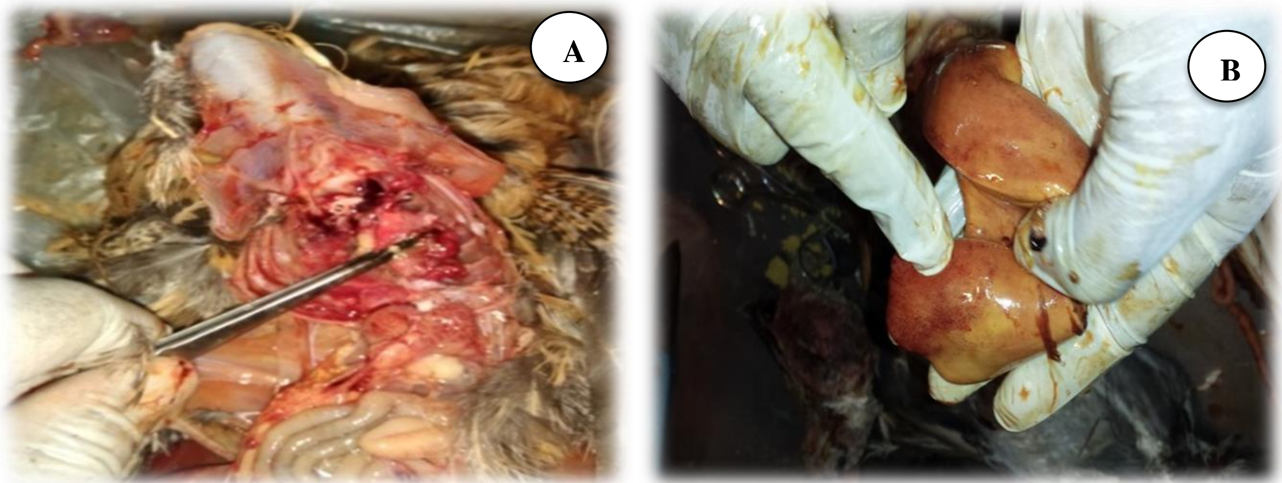

Figure 1. Necropsy findings of commercial layer chickens infected with *Mycoplasma gallisepticum* in Sylhet Sadar Upzilla, Bangladesh. **A:** The congested lung and air sacs, **B:** Hemorrhagic and pale-colored liver

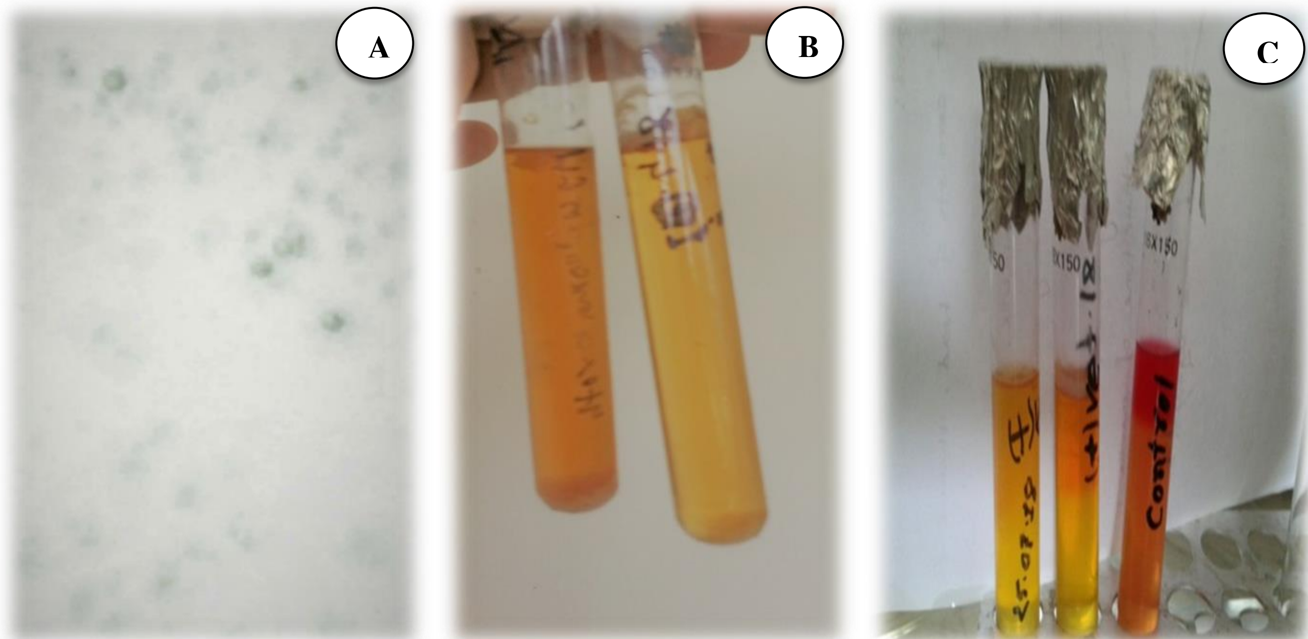

Figure 2. Culture characteristics of *Mycoplasma* isolated from layer chickens. **A**: Fried-egg appearance in stereomicroscope (25X), **B**: Changes of color from red to yellow in Freys broth, **C**: Glucose fermentation test shows changes of color from reddish yellow to yellow

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Molecular detection

During agarose gel electrophoresis PCR amplicon product visualized 185 base pairs that successfully targeted the respective gene by MG primers (Figure 3). PCR confirmed the mgc2 gene of MG from the tissue samples collected from Sylhet Sadar (75%), Dakshin Surma (60%), and Bishwanath (73.33%, Table 2).

Table 2. Comparative identification of *Mycoplasma gallisepticum* by PCR and culture methods in various Upazilas of Sylhet District, Bangladesh

Name of the Upazila	No. of samples	Culture (+ve) isolate	PCR (+ve) isolates
Sylhet Sadar	20	9 (45%)	15 (75%)
Dakshin Surma	15	5 (33%)	9 (60%)
Bishwanath	15	4 (26%)	11 (73.33%)
Total	50	18 (36%)	35 (70%)

No: Number

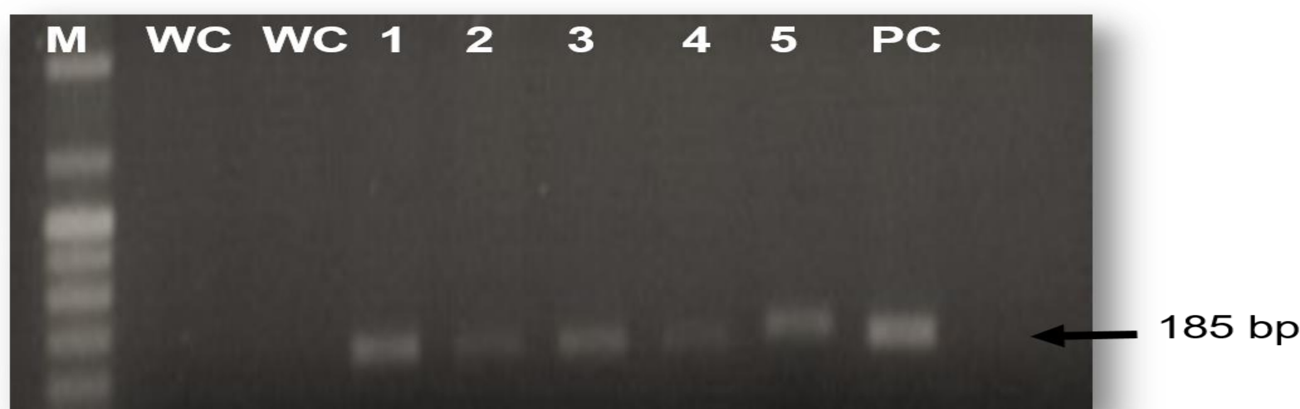

Figure 3. PCR amplicons of *Mycoplasma gallisepticum* isolated from layer chickens. Lane M: 100 bp DNA marker, Lanes 1-5: Cultural isolates, Lane PC: MG live vaccine as a positive control, Lane WC: Water control

DISCUSSION

Mycoplasma gallisepticum is a growing challenge in the poultry industry globally (Osman et al., 2009). Due to its fastidious nature of pathogenesis, MG is seldom identified as a key pathogen in poultry farms leading to concerns to isolate the pathogen from several farms. In this study, MG was detected from commercial layer flocks by both cultural and molecular approaches. Only clinical signs and postmortem findings cannot ascertain the confirmatory diagnosis of any disease hence, laboratory methods are crucial in case of the diagnosis of *Mycoplasma* infection (Jafar and Noomi, 2019). Frey's media was used in the current study for MG isolation which has been reported to be the most commonly used method for the isolation of mycoplasma. Some investigators have reported that this medium is superior for the isolation of mycoplasma-like fastidious organisms (Jafar and Noomi, 2019). Moreover, based on the report stated by (Gharaibeh and Al Roussan, 2008), it was expected that Frey's medium with the enrichments would provide superior efficacy for primary isolation of MG from infected chickens.

In the present study at Sylhet district, MG was isolated 36% and 70% using culture test and PCR, respectively (Table 2). The result of the culture test in the current study was closely similar to a study conducted in Iraq which was 36.6% (Jafar and Noomi, 2019). However, using PCR led to 33.3% and 31.6% MG in Egypt and Jordan, respectively, which were significantly lower than that of the present study (Gharaibeh and Al Roussan, 2008; Osman et al., 2009). Furthermore, around 50% of *Mycoplasma* was elucidated in Pakistan by PCR and approximately 55% seroprevalence of these organisms was reported in both Rajshahi and Bhola districts, Bangladesh (Hossain et al., 2007; Islam et al., 2014). This percentage may vary due to the variation of local environmental conditions as well as lower incidence of mycoplasmosis in that particular area. Another possible reason for the higher prevalence of *Mycoplasma*couldbe is its horizontal transmission from the infected chickens, eggs, wild birds, vehicles, or fomites to the healthy susceptible chicken flocks (Jordan, 1985). Poor management, cold air currents during winter, vaccination, high-density poultry farming, and rearing of multi aged-group chickens in the same premises may act as potential causative factors for

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immunity break down against MG infection in chickens (Prodhan, 2002). Furthermore, inadequate ventilation, contaminated litter, frequent movement of rodents, wild birds, pets, technical persons, professionals, visitors, and poor biosecurity measures are some of the contributing factors that are responsible for MG infection (Dulali, 2003).

PCR is a good technique for identifying *Mycoplasma* infected birds (Marois et al., 2002; Ghorashi et al., 2010; Saadh and Hasani, 2016). PCR-based nucleic acid detection is considered as an alternative method to that of conventional isolation technique (Ferguson et al., 2005; Raviv et al., 2007; Evans and Leigh, 2008). Cultural methods of MG might not isolate the organism and are laborious, time-consuming, and difficult, especially for chronic cases of disease and medicated layer chickens (Hyman et al., 1989). Moreover, anti-mycoplasmal substances, antiserum, and different types of inhibitors decrease the chances of isolation and soaring the time of isolation (Amin and Jordan, 1979). The viable organism is needed for success in isolation, alternatively, PCR detects the nucleic acid of MG even from medicated birds and more frequently than culture (Kempf et al., 1993). In the current study, it was revealed that 70% *Mycoplasma* were was detected by the PCR method, whereas the culture method detected only 36% *Mycoplasma* infection in layer chickens. This difference in these percentages could be due to the fastidious nature of the organism, and the high sensitivity of PCR (Chanie et al., 2009).

CONCLUSION

The present study concludes that MG infection in the selected areas may be an emerging concern in the future. Based on the results, it is suggested that the PCR technique may be a more reliable method to detect MG infection in chicken than the cultural method, although more sampling and DNA sequencing is needed for future studies. The present study can help to investigate epidemiology-based analysis, develop full genome sequence, prepare vaccine candidates for better control of mycoplasmosis in near future.

DECLARATIONS

Authors' contribution

The present study is a thesis part of the MS degree of Md. Shamsul Islam Basit. Monira Noor designed the study and Md. Shamsul Islam Basit did the research under the guidance of Monira Noor. Md. Masudur Rahman guided and critically reviewed the manuscript. All authors read and approved the final version.

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Competing interests

The authors declared that the present study was performed in absence of any conflict of interest

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

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