MOLECULAR CHARACTERIZATION OF *Mycoplasma gallisepticum* AND *Mycoplasma synoviae* FROM LAYER AND BREEDER CHICKEN IN BANGLADESH

A Thesis

By

Mohammad AL Amin

MASTER OF SCIENCE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA-1207

December, 2021

MOLECULAR CHARACTERIZATION OF *Mycoplasma gallisepticum* AND *Mycoplasma synoviae* FROM LAYER AND BREEDER CHICKEN IN BANGLADESH

By

MOHAMMAD AL AMIN

Reg. No.: 19-10073

A Thesis

Submitted to the Department of Microbiology and Parasitology

Sher-e-Bangla Agricultural University, Dhaka

In Partial Fulfillment of the Requirements

for the degree of

MASTER OF SCIENCE (M.S.) IN MICROBIOLOGY

SEMESTER: July - Dec/2021

APPROVED BY

Dr. Uday Kumar Mohanta

Supervisor
Professor
Dept. of Microbiology and Parasitology
Sher-e-Bangla Agricultural University

Mohammed Badrul Amin, PhD

Co- Supervisor
Assistant Scientist
Laboratory of Food Safety and One Health
Laboratory Sciences and Services Division,
icddr'b

Md. Kamrul Hassan

Chairman and Associate Professor
Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University

DEPARTMENT OF MICROBIOLOGY

AND PARASITOLOGY

Sher-e-Bangla Agricultural University

Sher-e-Bangla Nagar, Dhaka-1207

Memo No: SAU/

CERTIFICATE

*This is to certify that the thesis entitled "**Molecular Characterization of Mycoplasma gallisepticum and Mycoplasma synoviae from layer and breeder in Bangladesh**' submitted to the Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **Master of Science in Microbiology** ,embodies the result of a piece of bona fide research work carried out by **Mohammad AL Amin, Reg. No.: 19-10073** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dr. Uday Kumar Mohanta

Dated: *Supervisor*

Department of Microbiology and

Place: Dhaka, Bangladesh *Parasitology*

Sher-e-Bangla Agricultural University

DEDICATED TO MY BELOVED PARENTS

ACKNOWLEDGEMENTS

All praises are due to almighty God, who has created the universe and who enabled the author to complete this piece of research work and preparation of this thesis successfully.

*The author with a sense of respect, expresses heart felt gratitude to his Supervisor **Dr. Uday Kumar Mohanta,** Professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207, for his supervision, untiring guidance, invaluable suggestions, timely instructions, inspirations and constructive criticism throughout the tenure of research work.*

*The author with a sense of respect expresses his heartfelt gratitude to his Co-Supervisor **Mohammed Badrul Amin,** Assistant Scientist, Laboratory Sciences and Services Division, Laboratory of Food Safety and One Health.*

The author is also boundless grateful to all the staffs of icddr'b and Department of Microbiology and Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University Sher-e-Bangla Nagar, Dhaka-1207, for their co-operation.

The author takes the opportunity to express her indebtedness and profound respect to his beloved father and mother , for their love, blessings, prayers, sacrifices, moral support and encouragement for her study which can never be forgotten.The author deeply owes his whole hearted thanks to all the relatives, friends, well-wishers for their help and inspiration during the period of the study.

The Author

TABLE OF CONTENTS

TITLE	PAGE NO.
ACKNOWLEDGEMENT	**I**
LIST OF CONTENTS	**II-III**
LIST OF TABLES	**IV**
LIST OF FIGURES	**V**
LIST OF ABBREVIATION AND SYMBOLS	**VI**
ABSTRACT	**VII**

LISTS OF CONTENTS

NO. OF CHAPTER	TITLE	PAGE NO.
CHAPTER 1	**INTRODUCTION**	**1-3**
CHAPTER 2	**REVIEW OF LITERATURE**	**4-7**
CHAPTER 3	**MATERIALS AND METHODS**	**08-20**
3.1	Materials and Methods	8
3.1.1	Study area and population	8
3.1.2	Collection of samples	8
3.1.3	Transportation and Preservation of samples	9
3.2	Glassware and appliances	9
3.3	Methods	10
3.4	Media used for growth	11
3.4.1	Preparation of broth	11
3.4.2	Preparation of Frey's Medium supplemented with 15% swine serum	11
3.5	Preparation of reagents	11-12
3.6	Culture of *Mycoplasma* spp.	12
3.6.1	Purpose	12
3.6.2	Preparation Materials	12
3.6.3	Procedure	13

3.6.4	Isolation of *Mycoplasma* spp.	13
3.6.5	Suggested Protocol for *Mycoplasma* spp. isolation (Written Protocol)	14
3.7	DNA amplification	14
3.7.1	DNA Extraction	14
3.8	Polymerase chain reaction (PCR)	15
3.8.1	Principals of PCR	15
3.8.2	PCR amplification	16
3.8.3	Materials used for polymerase chain reaction	16
3.8.4	Preparation of Master Mix for *M. synoviae* and *M. gallisepticum*	16-17
3.8.5	Primers used in PCR for *M. gallisepticum* and *M. synoviae* identification	17
3.8.6	Thermal conditions	18
3.8.7	Precautions of PCR	18
3.8.8	Electrophoresis	18-19
3.8.9	Process of electrophoresis	19
3.8.10	Documentation of the DNA sample	20
3.9	DNA sequencing and phylogenetic analysis	20
CHAPTER-4	**RESULTS AND DISCUSSION**	**21-29**
4.1	Cultural characteristics of *Mycoplasma* spp**.**	21
4.1.1	Broth media	21
4.2	PCR amplification of *M. gallisepticum* DNA with specific Primers	22
4.3	PCR amplification of *M. synoviae* DNA with specific primers	22
4.4	DNA analyses	23
4.4.1	DNA sequences of *M. gallisepticum* and *M. synoviae*	23

4.5	Phylogenetic analysis of isolated *M. gallisepticum* and *M. synoviae*.	24
4.6	Prevalence of specific organism from sample	25
4.7	Prevalence of *M. gallisepticum* and *M. synoviae* in different types of chicken	26
4.8	*Prevalence (%) of M. gallisepticum* and *M. synoviae* in different areas of Bangladesh	27
	DISCUSSION	**28-29**
CHAPTER-5	**CONCLUSION**	**30**
	REFERENCES	**31-34**

LIST OF TABLE

NO. OF TABLE	TITLE	PAGE NO.
Table 1	PCR reaction mixture	16
Table 2	Primer sequence and their sources	17
Table 3	Thermal Condition of PCR for *M. gallisepticum* and *M. synoviae*	18
Table 4	Prevalence of specific organism from sample	25
Table 5	Prevalence of *M. gallisepticum* and *M. synoviae* in different types of chicken	26
Table 6	Prevalence (%) of *M. gallisepticum* and *M. synoviae* in different areas of Bangladesh	28

LIST OF FIGURES

NO. OF FIGURE	TITLE	PAGE NO.
Figure 1	(A) Sample collection from Layer (B) Sample collection from Breeder	8

Figure 2	Schematic illustration of experimental layout	10
Figure 3	Suggested protocol for *Mycoplasma* spp. isolation (Diagram protocol)	13
Figure 4	*Mycoplasma* spp. showed color change throughout the tube	21
Figure 5	Amplification of 185 bp DNA of *M. gallisepticum*	22
Figure 6	Amplification of 227 bp DNA of *M. synoviae*	23
Figure 7	Alignment of nucleotides in sequences of *M. gallisepticum* and *M. synoviae*	24
Figure 8	Phylogenetic analysis of isolated *M. gallisepticum* and *M. synoviae*	25
Figure 9	Prevalence of *M. gallisepticum* and *M. synoviae*	26
Figure 10	Prevalence of *M. gallisepticum* and *M. synoviae* in different types of chicken	27
Figure 11	Prevalence of *M. gallisepticum* and *M. synoviae* in different areas of Bangladesh	28

LIST OF ABBREVIATION AND SYMBOLS (CONT'D)

ABBREVIATION	FULL WORD
mg	Milligram
PCR	Polymerase chain Reaction
PBS	Phosphate buffered solution
R	Resistant
Rpm	Revolution Per Minute
S	Sensitive
SAU	Sher-e-Bangla Agricultural University
spp.	Species
UV	Ultraviolet
V	Voltage
yrs.	Years
°C	Degree Celsius
%	Percentage
~	Tilde
+	Positive
et al.	and others
etc.	Etcetra

Molecular characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from layer and breeder in Bangladesh

ABSTRACT

Mycoplasma is very important diseases in poultry. The economic value of Mycoplasma is very high in poultry industry. There are very few studies of Mycoplasma in Bangladesh. So it is an opportunity to identification of *Mycoplasma gallicepticum* and *Mycoplasma synoviae* and its prevalence area will be helpful for industry as well as the Nation. A total sum of 73 tracheal swab samples were collected from layer and breeder of Gazipur, Mymenshingh, Chattagram, Rangpur, Narshingdi and Keshoregong area. For molecular identification, PCR was done with 16S rRNA MG-13 and MG-14 primers and 16SrRNA MSL-1 and MSL-2 primers. From 73 of layer and breeder swab sample, 28 pure isolates of *M. gallicepticum* and *M. synoviae* were detected. Among the isolates, 24.65% were *M. gallicepticum* and 13.7% were *M. synoviae.* In this study, 18 isolates were positive for *M. gallicepticum* in which 9 isolates were from layer and 9 isolates were from breeder. Prevalence percentage of *M. gallicepticum* was 22% in both cases of layer and breeder. Out of 73 samples, 10 isolates were positive for *M. synoviae,* where 10 isolates were from layer but no isolate from breeder was found positive. Prevalence percentage of *M. synoviae* was 31% in case of layer chicken. Prevalence of *M. gallisepticum* was 9% in Gazipur, 33% in Mymensingh, 13% in Narshingdi 45% in Chattagram. 33% in Rangpur and 25% in Keshoregong area. On the other hand, prevalence of *M. synoviae* was 9% in Gazipur, 50% in Narshingdi, 42% in Keshoregong area but in Mymenshingh, Chattagram and Rangpur, no prevalence of *M. synoviae* was found.

Key words: Layer, Breeder, *Mycoplasma synoviae, Mycoplasma gallicepticum*

CHAPTER 1

INTRODUCTION

Avian mycoplasmosis is a disease which is distributed worldwide and is extremely important to both the broiler grower and the table-egg producer (Ley and Yoder, 1997; OIE, 2007). It is caused by Mycoplasma organisms. These organisms are different from other bacteria; they are of very small size (Kleven, 1998) and do not have a cell wall (Osman *et al*., 2009; Khan *et al*., 2010). *M. gallisepticum* has been reported to be the most economically significant mycoplasma pathogen of gallinaceous and certain non gallinaceous avian species and causes chronic respiratory disease (CRD) in chickens (Osman *et al.,* 2009). *M. gallisepticum* and *M. synoviae* belong to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae. These characteristics account for the "fried egg" type of colonial morphology exhibited by mycoplasmas, their complete resistance to antibiotics that affect cell wall synthesis and their complex nutritional requirements (Kleven, 1998). Avian mycoplasmas are also host specific (for instance, *Mycoplasma meleagridis* infects turkeys only) (Kleven, 1998).

Mycoplasma is the simplest and smallest bacterial cell. This organism can infect and grow in plant, animal, human and insect host. It should be noted that, *Mycoplasma meleagridis* and *Mycoplasma iowae* can also cause disease in poultry, but *M. gallisepticum* and *M. synoviae* are considered to be the most important of the pathogenic mycoplasmas.

Avian mycoplasmosis is one of the diseases that have a negative impact on the health and productivity of domestic chickens. *Mycoplasma* infection induces significant economic losses in poultry by reducing; body weight gain, meat quality, feed conversion rate; in broilers, causing a significant decline in egg output in layers, and increasing embryo mortality in breeders (Messa *et al.,* 2017).

M. gallisepticum infection is particularly important in chickens as a cause of decreased meat and egg production (Bradbury, 2001). In poultry the infection is spread vertically through infected eggs and horizontally by close contact (Marois *et al.,* 2000). Other methods of spread are less well documented.

M. gallisepticum may be associated with acute respiratory disease in chickens, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly

affected by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and or bacteria such as *Escherichia coli*.

The clinical signs of *M. gallisepticum* in infected poultry can vary from subclinical to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, rales and breathing through the partially open beak may occur. Respiratory signs and lesions are similar to those observed with *M. gallisepticum*, except that they are generally milder, and, as with *M. gallisepticum*, there is a synergistic effect with other respiratory age . It has been reported by (Oritz *et al*., 1995) that the treatment of mycoplasma infected breeders with anti microbials decreases the rate of clinical manifestations and consequently also decreases the risk of transovarian transmission.

Many Mycoplasma species are pathogenic for humans, animals, plants, and insects (Maniloff, 2002). In addition, Mycoplasmas have been a problem as intracellular contaminants in human cell therapy, and in the animal (poultry and swine farming) production as pathogens. Thus, rapid diagnostics and identification of Mycoplasmas is crucial for various activities.

Precise isolation and identification of *M. gallisepticum* and *M. synoviae* among poultry flocks improves prevention of pathogen's spreading. Due to the fastidious nature of *Mycoplasma* and the difficulty of isolation, molecular diagnostic tests are becoming the most common method for detection and characterization of *Mycoplasma* infections in poultry. Real time PCR is a sensitive, specific, and fast detection test for *M. gallisepticum* and *M. synoviae* and can be performed directly on clinical swabs taken from infected sites (eg, choana, sinuses, trachea, airsacs). Sequence typing by targeting and amplifying a specific sequence allows for differentiation between *M. gallisepticum* isolates and can be particularly useful for epidemiologic investigations and to identify the source of infection.

Mycoplasmas can be detected by direct culture techniques of the organisms and some indirect assays including DNA staining with the fluorescent dye, hybridization of nucleic acid, biochemical tests and polymerase chain reaction (PCR) (Stakenborg *et al.,* 2005). In most PCR methods, 16SrRNA sequences are used as the template sequences because this gene has some regions with commonly conserved sequences among Mycoplasmas (Tang *et al*., 2000; Quirk *et al*., 2001; Kong *et al.,* 2001).

There are very few studies of mycoplasma in Bangladesh. So it's an opportunity to work on it and serve the industry as well as the Nation in economical expect and securing the protein for building a sound Nation. Because of the fastidious nature of *Mycoplasma* and the difficulty of isolation, molecular diagnostic tests are becoming the most common method for detection and characterization of mycoplasma infections in poultry.

Objectives

The study was carried out considering the following objectives:

1. To identify the strains available in Bangladesh.
2. To enumerate the diseases prevalence caused by *Mycoplasma. gallisepticum* and *Mycoplasma. synoviae* in different area of Bangladesh.

CHAPTER 2
REVIEW AND LITERATURE

Avian mycoplasmosis is an important disease of poultry of great economic importance. It is caused by four (4) pathogenic mycoplasma species namely *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*; although other Mycoplasma species have also been incriminated in the disease (Thu *et al*., 2003; Bradbury, 2005; Hossain *et al*., 2007; Buim *et al*., 2009). The disease causes cough, rales, ocular and nasal discharges, decreased feed intake, decreased feed conversion, decreased egg production and hatchability.

Avian mycoplasmosis can lead to a significant reduction in egg production of between 10-20% in infected layer and broiler breeder flocks. It also causes infectious sinusitis in turkeys. It can be prevented and controlled by the acquisition of birds free from mycoplasma, maintenance of replacements from mycoplasma free sources in a single-age, all in all out management system, proper hygiene and biosecurity measures.

Mycoplasma. gallisepticum has been reported to have been isolated from infected falcons, parrots, pheasants, geese, quails, patridges, ducks and geese (Cookson and Shivaprasad, 1994; Garner *et al*., 2006; Poveda *et al*., 1990). Other species that have been incriminated in avian mycoplasmosis are *Mycoplasma anseris*(affects geese), *M. columbianum* (affects pigeons); *M. gallinarum, M. gallinaceum, M. lipofaciens* and *M. pullorum* which affect chickens (Whithear 1976). Others are *M. gallopavonis, M. iners, M. columbinasale. M. glycophilum, M. cloacale*. These are not pathogenic; therefore they are not of major concern to the poultry industry (Nascimento, 2000).

All ages of chickens and turkeys are susceptible to avian mycoplasmosis although young birds are more prone to infection than the older ones (Nunoya. *et al*., 1995); it seems that some resistance develops with age (Yoder. (1972a). In case of Veneral Transmission, Mycoplasmas are transmitted laterally by contact (Kleven, 1998) infectious aerosols coughed and sneezed by infected birds (Kleven . 1998; Nascimento. *et al*., 2005) through contaminated feed, water, contact personnel and communicant animals mainly birds (Nascimento *et al*., 2005) and vertically through the eggs (Kleven,1998). Veneral transmission is particularly important in the case of MM (Whithear 1976). *M. synoviae* infection can also be through the conjunctiva and

upper respiratory tract (McMullin P., 2004) . It has been reported by (Wang *et al.,* 1990) that *M. gallinarum* and *M. gallinaceum* have been isolated from the oviduct of chickens. This suggests that egg transmission of this species is possible. According to (OIE, 2007), infected birds carry *M. gallisepticum* for life and can remain asymptomatic until they are stressed.

Mycoplasma gallisepticum has been ascribed to be the most economically important of the pathogenic mycoplasma species affecting poultry due to the significant losses occurring from decrease in egg production, decrease in egg quality, poor hatchability (high rate of embryonic mortality and culling of day old birds), poor feed efficiency, an increase in mortality and medication costs. Economic losses in the poultry industry caused by this infection have been noted to be significant (Ahmad *et al*., 2008); the infection has been reported by to reduce egg production in layers and broiler breeder chickens by 10-20% (Bradbury, 2001).

In 1984 in the USA, *M. gallisepticum* infected chickens were found to lay 15.7 eggs less than healthy ones; this contributed to a loss of 127 million eggs corresponding to an annual loss of 125 million dollars (Mohammed H.O. *et al*., 1987). Also, losses over a 6 month period in 1999 in a North Carolina company were conservatively estimated to be between 500,000 and 750,000 dollars (Rhorer A.R., 2002).

Mycoplasma gallisepticum infection causes significant economic losses in the poultry industry due to downgrading of carcasses at slaughter because of airsacculitis, treatment costs, and due to its effect on flocks performance. *M. gallisepticum* infection mainly is transmitted through ovaries, and the *M. gallisepticum* infected breeder flocks should be depopulated; hence, the preferred method for *M. gallisepticum* control is to maintain *M. gallisepticum*-free flocks. However, in some situations such as multi-age production farms, maintaining the flocks free of *M. gallisepticum* may be difficult or impossible. Thus, the control programs and vaccination account for additional costs. The control programs, which may include surveillance (serology, culture, isolation, and identification) for *M. gallisepticum* and *M. synoviae*, must be performed, mainly in breeder flocks.

Previously reported that the seroprevalence of *M. gallisepticum* infection was higher (33.3%) in female than in male (10.14%), which it is indicating that the female birds significantly ($p < 0.05$) were more susceptible than male birds (Pradhan *et al*., 2000; Sarkar *et al.,* 2005; Hossain *et al.,*

2007). Isolation and identification of *M. gallisepticum* in Ghaemshahr town in north of Iran showed that 20% of broiler farms positive in case of Mycoplasma genus and 12 % of farms positive in molecular tests .

Researchers indicated that the highest prevalence of *M. gallisepticum* infection was 72.72% in 18-25 weeks age, whereas lowest prevalence was 44.00% in 66 weeks and above ages (Hossain *et al*., 2007) . Also there were similar reports, which were demonstrated highest *M. gallisepticum* infection at lower ages and lowest *M. gallisepticum* infection in later ages. Highest infection in the young chickens, maybe due to the vertical transmission of the organisms, and lowest rate of infection in adult chickens maybe due to infections or unsuitable environmental conditions in flocks. However, intensive nature of poultry farming provided opportunity for recycling of the pathogens due to population density. The other factors that contribute *M. gallisepticum* infection are poor ventilation, contamination of litters and no restriction on the movement of the technical personnel, visitors and such other persons as well as other biosecurity measures.

However, a research in Holland reported much higher rate of 73% positivity in commercial layers, while it was only 6% in both broiler parents and broiler farms. From Bangladesh, Sarkar *et al.* (2005) reported that sero-prevalence of *M. gallisepticum* infection was 58.9% in the study area.

In another experiment carried out by Fiorentin in Brazil, they housed a flock of broiler known to be free of *M. synoviae* and *M. gallisepticum* (using serology, culture and PCR techniques with farms known to be endemic for *M. synoviae*), detectable antibodies against *M. synoviae* using SPA test were 5% at 22 week of age. Silveira did an experiment in Brazil which showed that when 12 week-old layers were experimentally infected by *M. gallisepticum* and *M. synoviae* had a seropositivity rate of 100% for *M. gallisepticum* and 55.5% for *M. synoviae* after 32 day of infection. Kempf and Gesbert carried out an experiment in France on bird's 57-weeks-old free of *M. gallisepticum*, where they infected them with *M. gallisepticum*; signs of disease started to show after 3 days of the challenge. While it has been taken 10 days for antibodies in 100% of bird to appear when SPA was used.

Mycoplasma synoviae has always been considered less important than *M. gallisepticum* in poultry but during the last decade its importance has been highlighted in several studies and there

is an increased consciousness to generate *M. synoviae*-free poultry. There seems to be a large variability in the virulence of *M. synoviae* strains (Lockaby *et al.,*1999).

In both chickens and turkeys, *M. synoviae* can cause similar respiratory problems as *M. gallisepticum* as well as affecting the egg shell quality with typical eggshell apex abnormalities and decreased egg production (Feberwee *et al.,* 2009; Catania *et al*., 2010). Furthermore, arthropathic and amyloid-inducing strains may cause severe economic losses due to growth retardation and lameness induced by synovitis (Landman and Feberwee. 2001; Kleven, 2008).

This infection can be diagnosed by clinical signs and isolation and identification of the organism by culturing on mycoplasma media; mycoplasma colonies are tiny, circular, smooth and translucent having a "fried egg" appearance with a central dense mass. Mycoplasmosis can also be diagnosed by post mortem lesions (gross and microscopic), serological tests such as sero-agglutination reaction and hemagglutination inhibition test (HI); polymerase chain reaction (PCR), Enzyme linked immune sorbitant assay (ELISA), indirect immunofluorescence, immune peroxidase staining or growthinhibition test are also diagnostic for avian mycoplasmosis

Real-time PCR is a sensitive, specific, and fast detection test for *M. synoviae* and can be performed directly on clinical swabs taken from infected sites (eg, choana, sinuses, trachea, airsacs, and joints). Sequence typing by targeting and amplifying specific sequences allows for differentiation between *M synoviae* isolates and outbreaks and can be particularly useful for epidemiologic investigations and to identify the source of infection.

The Lauerman 16S rRNA PCR method is commonly used for confirmation of *M. synoviae* infection in chickens and turkeys in the United States. It is fast, sensitive, specific, and relatively inexpensive. However, since it is based on the 16S rRNA gene, identification of strains of *M. synoviae* from the PCR product is not possible because of the conserved nature of this gene. Identification of specific strains (fingerprinting) of *M. synoviae* and *M. gallisepticum* is helpful for identifying possible sources of infection and for identifying the presence of vaccine strains.

CHAPTER-3

MATERIALS AND METHODS

The present research work was conducted in the laboratory of icddr'b for molecular identification of Mycoplasma by different microbiological methods. The detailed outline of materials and methods are given below:

3.1 Materials and Methods

3.1.1 Study area and population

The samples were collected layer and breeder of from Gazipur, Mymensingh, Narshingdi, Chattagram, Rangpur, Kishoregang. Samples were collected in the laboratory of iccddr'b maintaining aseptic condition and kept in 4^0 C until processing was done

3.1.2 Collection of samples

A total of 73 swab samples were collected from the study population. Desired sample site (choanal cleft) was selected and samples were collected by cotton swab. After collecting, samples were inoculated by inserting cotton tip of swab into broth. It was swirled several times and then removed swab from tube by pressing the cotton tip on the edge of the tube to help squeeze residual sample into broth. Swab was discarded as biohazard waste.

(A) (B)

Figure 1: (A) Sample collection from Layer (B) Sample collection from Breeder

3.1.3 Transportation and Preservation of samples

During transportation, it was packaged in insulated containers with ice packs and sent overnight express. Then the samples were processed for the identification and characterization of *Mycoplasma* subsequently.

3.2 Glassware and appliances

The different types of important equipment used for this work are listed as follow down:

1. Distilled water
2. Sterile bent glass or plastic spreader Rods
3. Micropipette (1-5 µl,5-50 µl, 10-100 µl, 50-500 µl, 100-1000 µl)
4. Forceps
5. Measuring cylinder
6. Spirit lamp
7. Water bath
8. Vortex Mixture
9. Labeling tape
10. Experimental test tube
11. Conical flask
12. Electric balance
13. Cotton
14. Incubator

15. Autoclave etc.

3.3 Methods

The following methods were used for the identification of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from the key Flock (Figure 2)

Experimental layout

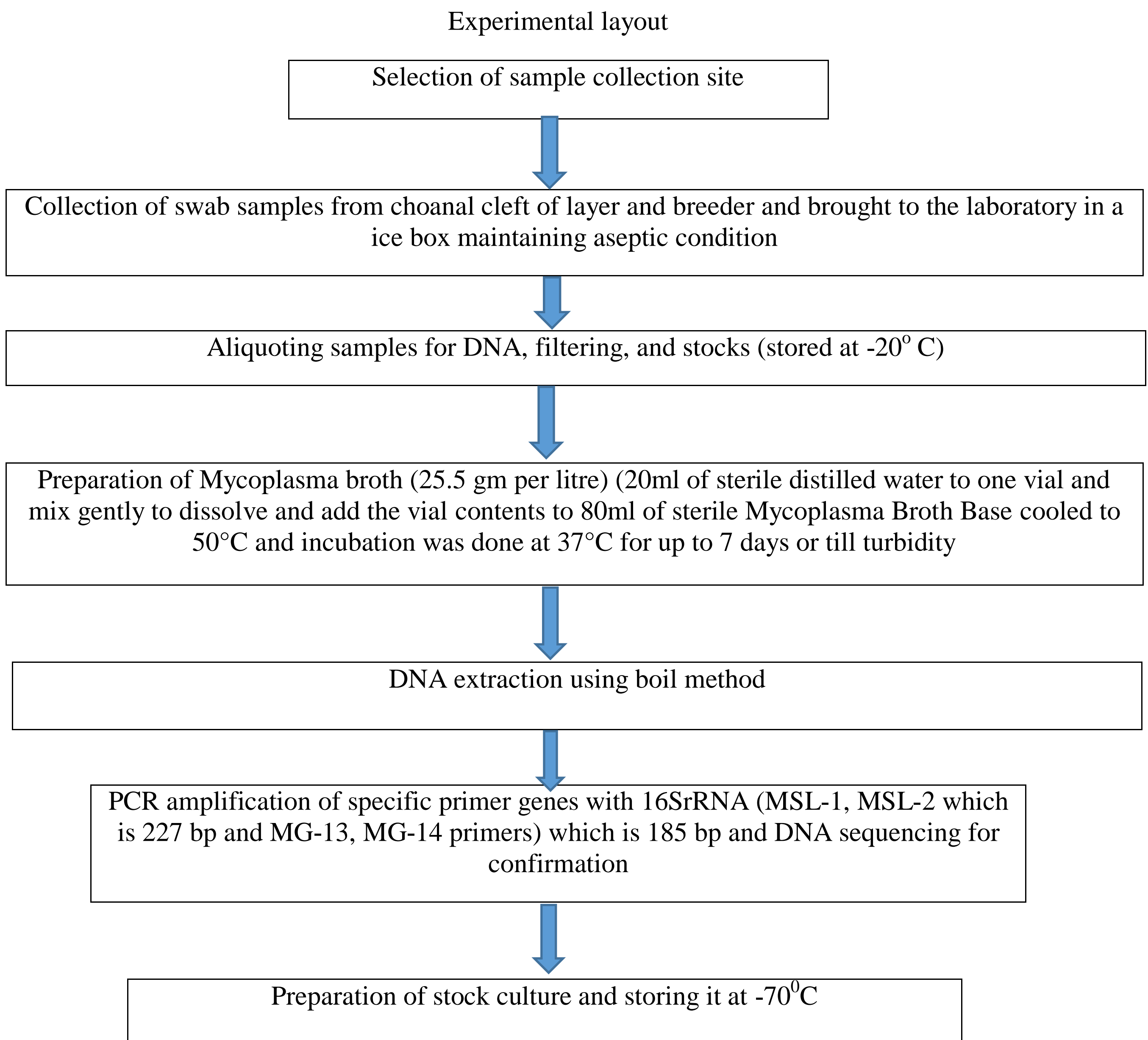


Figure 2: Schematic illustration of experimental layout

3.4 Media used for growth

3.4.1 Preparation of broth

25.5g was added to 1 litre (800ml+200ml supplements, 20 ml supplements per 80ml media) of distilled water. It was mixed well and distributed in 80ml volumes. Sterilization was done by autoclaving at 121°C for 15 minutes. It was cooled to 50°C and added the sterile supplement.

3.4.2 Preparation of Frey's Medium supplemented with 15% swine serum

This medium was used to isolate field cultures of avian mycoplasmas and propagate lab-adapted strains. *M. synoviae* and *M. gallisepticum* would ferment dextrose, turning the medium orange-yellow color.

Water bath was set at 56^0 C and Swabbing counter of laminar flow hood with 70% alcohol. 150 ml of swine serum was thawed, then it was heat inactivated at 56^0 C for 30 minutes. A sterile round bottom flask (1000 ml) was placed in laminar flow hood. To prepare 1000 ml of medium: 22.5 g Frey's Broth Base was added to 800 ml distilled H2O in sterile 1000 ml flask. pH was adjusted to 8.00 with 1 N NaOH while mixing solution on stir plate. It was autoclaved at 121^0 C for 15 minutes. It was allowed to cool to 56^0 C in water bath. Aseptically 150 ml swine serum, 50 ml 20% Dextrose, 20 ml 1% NAD/Cysteine , 5 ml 5% Thallium acetate , 5 ml Penicillin G (200,000 IU/ml), 2.5 ml 1% phenol red-for broth medium only wew added to flask. It was labeled, dated and filled out media preparation form. Then it was stored at 4^0 C. Note: Sterile, mycoplasma-free swine serum was used. For agar medium, mix sterile solutions from part 2 in a separate sterile 500 ml Glass bottle and heat in water bath to 560C for 15 minutes. Add heated mixture to the Frey's agar base, mix well (avoid bubbles or foam) and pour plates (in laminar flow hood) using approximately 15 ml molten medium per 100 mm diameter plate. Flame agar with Bunsen burner or torch to remove bubbles, then allow to cool with lids on and laminar flow on.

3.5 Preparation of reagents:

- **20% Dextrose**

100 g of dextrose was dissolved in 500 ml distilled H_2O and it was used in Frey's medium.

- **5% Thallium Acetate**

5g thallium acetate was dissolved in 100 ml of distilled H_2O.

- **1% NAD + Cysteine**

0.5 g of NAD and 0.5 g cysteine were dissolved in 100 ml distilled H_2O.

- **1% Phenol Red**

1 g phenol red powder was mixed in 100 ml distilled H_2O and autoclaved 121^0 C for 15 min. then it was dispensed into sterile bottles and stored at 4^0 C.

- **Penicillin (500,000 IU/vial)**

With a needle and syringe, 5 ml sterile distilled H_2O to a vial containing 1 million IU of Penicillin G. it was mixed thoroughly ad filter through 0.22 u filter into a sterile bottle. It was stored at -20^0 C (do not store in frost-free freezer).

3.6 Culture of *Mycoplasma* spp.

3.6.1 Purpose

This protocol was used to isolate mycoplasmas in the laboratory. Frey's media is most suited for the suspected species of mycoplasmas. Organism would ferment dextrose and turn broth media orange – yellow.

3.6.2 Preparation Materials

- Broth medium aliquoted into 1.8 ml volumes.
- Agar medium if direct agar inoculation is desired.

- Sterile cotton-tiped swabs, size determined to be optimal for age of birds and desired sample site.
- Container suitable for transport of materials to sampling location

3.6.3 Procedure

- **Broth :** inoculate sample by inserting cotton tip of swab into broth. Swirl several times, then remove swab from tube, pressing the cotton tip on the edge of the tube to help squeeze residual sample into broth. Do Not Break Swab in Tube. Discard swab as biohazard waste.
- **Agar:** inoculate swab directly onto agar medium by streaking cross and down the agar surface until all the area is covered. Discard swab as biohazard waste. Place samples on ice (if available) until they can be delivered to the laboratory If no ice is available, keep media at room temperature. When forwarding samples by mail, package them in insulated containers with ice packs and send overnight express.

3.6.4 Isolation of *Mycoplasma* spp.

1) After receiving field samples, incubate in a humidified 37oC incubator.

2) Keep samples at 37oC for up four weeks following the passing and plating diagram (on next page) as a guide(Figure 3)

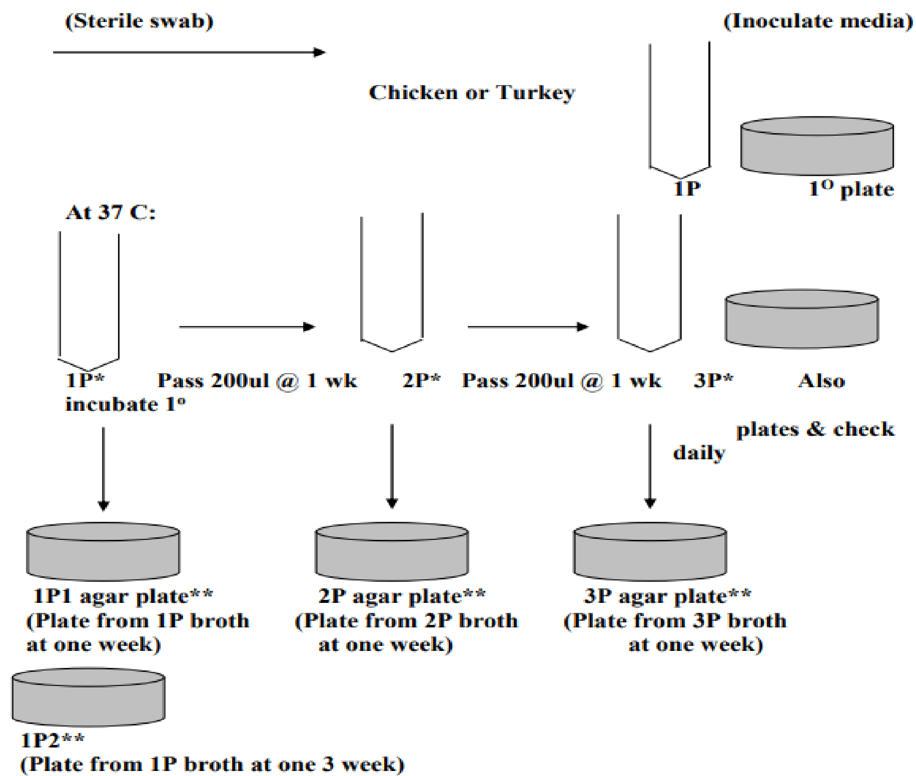


Figure 3: Suggested protocol for *Mycoplasma* spp. isolation (Diagram protocol)

* = Pass and plate to fresh broth and agar immediately if fermentation or hydrolysis is noted.

** = FA any mycoplasmas growing on agar with species specific FA conjugates. For chickens, include at least *M. gallisepticum* and *M. synoviae* conjugates.

3.6.5 Suggested Protocol for *Mycoplasma* spp. isolation

1) Samples were incubated for up to four weeks.

2) Broths were checked for indications of growth (i.e. color change) daily. If fermentation or hydrolysis is noted, pass 200 ul sample to fresh broth and plate to fresh agar immediately using cotton tipped swab.

3) Pass 200 µl of all broths not showing color change to fresh broth (2P) and agar (1P1) after 7 days. FA* any colonies on agar from step 2.

4) Follow instructions in step 2, plus check agar plates for growth. FA* any Mycoplasma colonies on agar with appropriate conjugate .

5) Pass 2P broths to fresh broth (3P) and agar (2P) after 14 days.

6) Follow instructions in step 2, plus check agar plates for growth. FA* if necessary (step 4 can be followed).

7) Plate 1P and 3P broths to fresh agar (1P2 and 3P respectively) after 21 days.

8) Check broth and agar samples for signs of growth, FA* any colonies growing on agar surface, close case on day 28. * = Save 500 ul (or 8-10 agar plugs) from positive samples in-80 freezer for future reference.

NOTE: A 5% CO2 environment may desirable for some mycoplasma spp.

3.7 DNA amplification

3.7.1 DNA Extraction

Materials:

- Microcentrifuge
- Sterile 1.5 ml screw-cap micro tubes or sterile 1.5 ml centrifuge tubes
- Sterile 0.5 ml snap-cap microcentrifuge tubes
- Inoculated broth field samples
- Mycoplasma strain extracted for Positive control depends on PCR.
- Rainin P-200 and P-10 pipets with appropriate size sterile, cotton-plugged tips
- Sterile, disposable transfer pipets
- Sterile distilled water or sterile high purity water.

Procedure: Rapid Boil method

- To sterile laminar flow hood, counter was wiped with 70% ETOH and turn on UV light for 10-15 min. prior to use.
- Dry bath was turned on to 110^0 C (maximum setting).
- 1 ml of inoculated broth sample was transferred into a screw-cap micro tube using a sterile transfer pipet and it was labeled appropriately.
- Centrifugation was done at maximum speed (13,200 or 14,000 RPM) for 10 min.
- Decanted medium from each tube into small biohazard bag, blot tube on paper towel (to remove remaining medium; precautions were taken for the pellet out from the tube), and resuspended each pellet with 1 ml sterile distilled water using a transfer pipet.
- Centrifugation was done as in step 3.

- Decanted medium from each tube into small biohazard bag, blot tube on paper towel (to remove remaining medium; precautions were taken for the pellet out from the tube), and resuspended each pellet with 1 ml sterile distilled water using a transfer pipet.
- Centrifugation was done as in step 3.
- Tapped out all liquid and dry side of tube with sterile cotton swab.
- Resuspended pellet in 20 - 25 µl sterile distilled water (volume added depends on size of pellet) using a PCR dedicated Rainin P-200 pipet and tip.
- Tubes were heated in dry bath for 10 min.
- Cooled on ice for 10 min.
- Centrifuged at maximum speed for 5 min.
- Removed 20 - 25 µl of supernatant, which contains released DNA and transfer each sample to a snap-cap microcentrifuge tube. Labelled appropriately with next consecutive PCR number and record information on master Lauerman PCR list.
- Extracted DNA was stored at 4^0 C until use.

3.8 Polymerase chain reaction (PCR)

3.8.1 Principals of PCR

PCR is based on the mechanism of DNA replication in vivo: dsDNA is unwound to ssDNA, duplicated and rewounded. This technique consists of repetitive cycle of:

- Denaturation of the DNA through melting at elevated temperature to convert double-stranded DNA to single – stranded DNA.

- Annealing (hybridization) of two oligonucleotides used as primers to the target DNA.

- Extension of the DNA chain by nucleotide addition from the primers using and DNA polymerase as catalyst in the presence of Mg2+ ions.

3.8.2 PCR amplification

3.8.3 Materials used for polymerase chain reaction (Table 1)

Table 1: PCR reaction mixture

Nuclease-free H_2O	17.5 µl
Green Promega Buffer	2.5 µl
$MgCl_2$	1.25 µl
d NTP's 0.5	0.5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
Promega Taq Polymerase	0.25 µl
Final volume	22.5 µl

3.8.4 Preparation of Master Mix for *M. synoviae* and *M. gallisepticum*:

1) Sufficient volume of master mix was prepared to include a diluted *M. synoviae* and *M. gallisepticum* positive control and a negative control respectively.
2) 2.5 µl of sample DNA was added to 22.5 µl PCR mix in thin walled snap-cap microcentrifuge tube. Close tube tightly and label appropriately.
3) 2.5 µl of diluted (WVU–1853) positive *M. synoviae* control DNA (optimum dilution of *M. synoviae* positive control) and (F strain or ATCC S6 strain) positive *M. gallisepticum* control DNA (optimum dilution of *M. gallisepticum* positive control) were added to 22.5 µl, respectively. PCR mix in thin walled snap-cap microcentrifuge tube. Close tube tightly and label appropriately.
4) Add 2.5 µl of nuclease-free water DNA for negative control (or PBS that you use in the DNA extraction step) to 22.5 µl PCR mix in thin walled snap-cap microcentrifuge tube. Close tube tightly and label appropriately.
5) Use thermal cycler to perform the amplification.

3.8.5 Primers used in PCR for *M. gallisepticum* and *M. synoviae* identification (Table 2)

Table 2 Primer sequence and their sources

M. gallisepticum **Primer sequences (5´- 3´)**	**PCR Product size**	**Source**
16SrRNA MG-14F 5' GAG CTA ATC TGT AAA GTT GGT C 3' (22 base pairs) 16SrRNA MG-13R 5' GCT TCC TTG CGG TTA GCA AC 3' (20 base pairs)	185 bp	Lauerman, (1998)
M. synoviae **Primer sequences(5´- 3´)**		
16SrRNA MSL-1 (5'-GAA GCA AAA TAG TGA TAT CA-3')-F (20 base pairs) 16SrRNA MSL-2 (5'-GTC GTC TCC GAA GTT AAC AA-3')-R (20 base pairs)	227 bp	Lauerman, (1998)

3.8.6 Thermal conditions (Table 3)

Table 3 Thermal Condition of PCR for *M. gallisepticum* and *M. synoviae*

Step	Temperature	Duration	Cycles
1.Initial denaturation	94^0C	5 min	01
2.Denaturation	94^0C	1 min	38
3.Annealing	56^0C	1 min	
4.Extension	72^0C	2 min	

5.Final extension	72^0C	5 min	01

3.8.7 Precautions of PCR

- PCR tubes were numbered carefully.
- All work was carried out in bio-safety cabinet class II to avoid contamination.
- Melt the vial containing all PCR reaction components.
- All components were taken in correct amounts into tube on PCR color box.
- All tubes were spin down or gently pipetting.

3.8.8 Electrophoresis

Agarose electrophoresis is a routiney used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined by the molecular weight where small weight molecules migrate faster than larger ones. In addition to size separation, nucleic acid fractionation using agars' gel electrophoresis can be an initial step for further purification of a band of interest.

Electrophoresis through agarose is a standard used to identity and purity of DNA fragments. The technique is simple, rapid to perform and capable of resolving fragments of DNA that cannot separated by other procedure.

3.8.9 Process of electrophoresis:

- Preparation of gel: Initially 1.4 gm agarose powder was weight out and placed into a 250 ml conical flask. Then 70 ml of electrophoresis buffer (1x TBE buffer) was added into the flask. The flask was then placed into microwave oven for 1 minute. The solution was heated again for 1 minute to dissolve small translucent agarose particles.
- The comb was then placed into the appropriate groove and slot of the casting tray.

- When the agarose solution was cooled to about 50°C (the flask was cooled enough to hold comfortably with bare band), 7µl to 10 *M. gallisepticum*/ml solution of ethidium bromide was added (the concentration of ethidium bromide in the melted agarose solution may be in the range of 0.5~1.0 µl/ml) and mixed well by gentle shaking to make DNA visible under ultraviolet light and poured into gel tray.
- The gel was allowed to solidify at room temperature for 20-30 minutes.
- The comb was removed carefully from the solidified gel and The casting daM. synoviae were removed from the edges of the gel tray, so that the gel does not slide off the tray.
- Sample application in the gel and sufficient amount of 1x TBE buffer (about 600 ml) was added to cover the gel. The volume of electrophoresis buffer should not be above maximum buffer mark on electrophoresis system.
- Adjustment of voltage or current (gel- electrophoresis about 70-100 volts). Set up run time about 30-60 minute.
- The separation process was monitored by the migration of the dye in the loading buffer. When the bromophenol blue dye had reached about three-fourths (3/4) of the gel length, the electrophoresis was completed and stopped.

The UV light of the system was switched on; the image was viwed on the monitor, focused, acquired and collected picture of gel.

3.8.10 Documentation of the DNA sample

After completion of electrophoresis the gel was taken out carefully from the electrophoresis chamber and placed on UV trans illuminator (WUV-L50, Korea) for primary checking the DNA bands and then placed into the high performance gel documentation chamber (UVD1-254) for further checking and picture storage.

3.9 DNA sequencing and phylogenetic analysis

The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model (Tamura *et al.,* 1993). The tree with the highest log likelihood (-2319.28) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and

BioNJ algorithM. synoviae to a matrix of pairwise distances estimated using the maximum composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch legs measured in the number of substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}+$ Noncoding. There were a total of 155 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,*2018).

CHAPTER 4

RESULTS AND DISCUSSION

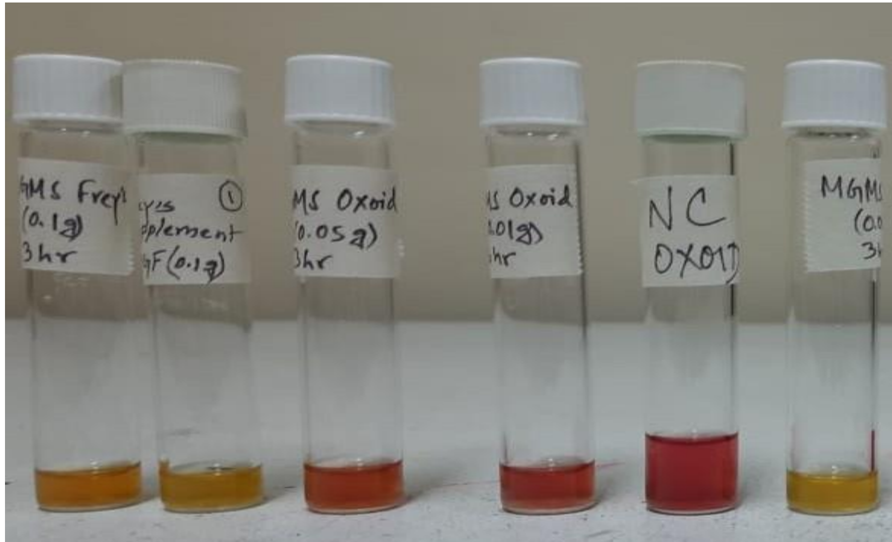
The present research was designed to determine the molecular characterization of *M. gallisepticum* and *M. synoviae* from layer and breeder chicken of different areas in Bangladesh. Out of 73 samples, 28 isolates were found to be positive.

4.1 Cultural characteristics of *Mycoplasma* spp.

After inoculation of swab sample on broth media, it was observed that maximum growth and color change were found in broth media.

4.1.1 Broth media

Liquid medium was used for the primary isolation of field cultures as well as for the propagation of laboratory adapted *Mycoplasma* spp. strains. *M. gallisepticum* and *M. synoviae* fermented dextrose and turned broth media orange – yellow (Figure 4).



Positive

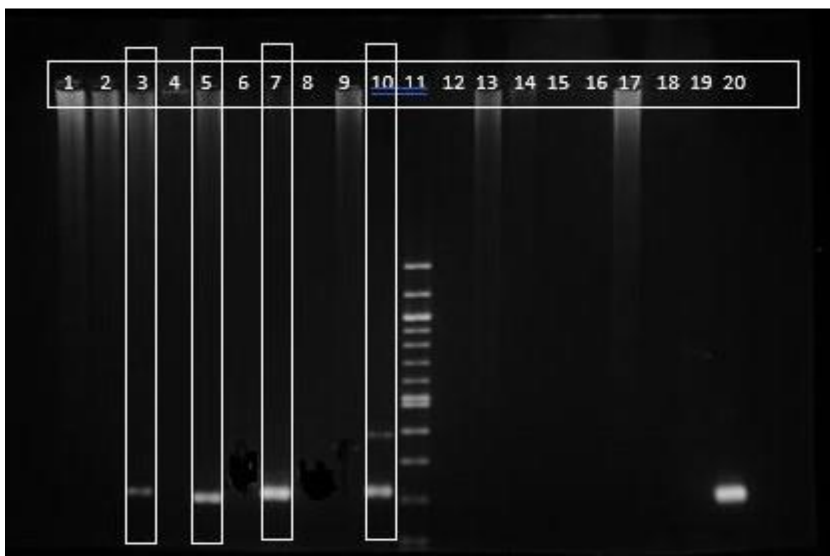
Negative

Figure 4: *Mycoplasma* spp. showed color change throughout the tube

4.2 PCR amplification of *M. gallisepticum* DNA with specific primers

In this study, 18 isolates were positive for *M. gallicepticum,* in which 9 isolates were from layer and 9 isolates were from breeder chicken. For molecular identification, PCR was done with 16S rRNA (MG-13 and MG-14) primers.

Gel image:



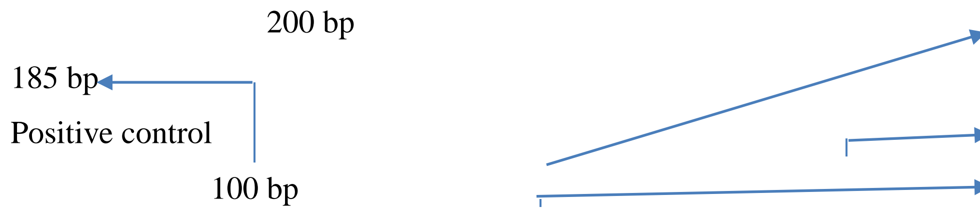


Figure 5: Amplification of 185 bp DNA of *M. gallisepticum*. Lane:2 to 10 and 12 to 19: test sample. Lane 11: DNA ladder (100 bp). Lane 1: negative control and Lane 20: positive control (Note: PCR=Polymerase Chain Reaction, bp= base pair)

4.3 PCR amplification of *M. synoviae* DNA with specific primers

In this study, 10 isolates were positive for *M. synoviae,* where 10 isolates were from layer but no isolate from breeder was found positive. For molecular identification, PCR was done with 16SrRNA MSL-1 and MSL-2 primers which are 227 base Pair

Gel image:

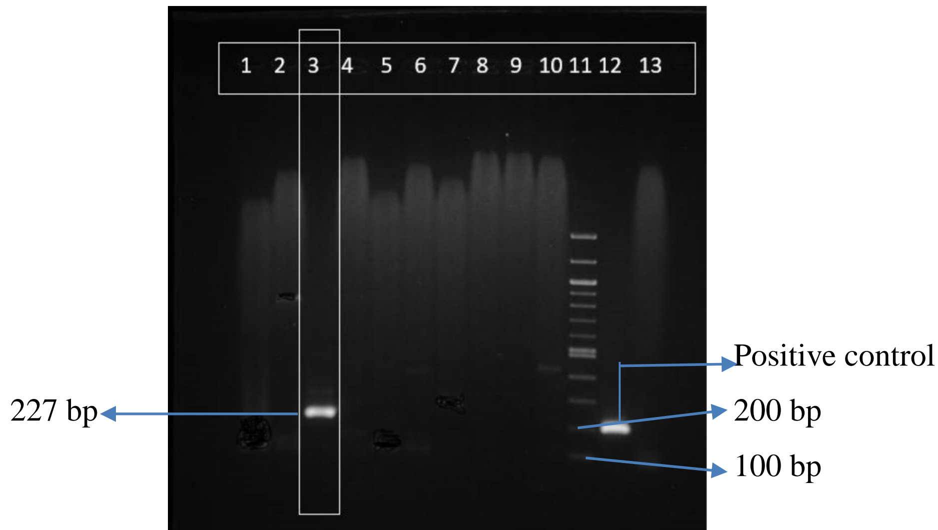


Figure 6: Amplification of 227 bp DNA of *M. synoviae*. Lane:1 to 10: test sample. Lane 11: DNA ladder (100 bp). Lane 12: positive control and Lane 13: negative control. (Note: PCR=Polymerase Chain Reaction, bp= base pair)

4.4 DNA analyses

DNA sequences of *M. gallisepticum* and *M. synoviae* were 185 bp and 227 bp, respectively. In case of *M. gallisepticum*, there were 4 nuclotide variation in between 2 haplotypes

MG_EM_05_F and MG_EM_07_F). On the other hand, *M. synoviae* yielded only one haplotype. There were 70 nucleotide variation in between MG_EM_05_F and MS_EM_19_F(2) which showed about 37% heterogenecity from each other. There were 71 nuclotide variation in between (MG_EM_07_F and MS_EM-19_F2) which showed about 38% heterogenecity.

4.4.1 DNA sequences of *M. gallisepticum* and *M. synoviae*

 # MG_EM_07_F

CCTGATAAGGCTGCATTCGCCCTCATGAGTCGGAATCACTAGTAATCGCGAATCAGC
CATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAACTATGAGA
GCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGC

#MG _EM_05_F

CCCGTATAGGCTGCATTCGCCCTCATGAGTCGGAATCACTAGTAATCGCGAATCAGC
CATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAACTATGAGA
GCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGC

#MS_EM_19_F(2)

TCCGCCCAAGAAGCTGACTTATCGGATTGTAGTCTGCAACTCGACTACATGAAGTCG
GAATCGCTAGTAATCGTAGATCAGCTACGCTACGGTGAATACGTTCTCGGGTCTTGT
ACACACCGCCCGTCACACCATGGGAGCTGGTAATGCCCGAAGTCGGTTTGTTAACTT
CGGAGACGAC

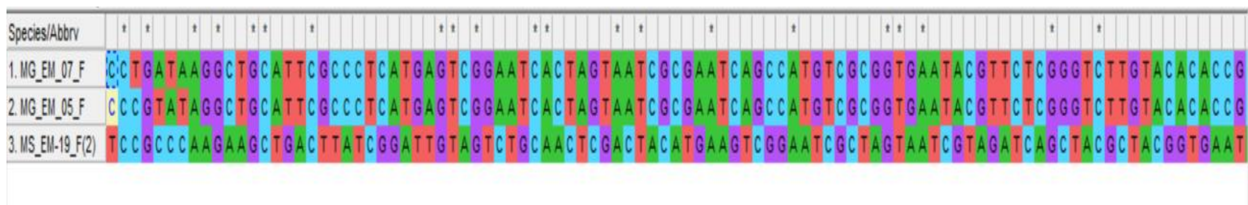


Figure 8: Alignment of nucleotides in sequences of *M. gallisepticum* and *M. synoviae.*

4.5 Phylogenetic analysis of isolated *M. gallisepticum* and *M. synoviae.*

Phylogenetic analysis was done by MEGA X. Both the haplotypes of *M. gallisepticum* (MG_EM_07_F and MG_EM_05_F) were included in one cluster which was sister to the clade

formed by *M. synoviae*. On the other hand, *M. synoviae* haplotype MS_EM-19_F(2) was sister to the clade formed by strains of *M. synoviae*.

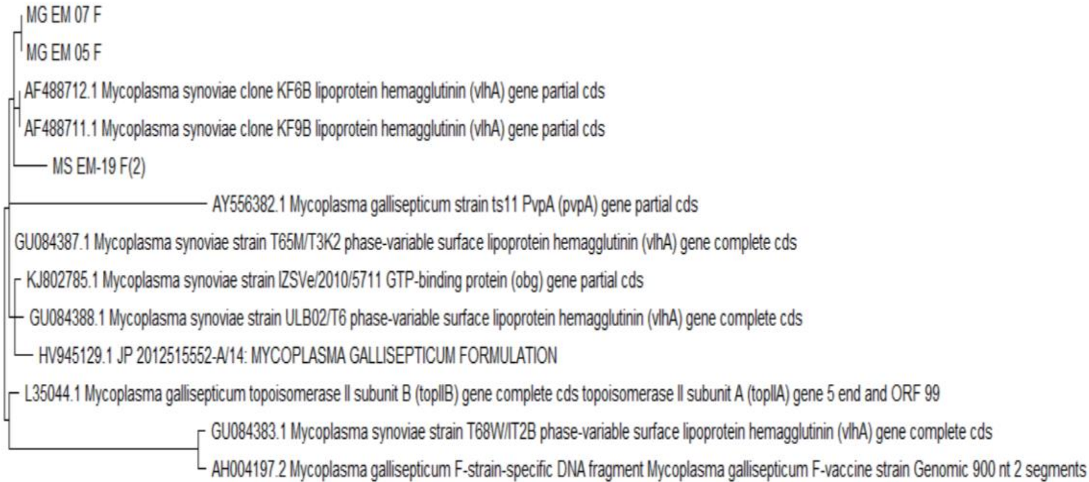


Figure 9: Phylogenetic analysis of isolated *M. gallisepticum* and *M. synoviae*

4.5 Prevalence of specific organism from sample

From 73 of layer and breeder swab sample, 28 pure isolates of *M. gallisepticum* and *M. synoviae* were detected. Among the isolates, 24.65% were *M. gallisepticum* and 13.7% were *M. synoviae.*

Table 4 Prevalence of specific organism from sample

No. of sample investigated	No. of pure isolates	No. of *M. gallisepticum* isolates with prevalence (%)	No. of *M. synoviae* Isolates with prevalence (%)
73	28	18(24.65%)	10(13.7%)

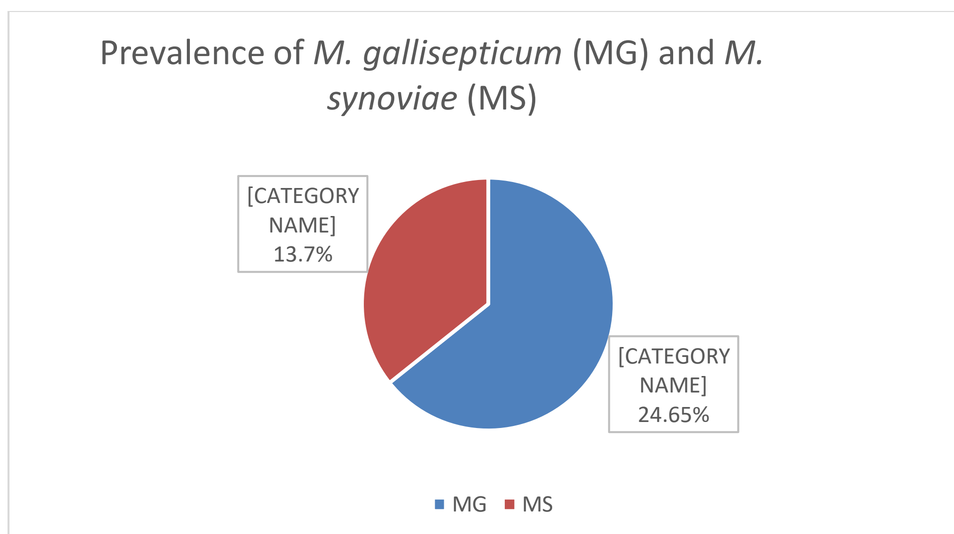


Figure 10: Prevalence of *M. gallisepticum* and *M. synoviae*

4.6 Prevalence of *M. gallisepticum* and *M. synoviae* in different types of chicken

In this study, 18 isolates were positive for *M. gallisepticum* in which 9 isolates were from layer and 9 isolates were from breeder. Prevalence percentage of *M. gallisepticum* was 22% in both cases of layer and breeder. 10 isolates were positive for *M. synoviae,* where 10 isolates were from layer but no isolate from breeder was found positive. Prevalence percentage of *M. synoviae* was 31% in case of layer chicken.

Table 5 Prevalence of *M. gallisepticum* and *M. synoviae* in different types of chicken

Name of the types of chicken	Isolated organism	No. of isolates with prevalence percentage (%)
Layer	*M. gallisepticum*	9 (22%)
	M. synoviae	10 (31%)
Breeder	*M. gallisepticum*	9 (22%)
	M. synoviae	Nil

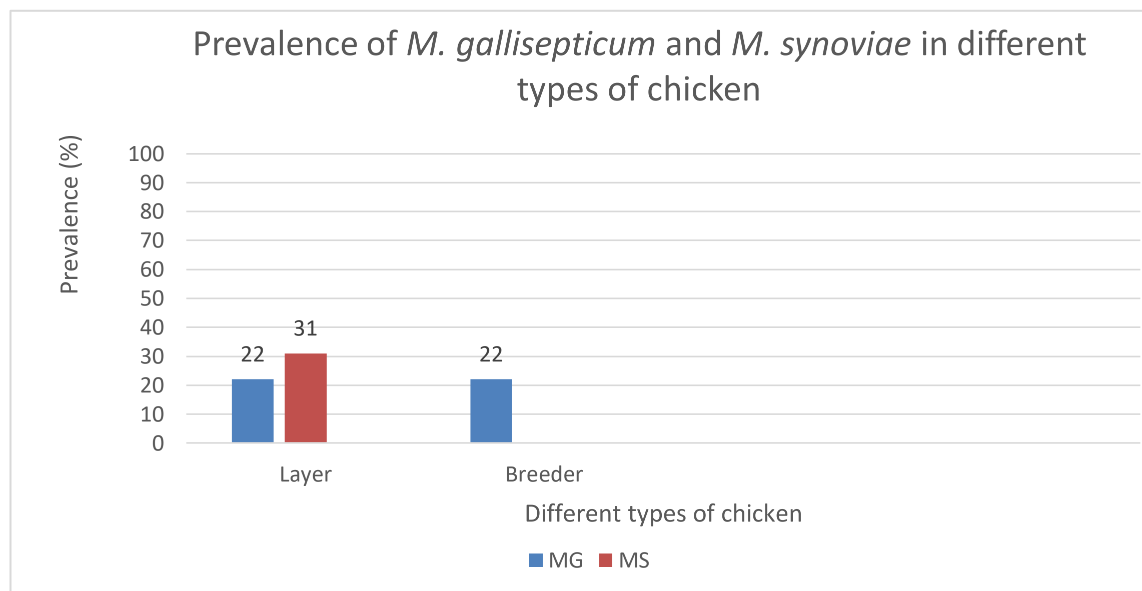


Figure 11: Prevalence of *M. gallisepticum* and *M. synoviae* in different types of chicken

4.7 Prevalence (%) of *M. gallisepticum* and *M. synoviae* in different areas of Bangladesh

Prevalence of *M. gallisepticum* was 9% in Gazipur, 33% in Mymensingh, 13% in Narshingdi 45% in Chattagram. 33% in Rangpur and 25% in Kishoregang area. On the other hand, prevalence of *M. synoviae* was 9% in Gazipur, 50% in Narshingdi, 42% in Kishoregang area but in Mymensingh, Chattagram and Rangpur, no prevalence of *M. synoviae* was found.

Table 6 Prevalence (%) of *M. gallisepticum* and *M. synoviae* in different areas of Bangladesh

	M. gallisepticum			*M. synoviae*		
Area	No. of isolates	No. of samples	percentage	No. of isolates	No. of samples	Percentage
Gazipur	1	11	9%	1	11	9%
Mymenshingh	2	6	33%	0	6	0%
Narshingdi	1	8	13%	4	8	50%
Chattagram	9	20	45%	0	20	0%
Rangpur	2	6	33%	0	6	0%
Kishoregang	3	12	25%	5	12	42%

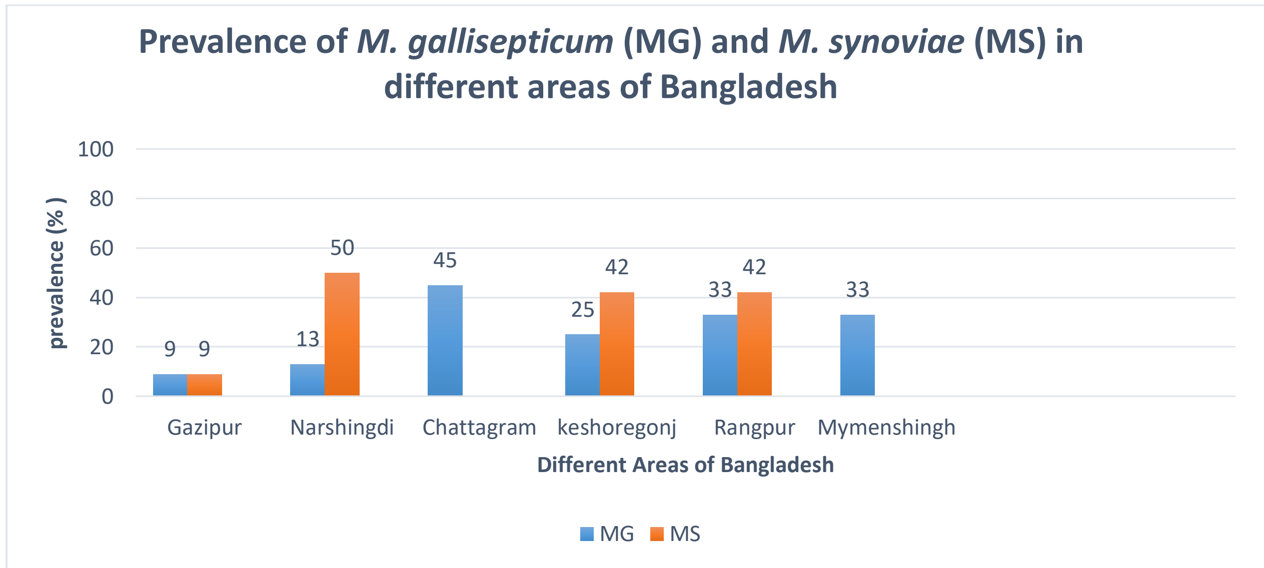


Figure 12: Prevalence of *M. gallisepticum* and *M. synoviae* in different areas of Bangladesh

DISCUSSION

The experiment was conducted for the molecular characterization of *M. gallisepticum* and *M. synoviae* which were isolated from swab samples of layer and breeder chickens of different areas of Bangladesh. Mycoplasma infections are important poultry disease that causes economic losses in poultry production, especially in layers (Singh *et al*., 2016). It is demonstrated that *M. synoviae* infection causes severe economic losses due to its vertical transmission of the germ, resulting in death of embryo, consequently a decrease in hatch rate, significant post-hatch mortality, bacteria diffusion in the hatchery, and quality degradation of day-old chicks.

Moreover, *M. synoviae* may induce transient immunosuppression, an increase in mortality of 1-4%, particularly in broiler chickens, a decrease of 5-10% in egg production rate, and a decrease of 5-7% in hatch rate (Stipkovits and Kempf, 1996). Mycoplasmosis due to *M. synoviae* occurred in layer hens flocks are resulting in a decrease in the egg quality.

Molecular characterization of *M. gallisepticum* was done by PCR amplification of 16SrRNA(185 bp) with MG 13 and MG 14 primers. On the other hand, amplification of 16SrRNA (227 bp) of *M. synoviae* was done by MSL-1 and MSL-2 primers (Lauerman, 1998). In the present study, 16S rRNA gene of *M. gallisepticum* and *M. synoviae* were sequenced and analyzed. The isolates were clustered with respective organisms from different countries of the world. Both the haplotypes of *M. gallisepticum* (MG_EM_07_F and MG_EM_05_F) were included in one cluster which was sister to the clade formed by *M. synoviae*. The sequences of *M. gallisepticum* were in the different clades along with other sequence reported from China (AY556382) which was found in poultry. On the other hand, *M. synoviae* haplotype MS_EM-19_F(2) was sister to the clade formed by strains of *M. synoviae*. The sequences were in the same clade along with other sequence reported from Georgia (AF488711, AF488712) which were found in chicken and turkey and Egypt (GU084387, GU084388) which were found in breeder chicken. The sequences from other countries were present in different clads including Italy (KJ802785) which was found in lesser flamingo.

In this study, From 73 of layer and breeder swab sample, 28 pure isolates of *M. gallicepticum* and *M. synoviae* were detected. Among the isolates, 24.65% were *M. gallicepticum* and 13.7% were *M. synoviae* with overall prevalence 19.17% which is comparatively lower than previous reports. S.R. Barua *et al.* (2006) observed that the overall sero-prevalence of mycoplsmosois was 66.50 % in layer which was higher than that of earlier report (57.15%; Prodhan, 2002 and 13-22% ; Biswas *et al*., 1992). It may be due to the age of the chickens because highest infection in the young chickens, maybe due to the vertical transmission of the organisms, and lowest rate of infection in adult chickens maybe due to infections or unsuitable environmental conditions in flocks.

In this study, 18 isolates were positive for *M. gallisepticum* in which 9 isolates were from layer and 9 isolates were from breeder. Prevalence of *M. gallisepticum* was 22% in both cases of layer and breeder which are comparatively lower than some previous results. Previous studies on

broiler breeder farms in Iran also demonstrated high seroprevalence (21.4%) of *M. gallisepticum* (May M and Brown, 2011; Seifi and Shirzad, 2013) . Overall prevalence of *M. gallisepticum* in 2012 was 49.38% in Pakistan (Raviv and Kleven, 2008). It was reported that the 58.9% of layer chickens was seropositive for *M. gallisepticum* infection in Feni District of Bangladesh (Dulali, Unpublished). Furthermore high seroprevalence rates 45.10% and 81.15 % of positive in laying hens of Rajshahi and Batna Districts of Eastern Algeria and its surroundings, respectively, was reported (Catania S. *et al.,* 2010; Heleili *et al.,* 2012) . However, intensive nature of poultry farming provided opportunity for recycling of the pathogens due to population density. The other factors that contribute *M. gallisepticum* infection are poor ventilation, contamination of litters and no restriction on the movement of the technical personnel, visitors and such other persons as well as other biosecurity measures.

Out of 73 samples, 10 isolates were positive for *M. synoviae,* where all the isolates were from layer but no isolate from breeder was found positive. Prevalence of *M. synoviae* was 31% in case of layer chicken. Kursa *et al.* (2019) observed 29% *M. synoviae* infection in layer in Poland which is lower than this results. Lack of proper management and vaccination may cause it.

Prevalence of *M. gallisepticum* was 9% in Gazipur, 33% in Mymensingh, 13% in Narshingdi 45% in Chattagram. 33% in Rangpur and 25% in Kishoregang area which was highest in Chattagram and lowest in Gazipur. On the other hand, prevalence of *M. synoviae* was 9% in Gazipur, 50% in Narshingdi, 42% in Kishoregang area but in Mymensingh, Chattagram and Rangpur, no prevalence of *M. synoviae* was found which was highest in Narshingdi and lowest in Gazipur. This variation was occurred due to variation of management practices, treatment, maintenance of biosecurity etc. It is suggested that proper management practices and improvement of biosecurity should be properly managed for controlling of Mycoplasma infection.

CHAPTER-5

CONCLUSION

The prevention of mycoplasmosis in poultry includes the acquisition of birds free from *Mycoplasma* spp. and constant monitoring of breeder flocks. These flocks free of *M. gallisepticum* should be sustained by maintaining replacements from mycoplasma-free sources in

a single-age, all in all out management system. Control of avian mycoplasmosis consists of good biosecurity and proper hygiene. Although medication can be very useful in preventing clinical signs and lesions as well as economic losses, it cannot eliminate infection from a flock, it is not a satisfactory long term solution. Control by medication is necessary to compliment biosecurity measures to minimize economic losses, lateral and vertical transmissions. The results of this study suggest that chickens should be checked periodically to investigate the status of Mycoplasma infection. Moreover, the implementation of biosecurity measures in poultry farms are needed. More exhaustive studies including attempts at isolation, in vivo pathogenicity studies and molecular analysis may be useful to better investigate the molecular profile and the potential epidemiological role of Mycoplasma strains circulating in poultry farms. There are several research on Mycoplasma in Bangladesh. The culture of Mycoplasma is so tough. So further work should be on Isolation and identification of Mycoplasma strain available in Bangladesh and determination of Minimum Inhibitory Concentration of locally used antibiotics should be determined.

REFERENCES

Ahmad, A., Rabbani, M., Yaqoop, T., Ahmad, A., Shabbir, M.Z. and Akhtar, F. (2008). Status of IgG antibodies against Mycoplasma gallisepticum in non-vaccinated commercial poultry breeder flocks. *Journal Animal Plant Science*. Volume **18** Pages 2-3.

Biswas, H.R., Hellana, G.M., Mostafa, Afzal, H.M. and Haque, M.M. (1992). Chicken Mycoplasma in Bangladesh. *Asian Australasian Journal of Animal Science*. **6**: 249-251.

Bradbury J.M. (2005). Gordon Memorial Lecture. Poultry mycoplasmas: sophisticated pathogens in simple guise. *British Poultry Science*. **46** (2): 125-136.

Bradbury J.M. (2001). Avian mycoplasmosis. In: poultry diseases. 5 th ed. Iowa, 178-193.

Buim, M.R., Mettifogo, E., Timenetsky, J., Kleven, S. and Ferreira, A.J.P. (2009). Epidemiological survey on *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by multiplex PCR in commercial poultry. *Pesq. Vet. Bras*. **29**(7): 552-556.

Catania, S., Bilato, D., Gobbo, F., Granato, A., Terregino, C., Iob, L. and Nicholas, R.A. (2010). Treatment of eggshell abnormalities and reduced egg production caused by *Mycoplasma synoviae* infection. *Avian Dis.* **54**(2):961–964.

Cookson, K.C. and Shivaprasad, H.L. (1994). *Mycoplasma gallisepticum* infection in chukka patridges, pheasants and peafowl. *Avian Diseases*. **38**:914-921.

Dulali, R. (2003). Seroprevalence and pathology of mycoplasmosis in sonali chickens. MS Thesis, Department of Pathology, Faculty of Veterinary Science. Mymenisngh: Bangladesh Agricultural University.

Feberwee, A., De Wit, J. and Landman, W.J. (2009). Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: Field and experimental studies. *Avian Pathol*. **38**(1): 77-85.

Garner, G., Saville, P. and Fcdiaevsky, A. (2006). Manual for the recognition of exotic diseases of livestock. A reference guide for animal health staff (online). FAO. 2004. Mycoplasmosis (*M. gallisepticum*).

Heleili, N., Ayachi, A., Mamache, B. and Chelihi, A.J. (2012). Seroprevalence of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* at Batna commercial poultry farms in Algeria. *Vet. World.* **5**(12):709–712.

Hossain, K.M.M., Ali, M.Y. and Haque, M.I. (2007). Seroprevalence of *Mycoplasma gallisepticum* infection in chicken in the greater Rajshahi District of Bangladesh. *Bangladesh Journal of Veterinary Medicine* . **5** (1 &2): 09-14.

Kempf, I., Gesbert, F., Guittet, M., Bennejean, G. and Stipkovits, L. (1994). Evaluation of two commercial enzyme-linked immunosorbent assay kits for the detection of *Mycoplasma gallisepticum* antibodies. *Avian Pathology*. **2**:329 - 338.

Khan, J., Farzand, R. and Ghumro, P.B. (2010). Antibiotic sensitivity of human genital mycoplasmas. *African Journal of Microbiology Research*. **4**(9): 704-707

Kleven, S. H. (2008). Control of avian mycoplasma infections in commercial poultry. *Avian Diseases*. **52**(3): 367-374.

Kleven, S.H. (1998). Mycoplasmas in the aetiology of multifactorial respiratory diseases. *Poultry Science.* **77**: 1146- 1149.

Kong, F., James, G., Gordon, S., Zelynski, A. and Gilbert, G.L. (2001) Species-specific PCR for identification of common contaminant Mollicutes in cell culture. *Appl Environ Microbiol.***67**:3195–3200.

Kursa, O., Tomczyk, G. and Sawicka, A. (2019). Prevalence and phylogenetic analysis of *Mycoplasma Synoviae* strains isolated from Polish chicken layer flocks. *Journal Veterinary Research*. **63**(1): 41-49.

Kumar, S., Stecher, G., LiM., Knyaz, C. and Tamura, K. (2018). MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms. **35**: 1547-154.

Lauerman, Lloyd, H. (1998). Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases. Development and Application of a Polymerase Chain Reaction Assay for *Mycoplasma synoviae*. *Avian Diseases.* **37**: 829-834.

Landman, W.J.M. and Feberwee, A.(2001). Field studies on the association between amyloid arthropathy and *Mycoplasma synoviae* infection, and experimental reproduction of the condition in brown layers. *Avian Pathol*. **30**:629–639.

Ley, D.H. and Yoder Jr, H.W. (1997). *Mycoplasma gallisepticum* infection. In: Calnek, B.W., Barnes, H., Beard, C.W., Mcdougald, L.R., Saif, Y.M. (eds). Disease of poultry 10th Edition. Ames, Iowa, USA. 194-207.

Lockaby, S. B., Hoerr, F.J., Lauerman, L. H., Smith, B.F., Samoylov, A.M., Toivio-Kinnucan, T.A. and Kleven, S.H. (1999). Factors associated with virulence of *Mycoplasma synoviae*. *Avian Dis*. **43**:251–261.

McMullin P. (2004). A pocket guide to poultry health and disease.

Maniloff, J. (2002). Phylogeny and evolution. In: Razin, S., Herrmann, R. (Eds.), Molecular Biology and Pathogenicity of Mycoplasmas. Kluver Academic/Plenum Publishers, New York, pp. 31–43.

Marois, C., Oufour-Gesbert, F. and Kempf, I. (2000). Detection of *Mycoplasma synoviae* in poultry environment samples by culture and polymerase chain reaction. *Veterinary Microbiology*. **73**: 311-318.

May, M. and Brown, D.R.(2011). Diversity of expressed vlhA adhesin sequences and intermediate hemagglutination phenotypes in *Mycoplasma synoviae*. *J. Bacteriol.***193**(9):2116–2121.

Messa Júnior, A., Taunde, P., Zandamela, A.F., Junior, A.P., Chilundo, A. and Costa, R. (2017). Serological Screening Suggests Extensive Presence of *Mycoplasma gallisepticum* and

Mycoplasma synoviae in Backyard Chickens in Southern Mozambique. *Journal of veterinary medicine*. **2017**:2743187

Mohammed, H.O., Carpenter, T.E. and Yamamoto R. (1987). Economic impact of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in commercial layer flocks. **31**: 477-82.

Nascimento, E.R. (2000). Mycoplasmoses. In: Macari M., Berchieri Jr. A, (editors). Doenças das aves. Campinas: FACTA. 217-24.

Nascimento, E.R., Pereira, V.L.A., Nascimento, M.G.F. and Barreto, M.L. (2005). Avian mycoplasmosis update. Brazilian Journal of Poultry Science. **7**(1): 01-09

Nunoya, T., Yagihashi, T., Tajima, M. and Nagasawa, Y. (1995). Occurance of keratoconjunctivitis apparently caused by *Mycoplasma gallisepticum* in layer chickens. *Veterinary Pathology*. **32**: 11-18.

OIE, (2007). Avian mycoplasmosis (*Mycoplasma gallisepticum*).

Oritz, A., Froyman, R. and Kleven, S. H. (1995). Evaluation of enrofloxacin against egg transmission of *Mycoplasma gallisepticum*. *Avian Diseases*. **39**: 830-6.

Osman, K.M., Aly, M.M., Amin, Z.M.S. and Hassan, B.S. (2009). *Mycoplasma gallisepticum*: an emerging challenge to the poultry industry in Egypt. *Rev. Sci. Tech*. Office International Epizootics. **28** (3): 1015-1023.

Poveda, J.B., Carranza, I., Miranda, A., Hermoso, M. and Comenech, I. (1990). An epizootiological study of avian mycoplasmas in Southern Spain. *Avian Pathology*. **19**: 627-633.

Pradhan, MaM, Amin, MM, Taimur, MJF 2000. A seroprevalence study of avian Mycoplasma in Bangladesh. 7th BSVER.

Prodhan, M.A.M. (2002). Studies on avian Mycoplasmosis: Prevalence, Isolation, Characterization and Antigenic properties. A Ph.D. thesis offered from Department of Microbiology and Hygiene. Bangladesh Agricultural University, Mymensingh, pp. 178.

Quirk, J.T., Kupinski, J.M. and Dicioccio, R.A. (2001). Detection of Mycoplasma ribosomal DNA sequences in ovarian tumors by nested PCR. *Gynecol Oncol.* **83**:560–562.

Raviv, Z. and Kleven, S.H. (2008). The Development of Diagnostic Real-Time Taqman PCRs for The Four Pathogenic Avian Mycoplasmas. *Av. Dis.* **53**(1):103–107.

Rhorer, A.R. (2002). USDA, APHIS, National Poultry Improvement Plan Conyers, GA. Personal Communication. 5 th ed. edited by Jordan et al., W.B. Saunders Company, Iowa, Pages 178-193.

Barua, S.R., Prodhan, A.M., Islam, S. and Chowdhury, S. (2006) . *Bangl. J. Vet. Med*. **4** (2): 141–142.

Sarkar, S., Rahman, M., Rahman, M., Amin, K., Khan, M. and Rahman, M. (2005). Sero-prevalence of infection of chickens in model breeder poultry farms of Bangladesh. *Int. J. Poult. Sci.* **4**(1):32–35.

Seifi, S. and Shirzad, M. (2013). Risk factors and seroprevalence of *Mycoplasma synoviae* infection in broiler breeder farms. *Acta Sci. Vet.* **41**:1139.

Singh, N., Shukla, S. and Sharma, V. (2016). Detection of anti'*Mycoplasma gallisepticum'*antibodies in different age group of chicken by Enzyme linked immunosorbant assay. *Journal of Animal Research*. **1**:49.

Stakenborg, T., Vicca, J., Verhelst, R., Butaya, P. and Maes, D. (2005). Evaluation of tRNA gene PCR for identification of Mollicutes. *J Clin Microbiol.* **43**:4558–4566.

Stipkovits, L. and Kempf, I. (1996). Mycoplasmoses in poultry. *Rev. Inform. Sci. Technol*. **15**(4): 1495-1525.

Tang, J., Hu, M., Lee, S. and Robin, R.A. (2000) Polymerse chain reaction based method for detecting *Mycoplasma/ Acholeplasma* contaminants in cell culture. *J Microbiol Methods.* **39**:121–126.

Thu, N.V., Thuy, L.T. and Phuong P.T. (2003). Investigation of avian mycoplasma infection in Vietnam by molecular tools.

Tamura, K. and Nei, m. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution.* **10**: 512-526.

Wang, Y., Whithear, K.G. and Ghiocas, E. (1990). Isolation of *Mycoplasma gallinarum* and *Mycoplasma gallinaceum* from the reproductive tract of hens. *Australian Veterinary Journal.* **67**: 990.

Whithear, K.G. (1976). Avian mycoplasmosis. Bacteriology. First published as: Avian mycoplasmosis; by Eggleton D., Lewis P.F. and Hall W.T.K. by the Australian Bureau of Animal Health.

Yoder, H.W. (1972a). *Mycoplasma gallisepticum*. In: Diseases of poultry, 6th Edition. Edited by Hofstad, M.S., Calnek, B.W., Helmboldt, C.F., Reid, W.M. and Yoder, H.W., Ames, Iowa: State University Press. 287-307.