SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF *MYCOPLASMA GALLISEPTICUM* FROM LAYER POULTRY IN NORTHERN PART OF BANGLADESH

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

SUBMITTED BY
Mahfuja Khatun
Registration Number: 1405163
Session: 2014

Department of Microbiology
Hajee Mohammad Danesh Science and Technology University
Dinajpur – 5200, Bangladesh

September, 2018

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DOCTOR OF PHILOSOPHY

Approved as to style and content by

_____	_____
(Professor Dr. Md. Mostafizer Rahman)	**(Professor Dr. Md. Shahidur Rahman Khan)**
Supervisor	Co-Supervisor

_____
(Professor Dr. Md. Khaled Hossain)
Member
Supervisory Committee

_____
(Professor Dr. Md. Fazlul Hoque)
Chairman
Examination Committee

Department of Microbiology
Hajee Mohammad Danesh Science and Technology University, Dinajpur–5200, Bangladesh
September, 2018

Dedicated
To
My Respected Parents,
Beloved Husband & Sons

Department of Microbiology
Faculty of Veterinary and Animal Science
Hajee Mohammad Danesh Science and Technology University
Dinajpur-5200

CERTIFICATE

This is to certify that the thesis entitled "**Serological and molecular characterization of *Mycoplasma gallisepticum* from layer poultry in northern part of Bangladesh"** is prepared by Mahfuja Khatun, Registration No. 1405163, Session: 2014, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, for the degree of **Doctor of Philosophy** under my guidance and supervision. The research work is an original and to the best of my knowledge, no part of the thesis has been produced elsewhere for any degree.

Professor Dr. Md. Mostafizer Rahman
Supervisor
Department of Microbiology
Faculty of Veterinary and Animal Science
Hajee Mohammad Danesh Science and
Technology University (HSTU)
Dinajpur-5200

DECLARATION

The dissertation entitled **"Serological and molecular characterization of *Mycoplasma gallisepticum* from layer poultry in northern part of Bangladesh"** submitted to Hajee Mohammad Danesh Science and Technology University, Dinajpur in partial fulfillment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in Microbiology is a bonafide research work and carried out by me. I solemnly declare that this thesis is not submitted to any other University or institution anywhere for the award of any academic degree.

September, 2018 Mahfuja Khatun

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SEROLOGICAL AND MOLECULAR CHARACTARIZATION OF *MYCOPLASMA GALLISEPTICUM* FROM LAYER POULTRY IN NORTHERN PART OF BANGLADESH

Mahfuja Khatun

ABSTRACT

Poultry industry targets the production of animal protein of excellent quality with low costs and helps in national economy for generating jobs and foreign exchange credits for the balance of trade. But outbreaks of different infectious diseases are one of the major constraints of poultry farming. *Mycoplasma gallisepticum* organism is one of the most infectious organism and economically significant throughout the world. The present research was performed with the objective of serological and molecular characterization of *Mycoplasma gallisepticum* organism from field samples in order to get pure culture of the etiological agent. The epidemic behavior of the etiological agents were studied based on age and breed of birds, seasons and location where the birds were rearing. The incidence rate of infections were recorded as per information collected from farmers by using a set of questionnaire, symptoms of affected birds and post mortem lesions. In association with the epidemiological investigation, the overall incidence rate was 11.54%. For detection of specific antibody of *Mycoplasma gallisepticum*, 920 sera were tested from ten selected farms based on location, age groups, seasons, breeds and flock size, by SPA test and found 526 sera were positive. The overall incidence was 57.17%. The highest incidence rate (61.96%) was found at Dinajpur and the lowest (51.09%) at Rangpur in comparison to other districts. The highest incidence (64.78%) was found in above 40 wks followed by 59.30% in 21-40wks, 56.52% in 9-20wks and 48.26% in 0-8wks respectively. The prevalence was found 61.96% in winter and 52.39% in summer season, 59.78% in Sonali and 56.52% in Isa brown breed, 61.96% in large and 51.44% in small flock. No significant difference was observed as their location and breed but significant was in age and season variation. Furthermore 526 SPA positive sera were tested by iELISA test and 164 (31.18%) sera were positive. 80 representative sera were tested by HI from iELISA positive sera and found that 15 (18.75%) sera were positive. A total of 156 different organs of trachea, lungs and air sacs were cultured for isolation of etiological agent of Mycoplasmosis and the positive cases were 5.77%. The highest rate (13.46%) of organism was found in tracheal swabs followed by 1.92% in air sac and 1.92% in lung. For molecular characterization of *Mycoplasma gallisepticum*, 48 different organs were tested by direct PCR without culture and overall positive was 12.5% (6 bands). Among them 25%, 6.25% and 6.25% were found from tracheal swabs, air sacs and lungs respectively and 0.64% (1 band) from culture positive isolates. The DNA sequencing of the present study showed 99 to 100% per cent similarity with the sequence of the duck isolate from South Africa, chicken isolate of Spain and USA as demonstrated by blast (NCBI). It is recommended that the samples from live birds are sufficient enough to identify the organism and tracheal swab samples gave more isolations than from lung and air sac by molecular technique. It is justified that PCR is a serviceable tool in the accurate diagnosis of *Mycoplasma* infections, not only for its sensitivity but also for its high specificity. This technique overcome culture method as it depends on the direct detection of the micro organism's DNA without the need for cultivation. PCR has allowed the study of microbial genes, directly amplified from samples. It is a sensitive, easy, rapid and inexpensive technique and the most important advantage is eliminating the need for isolation of *Mycoplasma gallisepticum*.

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LIST OF ABBREVIATIONS

$	:	Dollar
-	:	Minus
%	:	Percentage
+	:	Plus
=	:	Equal
µg	:	Milligram
µl	:	Microliter
µm	:	Micrometer
AFLP	:	Amplified fragment length polymorphism
BBS	:	Bangladesh bureau of statistics
BER	:	Bangladesh economic review
bp	:	Base pair
CI	:	Confidence interval
cm	:	Centimeter
CO_2	:	Carbon dioxide
cRBC	:	Chicken red blood cell
CRD	:	Chronic respiratory Disease
DNA	:	Deoxyribonuclic acid
E. coli	:	Escherichia coli
EDTA	:	Ethylene Diamine tetra acetic acid
eg	:	Example
ELISA	:	Enzyme Linked immune sorbent assay
et al	:	Associates with
Etc	:	Et cetera
G	:	Gram
h	:	Hour
HA	:	Haemaglutination
HI	:	Haemaglutination inhibition
hr	:	Hour
HSTU	:	Hajee Mohammad Danesh Science and Technology University
IB	:	Intectious Bronchitis
iELISA	:	Indirect Enzyme Linked immune sorbent assay

LIST OF ABBREVIATIONS (Contd.)

IP	:	Immune peroxidase
IU	:	International unit
kb	:	Kilo byte
M. gallisepticum	:	*Mycoplasma gallisepticum*
M. synoviae	:	*Mycoplasma synoviae*
MG	:	*Mycoplasma gallisepticum*
MI	:	*Mycoplasma iowae*
Min	:	Minute
ml	:	Milliliter
MM	:	*Mycoplasma meleagridis*
MM	:	Milimeter
MS	:	*Mycoplasma synoviae*
N	:	Number
N.B	:	Nota bene
NaOH	:	Sodium hydroxide
NCD	:	New castle disease
NIB	:	National Institute of Biotechnology
nm	:	Nanometer
NPIN	:	National poultry improvement plan and auxiliary provision
NPIP	:	National poultry improvement program
ºC	:	Degree Celsius
OIE	:	World organization for animal health
op	:	Optical density
OR	:	Odd ratio
PBS	:	Phosphate buffer saline
PCI	:	Phenol chloroform isoamyl alcohol
PCR	:	Polymerase chain reaction
PNNP	:	P-Nitrophenyle phosphate
PNSA	:	Program a National de sanidade avicola
PPLO	:	Pleuropneumonie like organism
RAPD	:	Random amplified polymorphic DNA
RBC	:	Red Blood Cell

LIST OF ABBREVIATIONS (Contd.)

RFLP	:	Restriction fragment length polymorphism
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
rRNA	:	Ribosomal RNA
RSA	:	Rapid serum agglutination
SDS	:	Sodium dodecyl sulfate
SDS	:	Sodium dodecyl sulfate
Sec	:	Second
Sl. No.	:	Serial number
sp	:	Species
SPA	:	Standard plate agglutination
SPSS	:	Statistical package for social science
TAE	:	Tris acetate EDTA
TM	:	Trade mark
UK	:	United Kingdom
US	:	United State
USA	:	United state of America
USDA	:	United states department of Agriculture
UV	:	Ultraviolet
w/v	:	Weight/ volume
wk	:	Week
χ^2	:	Chi-squre

CHAPTER 1

INTRODUCTION

The Poultry industry is one of the organized and vibrant sectors of Bangladesh and represents one way of accomplishing several national goals under a single banner. Employment, poverty alleviation and improved nutrition are all potential benefits from continued support and encouragement of poultry development. In the agriculture sector, it contributes 39% of employment and represents 15% of job opportunities in the national economy (BBS, 2007). Overall, the annual average growth in poultry production from 2005 to 2011 was 3.7% (BER, 2011). Poultry is usually the first step in the drive towards increased incomes through the acquisition of larger animals like goats, sheep and cattle (Saleque and Mustafa, 1996).

Poultry sector has tremendous opportunities in Bangladesh. The demand and supply gap of animal protein in Bangladesh is huge; and poultry industry is playing a major role in minimizing the nutrition deficiencies of this country. Especially in case of animal protein supply. The poultry meat alone contributes 37% of the total meat production in Bangladesh (Begum *et al*., 2011) and poultry contributes significantly to the welfare of the people both at household and national level. Poultry sector is a key actor, but the growth and profitability is being challenged by many reasons (Chowdhury, 2013). The total chicken population was 253.79 million in January 2015 (Bangladesh Economic Review, 2014). The yearly layer chicken production in Bangladesh, during the year 2014, only by 11 mainstream poultry hacharies were 26.97 million egg production in 2010, 2011, 2012 was 27.50, 26.00, 23.50 million/day respectively. The numbers of commercial poultry farms in Bangladesh were 114,00 in 2010, 98,000 in 2011 and 75,000 in 2012 (Chowdhury, 2013).

The poultry sub-sector is crucially important in the context of growth and improvements of diets of people in Bangladesh. According to BBS (2011), there are 43.2% (urban) and 39.5% (rural) population live below the poverty line and suffer from serious malnutrition. In Bangladesh; standard egg requirement per person/year is 104; availability of eggs per person/year is 31 and there is a deficit of egg production by 73 eggs which is 65.38%.

Protein deficiency has been taken as the major contributory factor in malnutrition. The per capita consumption of animal protein in Bangladesh is only 11.80gm per day (BBS, 2011),

whereas the standard requirement is 36 gm. According to Bhuyian (1999), 37% of the total animal protein supplied in the country comes from poultry meat. Thus, the poultry sub-sector can contribute in combating this problem.

Poultry meat is the fastest growing component of global meat production, consumption, and trade, with developing and transition economies playing a leading role in its expansion. Expansion of poultry industry in Bangladesh is being driven by rising incomes of the consumers and a shift in industry structure towards integrated ownership and coordination of the input, production and marketing operations involved in poultry production.

In Bangladesh, however, outbreaks of different infectious diseases are one of the major constraints of poultry farming. Diseases and other factors are rampant and hindered the development of poultry production in the country and mortalities due to diseases are estimated to be 20% to 50% but can go as high as 80% during times of epidemics (Alemu, 1995). Despite rapid growth of this industry in Bangladesh, it is vulnerable to certain infectious agents (Sarker *et al*., 2005). Among them microbial diseases are major health hazards being faced by poultry birds. Mycoplasmosis, one of the major avian diseases in Bangladesh which is economically important and emerging problems for raising poultry industry (Prodhan, 2002). It affects both commercial exotic breeds and indigenous local breeds.

Mycoplasmosis is caused by four commonly recognized pathogens namely: *Mycoplasma gallisepticum, M. synoviae, M. melegridis and M. iowae* (Bradbury, 2001). Among them, *Mycoplasma gallisepticum* (MG) is the most pathogenic and economically significant throughout the world (Raviv and Ley, 2013). MG infections are known as "chronic respiratory disease" (CRD) of chickens and "infectious sinusitis" of turkeys (Ferguson and Noormohammadi, 2013; Raviv and Ley, 2013). Respiratory rales, coughing, nasal discharge and conjunctivitis are the clinical manifestations of chickens caused by MG (Lay and yoder, 1997) and frequently causes infra orbital sinusitis in turkeys (Ley, 2008). In breeders and layers, the disease causes tremendous drop in egg production and an increase in embryo mortality (Ley, 2003). Vertical transmission of MG has been documented and it leads to infected progeny flocks (Bradbury, 2001). Reduced feed consumption and loss of body weight are the most characteristic signs of naturally-occurring MG disease in adult flocks (Kleven and Noel, 2008; Ley, 2008). Therefore, in

order to increase the output of poultry farm, it is very important to prevent mycoplasmosis. There are three normal aspects (prevention, vaccination and medication) being applied to control mycoplasmosis. Although the farmers have been taking above measures, recurrent of infection and disease outbreaks are still present (Kartini, 2012). Moreover, in many cases antibiotic resistance was observed (Gautier-Bouchardon *et al.*, 2002).

Laboratory methods are essential for diagnosing MG, as in many cases clinical signs or pathological lesions are not adequate for a confirmatory diagnosis. There are three approaches to diagnosis of MG which are; isolation and identification of the organism, detection of specific antibodies and molecular detection (Bradbury and Kleven, 2008).

The cultivation technique (used for isolation and identification of the organism) has a number of limitations besides being laborious, time consuming, slow, expensive and require sterile conditions. Problems experienced with culturing include overgrowth by faster-growing Mycoplasma species and other organisms or no growth in subcultures. It can take three- four weeks and even then the result can be negative or be hampered by over growth of non-pathogenic mycoplasma and mixed infections (Bradbury and Mc Lenaghan, 1982; S. Zhao *et al.*, 1993; Ferguson-Noel and Williams, 2015).

For many years, detection of specific antibodies of MG was based on serological assays. Serological tests include serum plate agglutination test (SPA), haemaglutination inhibition test (HI) and enzyme linked immunosorbant assay (ELISA) (Khalifa *et al.*, 2013). These are nonspecific and unconvincing due to the possibility of cross reaction between MG field strains and the antigenic variation among MG strains, which can affect the sensitivity of the serological tests (Avakian *et al.*, 1990; Ben Abdelmoumen *et al.*, 1995).

To overcome these problems, the best approach would be molecular detection. Definite MG diagnosis with genotype identification represents the first step for subsequent-successful control measurements. Nucleic acids/genomes (DNA or RNA) are rapidly deployed next generation vaccine candidates and in trials towards prevention and management of a number of deadly infectious and non-infectious diseases (Doolan *et al.*, 2010). On the other hand, it has been reported that molecular characterization enables epidemiological tracing and classification of new MG field strains, but limited information is available about molecular characterization of local field MG strains in Bangladesh. To solve these problems, species specific recombinant DNA probes for the diagnosis of MG have been developed (Khan *et al.*, 1987).

Though there are many reports regarding the detection of DNA and ribosomal RNA gene probes for MG diagnosis, PCR based methods are more suitable in the majority of applications due to its simplicity, accuracy, rapidity, better sensitivity and specificity facilitating the detection of pathogens in or even alternative diagnostic tool for cultivation and serological tests and for amplifying low amount of nucleic acid to a level that cannot be easily detected by other methods (Harasawa *et al.*, 2004; Evens *et al*., 2005; Peebles *et al*., 2014). This method also diagnosed those who are under treatment with antibiotics (Buim *et al*., 2009). PCR tests can be completed in 1 day or less (Afshrifar, 2005). Sequencing also offer the possibility for typing of uncultivable pathogens, as well as inter laboratory data exchange (Armour *et al*., 2013; Bayatzadeh *et al*., 2014; Ferguson *et al*., 2005; Gharaibeh *et al*., 2011).

As per literature review in Bangladesh many investigators like Sarker *et al.* (2005); Hossain *et al.* (2007); Jalil *et al.* (2010); Zulfekar *et al.* (2015) etc covering seroprevalence and cultural studies. Seroprevalence study is more advantageous due to serological characterization by using SPA as well as indirect ELISA and HI test. It is noted that molecular characterization in this study is a new approach and confirmatory diagnostic tool of any *Mycoplasma* spp. identification.

Thus, to clarify the above issues, the present study was undertaken with the following objectives

1. To conduct serological characterization of *Mycoplasma gallisepticum* considering age, breed, season, flock size and location using SPA, iELISA and HI test.
2. To isolate and characterize *Mycoplasma gallisepticum* organism from suspected to be infected and dead layer chickens reared in various selected farms using cultural, morphological, biochemical and molecular techniques.
3. To perform sequence analysis of selected PCR positive DNA for confirmation of PCR results.

CHAPTER 2

REVIEW OF LITERATURES

2.1 Historical overview

More than a century ago in 1898 a bovine pleuropneumoniae like organism was successfully cultivated by Nocard and Roux which set the stage for mycoplasmology, a new area in science and cattle showing signs of contagious bovine pleuropneumonia, from which the "Pleuropneumonia like organisms (PPLO)" was named. These organisms have long been known, their taxonomy has been contentious including the simplest classification; bacteria or a virus? In 1930 the virus status of mycoplasma was refuted. Klienenberger was isolated an L-form (a laboratory strain after partial or complete removal of cell wall) of *Streptobacillus moniliformis*, in 1935, which significantly resembled mycoplasmas in their morphology, filterability and colony formation. About 30 years until in 1969 it was found that mycoplasmas are nothing but L-form variants of common bacteria where the genomic analysis data containing guanine and cytosine became available and this conception was ruled out (Razin, 1969). From an evolutionary perspective, mycoplasmas lost their cell wall, many more genes that are responsible for cytochrome mediated electron transport chain system, biosynthesis of purines and functional tri-carboxylic acid cycle led to a significantly diminished genome size, differentiating them from the L-forms (Dybvig and voelker, 1996).

Now mycoplasmas are recognized as the smallest self-replicating bacterium. The name 'mycoplasma' was derived from Greek word *mykes* or fungus and *plasma* for something molded to denote the fungus like morphology of *Mycoplasma mycoides,* the first identified mycoplasma. In 1956 current classification of mycoplasma was done by Edward and Freundt and consists of phylum Firmicutes, class Mollicutes, order *Mycoplasmatales*, family *Mycoplasmatacae*, genera *Mycoplasma* (Edward and Freundt 1956, Yoder *et al*., 1984; Razin *et al*., 1998; Quinn *et al*., 2002).

2.2 Avian mycoplasmosis

The termed "epizootic pneumo enteritis of the turkeys" was the first avian *Mycoplasma* reported in 1905 by English scientist Dodd (Lancaster and Fabricant, 1988). Avian mycoplasmosis caused by *Mycoplasma* species described in turkeys and chicken in 1926

and 1936 respectively (Nascimento *et al*., 2005; Kleven, 2008). "Chronic respiratory disease'' (CRD) was first introduced in 1943 by Delaplane and Stuart for the clinical manifestations of the disease in chicken. There are more than 120 named *Mycoplasma* spp. and more than 20 are known to infect avian hosts (Nascimento *et al*., 2005; Purswell *et al*., 2011). Of these, *Mycoplasma gallisepticum* (MG) *and Mycoplasma synoviae* (MS) are the major pathogens, and *Mycoplasma meleagridis* (MM), and *Mycoplasma iowae* (MI) are important in turkeys (Bradbury, 2001; Sprygin *et al*., 2011).

2.3 *Mycoplasma gallisepticum* (MG)

2.3.1 Characteristics features

In worldwide poultry industry MG is the most pathogenic costliest diseases of avian *Mycoplasma* species which have significant economic impact (Raviv and Ley, 2013). Cell shape being spherical, pear shaped, spiral and filamentous forms. The size of spherical or coccoid form ranges from 0.25 to 0.45μm. They are extremely pleomorphic, small prokaryotic, have a single trilaminar membrane composed of glycolipid, phospholipid, protein, and glycoprotein being devoid of cell wall and lacking the genetic capacity to synthesize one (Bradbury, 2001).The base composition is poor in guanine and cytosine with mol % G + C of DNA ranging from 23% to 40%, genome size is small with 5×10^8 to 1×10^9 daltons (Hirsh *et al*., 1999; Quinn, 2002). Most of the disinfectants, heat, and sunlight killed the MG because they have no cell wall. They cannot survive long time from outside of their natural host (Ley *et al.*, 1997). It may be stable normally in the environment up to 3 days (Bradbury, 2001). MG can remain viable in egg yolk at 37°C for 18 weeks or at 20°C for 6 weeks, in chicken feces at 20°C for 1 – 3 days (Chandiramani *et al.*, 1966).

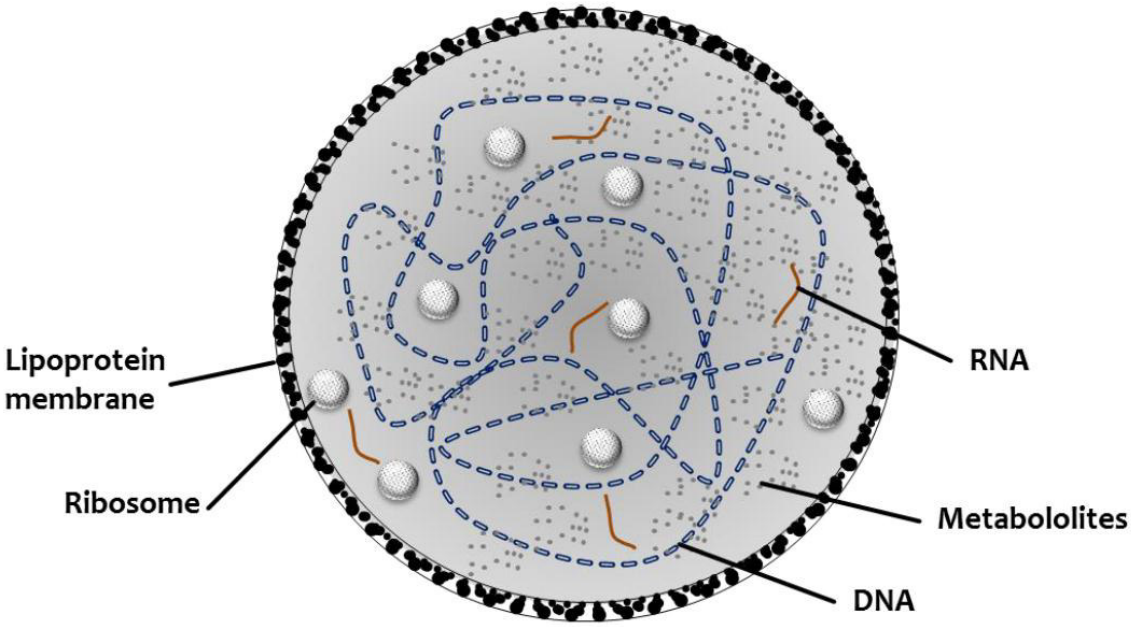


Figure 1: Structure of *Mycoplasma gallisepticum*

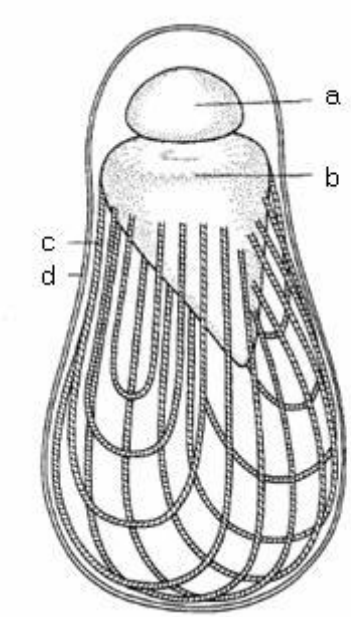


Figure 2: A hypothetical MG scheme cell.

(a) The specialized terminal structure, (b) the electron-dense area (infrableb), (c) loop-shaped tubules and (d) plasma membrane (Korolev *et al*., 1994).

Table 1: Taxonomy of MG (Morowitz and Maniloff, 1966).

Taxonomy of ID33150	233150Ta22xonomy ID 233150222
Kingdom	Bacteria
Intermediate rank 1	Firmicutes
Intermediate rank 2	Mollicutes
Intermediate rank 3	Mycoplasmataceae
Intermediate rank 4	Mycoplasma
Intermediate rank 5	*Mycoplasma gallisepticum*

2.3.2 Epidemiology

2.3.2.1 Distribution of the disease

MG infection has become an important flock problem in chickens and turkeys in all areas of the world (Calnek *et al*., 1991; Ley and Yoder, 1997). In 1984, the prevalence of this organism in commercial pullet and layer flocks of southern and central California of USA was estimated by testing serum and egg-yolk samples through ELISA. In the United States, this organism has been eradicated from most commercial chicken and turkey breeding flocks, but present in other poultry operations (Calnek *et al*., 1991).

Literature on epidemiology of avian mycoplasmosis in backyard chickens of Africa is scanty, with few reports in Zimbabwe (Kelly *et al*., 1994), Botswana (Mushi *et al*., 1999), Benin (chrysistone *et al*., 1995), South Africa (Thekisoe *et al*., 2003), and Ethiopia (Chaka *et al.*, 2012).

Complicated CRD is a disease of major importance in most Asian countries. It is commonly encountered as a result of vaccination and other stress factors. Uchida *et al.* (1987) isolated a total of 280 MG strains from 228 diseased layer chickens housed in 51 commercial farms of various geographical areas of Japan during the period 1979 to 1985. The incidence of MG infection among 20 breeder flocks from six regions of Korea was reported as ranging from 20.0% to 67.0%. The organism has been isolated from natural infections of the gallinaceous birds, especially chickens and turkeys. It also isolated from ducks in England (Jordan *et al.*, 1980) and in Yugoslavia (Bencina *et al.,* 1988a), geese which have come from France (Buntz *et al*., 1986) and from Yuogoslavia (Bencina *et al.*, 1988b), song birds (Morishita *et al*., 1999) as well as from peafowl (Cookson and

Shivaprasad, 1994) and also bobwhite quail, Japanese quail, yellow-naped Amazon parrots in America (Bozeman *et al.*, 1984; Makarami *et al*., 2002), pigeons and greater flamingos. It has been found in wild peregrine falcons in Spain (Calnek *et al*., 1991; Kollias *et al*., 2004; Farmer *et al*., 2005).

Prevalence of avian mycoplasma in different states of India has been reported by several workers (Asnani and Pathak., 1974; Shukla *et al*., 1985; Mahajan *et al*., 1994; Srithar *et al*., 1997; Chakraborty *et al*., 2001) and tested 195 birds in 18 flocks for MG antibodies by rapid slide agglutination and found 42.5% birds were seropositive to MG.

Mahajan *et al.* (1994) carried on experiment on the mortality of 73 outbreaks from January 1987 to December 1990 in Haryana of India and found that the mortality was 7.94% caused by CRD.

Srithar *et al.* (1997) analysed the post mortem records of layer birds over 10 years and found that major respiratory diseases were caused by *Mycoplasma sp.* and *Haemophilus paragallinarum*.

Biswas *et al.* (2003) studied the seroprevalence of MG in small scale commercial broiler farms and found 46.10% birds were serologically positive.

Outbreak of MG infection in different areas of Bangladesh were serologically identified by several scientists and found 32% in Chittagong district (Islam *et al*., 2015), Zulfekar *et al.* (2015) showed 64.47% and 56.13% MG antibodies using iELISA and SPA test respectively in Bogra district, 55.83% in Bhola district (Mahfuzul *et al*., 2014), 67.4% in Khulna district (Jalil *et al*., 2010), 58.90% in Fani district (Sarker *et al*., 2005), 46.88% in Patuakhali district (Sikder *et al*., 2005), 73% at Lohagara and 60% at Satkania Upazilla in Chittagong district (Barua *et al*., 2006) by SPA test.

2.3.2.2 Transmission

MG is transmitted vertically through eggs (transovarian) from infected breeders to progeny (OIE, 2007; Kleven, 1998) and epidemiologically play a significant role (Armour and Ferguson-Noel, 2015; Raviv and Ley, 2013). The organism also transmitted horizontally via infectious aerosols and through contamination of feed, water, environment and by human activity on fomites (shoes, equipment, etc) (Nascimento *et al*., 2005; Pitcher and Nicholas, 2005; Dhondt *et al*., 2007 and Razin and Hayflick, 2010). Infection may be

sustain or latent in some birds for days to months, but when birds remain under stress condition, horizontal transmission may occur rapidly via aerosols and clinical disease spread through the flock. Flock-to-flock transmission occurs readily by direct or indirect contact from the movement of birds, people or fomites from infected to susceptible flocks (Okwara, 2016). Some potential reservoirs of MG in USA are noncommercial (backyard) flocks, multiple-age layer flocks and some free-ranging songbird species.

2.3.2.3 Predisposing factors

The severity and appearance of the disease may be influenced by concomitant infection of other pathogens and/or by predisposing factors. Predisposing factors include age, sex, season, breed, nutritional deficiencies and poor management with excessive ammonia, dust (Donald and Mack, 1990). Presence of concurrent infection with ranikhet disease virus, infectious bronchitis virus, colibacillosis or other pathogens make disease more severe (Matilda *et al.,* 2018; Okwara, 2016).Vaccine strains and pathogenic strains of *E. coli* and *Haemophilus paragallinarum* enhance the severity of MG infection (Omuro *et al.*, 1971; Gross, 1990). The organism may be associated with acute respiratory disease in chickens and turkeys, especially in young birds. Some live virus vaccinations, crowding, poor air and ventilation, cold weather, and concurrent infections may facilitate infection, disease and transmission (Ley, 1997; Talha, 2003 and Bukhari, 2005).

2.3.2.3.1 Age

Rajkumar *et al.* (2018) analyzed 635 sera samples in India by ELISA and showed that the seroprevalence of MS and MG is very high (80-100%) in older flocks (above 50 weeks) as compared to younger flocks (<25 weeks).

Shanmugasundaram *et al.* (2016) collected 103 sera were from 6 commercial layer farms to detect MG antibody of Namakkal region of Tamil Nadu, India and tested by iELISA test and overall 53.40% seroprevalence was found. The highest (100%) sero-prevalence of MG was recorded at 32 weeks but none was recorded at 68 weeks.

All the age groups of turkeys and chickens are susceptible but disease is more common in upto 32 weeks old commercial layer chicken (Udhayavel *et al.,* 2016; Singh *et al.,* 2016).

Islam *et al.* (2015) conducted a cross sectional study was from September 2010 to October 2012 to identify the potential risk factors for Mg seroprevalence in commercial chicken

production in Chittagong area, Bangladesh. A total of 5589 serum samples were collected from one hundred chicken flocks of different production systems (commercial layer, broiler and layer breeder) and determined by iELISA kit and identified a strong effect of 'age' on the Mg antibody level where birds of more than 53 weeks of age showed the greatest risk of being positive for Mg compared to birds less than 16 weeks of age.

Zulfekar *et al.* (2015) detected MG antibody from 563 blood samples of five upazilas of Bogra District which were randomly collected from selected commercial layer chickens at laying period during the period from July to December, 2013 and performed by iELISA and SPA. Prevalence of MG was recorded the highest (69.63%) at 50-55 weeks of age compared with lowest (53.26%) at 56-61 weeks of age.

Mahfuzul *et al.* (2014) revealed the seroprevalence of MG infection in the chicken population of Bhola district, during the period from April 2011 to March 2012. A total of 480 blood samples from chickens were collected from different *upazilas* of Bhola district. On the basis of SPA test, 55.83% chickens were found positive for MG. With respect to age groups, the prevalence was highest in pullets (60.63%) followed by adults (55.63%) and old chickens (51.25%).

Ayim *et al.* (2012) collected sera from apparently healthy individual chicken from forty-nine (49) layer flocks and using SPA test and obtained overall 59.1% MG seropositive. Sero-prevalence of MG was high in all the different age groups (18-75 weeks). Infection was significantly higher in older birds (100.0%) than younger birds (15.6%).

Mukhtar *et al.* (2012) were collected a total of 640 sera samples for prevalence study of MG in layer flocks of District Faisalabad of Pakistan and using SPA test found the highest prevalence (54.84 %) was found in pullets, followed by 46.34 %, and 44.44% in adult and old laying flocks, respectively.

Jalil *et al.* (2010) a cross-sectional study was carried out by SPA test on 1268 sera which were collected from 96 commercial layer farms of six upazilas of Khulna district to know the seroprevalence of MG infection during the period from August 2009 to July 2010 and found overall 67.4% prevalence of MG infection was recorded. Age-wise analysis revealed that there was significant relationship ($p<0.001$) between MG infection and age of birds. The highest prevalence of MG infection was 71.2% in 21-56 weeks age group followed by 66.8% in 8-20 weeks age group.

Hossain *et al.* (2010) survey on the prevalence of antibodies against *Salmonella* and MG in layer chickens in Rajshahi and surrounding districts of Bangladesh. A total of 605 sera samples were examined by RPA test and prevalence of *Salmonella* was recorded the highest (37.6%) in adult compared to young (16.7%). On the contrary, MG antibody were recorded the highest (71.7%) in young compared to adult (50.4%).

Osman *et al.* (2009) were investigated seroprevalence of MG antibodies in chickens and chicks using SPA and ELISA test in Egypt. SPA assay detected antibodies against MG in 69.9% of chicken and 48.7% in chick samples whereas by ELISA assay 58.3% in chicken and 60% in chick samples.

A total of 2777 sera were investigated from unvaccinated flocks of different areas surrounding Lahore District and tested by i-ELISA to detect the status of IgG antibodies produced due to MG field exposure (Ahmed *et al*., 2008). They isolated highest (74.60%) positive was in breeding stock aging from 6 to 23 wks while lowest was 33.17% in flocks of 60 to 76 wks of age.

575 sera samples were collected from 115 flocks for serological investigation of MG antibody in the greater Rajshahi district during the period of July 2006 to June 2007 using SPA test and overall 55.13% seroprevalence was recorded (Hossain *et al*., 2007).They also reported that the highest MG antibody (72.72%) was found in young chickens (18-25 wks) and the lowest antibody (44.00%) were found in adult chickens (above 66 wks).

Sikder *et al.* (2005) were collected total of 364 sera samples from chickens belonging to six model breeder poultry farms located at kalapara Upazilla under Patuakhali district, Bangladesh. All sera samples were examined by SPA test. Mycoplasma infection was recorded highest 71.42% at 18 weeks of age and lowest 50% at 22 weeks of age.

2.3.2.3.2 Season

Vadivalagan *et al.* (2018) studied was conducted from October 2008 to September 2010 to detect the presence of antibodies against MG in commercial layer farms at few districts of Tamil Nadu, India**.** 1350 sera was tested using iELISA and SPA test in two seasons and found that, 33.6% and 26.0% were positive in SPA and ELISA test in winter and 22.0% and 16.2% were positive in SPA and ELISA test in summer season respectively.

Islam *et al.* (2015) obtained a cross sectional study from September 2010 to October 2012 to identify the potential risk factors for Mg seroprevalence by iELISA kit in commercial chicken production in Chittagong area, Bangladesh in different seasons and found 20, 27 and 45% in winter, summer and monsoon respectively.

Zulfekar *et al.* (2015) detected MG antibody from 563 blood samples of five upazilas of Bogra District which were collected randomly from selected commercial layer chickens at laying period during the period from July to December, 2013 and performed by iELISA and SPA. Prevalence of MG was recorded the highest (70.13%) in December followed by November (68%), October (65.67%), August (63.46%), September (58.54%) and July (51.78%) month.

Mahfuzul *et al.* (2014) were reported the seroprevalence of MG infection in the chickens reared in winter showed higher prevalence of MG (60.42%) as compared to those reared in summer (51.25%) season in different *upazilas* of Bhola district by SPA test.

Rachida *et al.* (2013) were examinated 540 sera by SPA test and showed the presence of antibodies against MG was 27.9% in summer and 43.8% in winter.

Mukhtar *et al.* (2012) were collected a total of 640 sera samples using SPA test for prevalence study of MG in layer flocks of District Faisalabad of Pakistan. They found the disease was more prevalent in winter season (45.13 %) in comparison with the summer season (36.30 %).

Heleili *et al.* (2011) tested 237 sera in Eastern Algeria using SPA test and prevalence of MG antibody was found 73.91% in winter season and 91.13% in summer season.

Jalil *et al.* (2010) a cross-sectional study was carried out on 96 commercial layer farms of six upazilas of Khulna district to know the seroprevalence of MG infection during the period from August 2009 to July 2010. A total of 1268 sera samples were tested by SPA test. The prevalence of MG infection was the highest (75.6%) in winter season followed by summer (66.3%) and rainy (60.8%) seasons.

Hossain *et al.* (2010) a serological survey on the prevalence of antibodies against *Salmonella* and MG was carried out in layer chickens in Rajshahi and surrounding districts of Bangladesh. A total of 605 sera were examined by RPA test. The prevalence of MG

infection was the highest (61.6%) in winter followed by autumn (56.9%), rainy (55.0%) and summer (49.6%).

Hossain *et al.* (2007) identified the highest (72.72%) MG antibody in winter season from 575 sera samples in the greater Rajshahi district during the period of July 2006 to June 2007 using SPA test and the lowest antibody (44.00%) were found in summer season.

Sarker *et al.* (2005) a total of 382 sera samples were collected in selected Model Breeder Poultry Farms from two Thana of Feni districts, namely Feni Sadar and Chhagoalnaiya to detect the presence of MG antibodies of chickens during the period January to May, 2004 using SPA test. The overall sero-prevalence of MG infection was 58.90% in the study area. The highest prevalence (62.44%) of MG infection was found in winter season followed by summer season (53.10%).

Sikder *et al.* (2005) studied seasonal prevalence on 364 sera at kalapara Upazilla Patuakhali District, Bangladesh using SPA test and infection was recorded highest in winter season (61.45%) than the rainy season (51.74%).

2.3.2.3.3 Breed

Zulfekar *et al.* (2015) detected MG antibody from 563 blood samples which were collected randomly from selected commercial layer chickens at laying period of five upazilas of Bogra District from July to December, 2013 and performed by iELISA and SPA. They revealed prevalence effect of breed in the seroprevalence of MG infection in layer birds were found 68.77%, 63.74% and 59.37% in sonali, ISA Brown and White leg horn respectively.

2.3.2.3.4 Flock size

Zulfekar *et al.* (2015) were randomly collected 563 blood samples from selected commercial layer chickens of five upazilas of Bogra District at laying period during the period of July to December, 2013 to detect MG antibody and performed by iELISA and SPA test. They found seroprevalence of MG antibodies was higher (69.63%) in most of the large flocks (3000-4200 birds) and lower (56.82%) in small flocks (1300-1600 birds).

Ayim *et al.* (2012) collected 719 sera from apparently healthy individual chicken from forty-nine (49) layer flocks in Ghana and obtained overall 59.1% MG seropositive using SPA test and. They revealed sero-prevalence was found to be significantly higher (81.3%)

in larger flock sizes (above 1000 birds) than (50.9%) in smaller flock sizes (100-400 birds).

Mukhtar *et al.* (2012) were collected a total of 640 sera samples for prevalence study of MG in layer flocks of District Faisalabad of Pakistan and using SPA test. With respect to flocking density, the higher prevalence rate (48.11%) was recorded in flocks having high bird density as compared to those with lower one (27.27 %).

Jalil *et al.* (2010) a cross-sectional study was carried out on 96 commercial layer farms of six upazilas of Khulna district to know the seroprevalence of MG infection during the period from August 2009 to July 2010 and 1268 sera tested by SPA test and detected lowest (63.25%) MG antibodies in (500-1500 birds) flocks and highest (100%) in (3501-above birds) flocks.

Hossain *et al.* (2010) a serological survey for the prevalence of antibodies against *Salmonella* and MG was carried out in layer chickens in Rajshahi and surrounding districts of Bangladesh. A total of 605 sera samples were examined by RPA. Out of 605 sera showed 14.1%, 45.1% and 11.2% *Salmonella*, MG and concurrent infection respectively. The prevalence of MG was recorded the highest 68.6% in large flocks compared to small flocks 50.0%.

Hossain *et al.* (2007) obseved MG antibody in their study in the greater Rajshahi district during the period of July 2006 to June 2007 using SPA test from 575 sera samples. They reported that the highest MG antibody (62.86 %) was found in large flocks (5001-above birds) and the lowest (52%) were found in small flocks (500-1000 birds).

2.3.3 Pathogenesis

The pathogenesis of MG infection is a complex process which is influenced by the genetic background both from the host and from the organism, environmental factors, and the presence of other infectious agents (Simecka *et al*., 1992). The infectivity, tissue tropism and pathogenicity among various MG strains differ significantly (Domanska-Blicharz *et al*., 2008). The ability of organism to attach to the host cells, cell injury, and high frequency phenotypic variation and to modulate and resist the host immune response is the influencing matters to produce the infection.

2.3.3.1 Attachment

The organism attach to specific target cells via sialoglycoproteins along the respiratory epithelium is required prior to initiation of the disease processes (Glasgow & Hill, 1980).This is led by a complex multi factorial process which mediates cytadherence. Attachment is also important to avoid rapid clearance by innate host defence mechanisms. MG is metabolically deficient, so for its survival require essential nutrients from the host cells (Simecka *et al.*, 1992). An apparent attachment organelle or tip structure was identified with the help of microscopy by Razin *et al.* (1980) and shown to bind to sialoglycoproteins (Kahane *et al*., 1984). MG bears narrow tip flask-shaped structure. It has gliding motility and mediates tight attachment to host cell surface. For disease pathogenesis, cytadhesion to host cells is a necessary step and supported by P1 and P30 proteins (Rottem, 2003; Fürnkranz *et al*., 2013; Majumder, 2014). Protein P1 is primary adhesion molecule which is densely aggregated near the tip structure along with an associated molecule P30, which enables P1 to function. Gliding motility is yet another unique phenomenon observed in mycoplasma and involves the polarized tip structure, which enables them to bind and move on solid surfaces (Manafi *et al*., 2015). Gliding motility allows mycoplasmas to escape muco-ciliary clearance, enabling them access the mucosal epithelial cells (Indiková *et al*., 2014; Wijesurendra *et al*., 2015). Therefore these properties play a significant role in virulence and disease pathogenesis upon infection. During attachment they can cause the release of mucus from goblet cells (Rottem, 2003; Majumder, 2014; Xu *et al*., 2015). Pathogenicity of MG may be further complicated by its ability to penetrate and survive within non-phagocytic host cells resulting evasion of host immune response and dissemination throughout the host body (Gharaibeh and Al-Rashdan, 2011; Indiková *et al*., 2013).

2.3.3.2 Cell injury

Parasitism of MG with host cells may contribute to cell injury through deprivation of nutrients, alteration of host cell components and metabolites, and the production of toxic substances. Enzymes are produced that play a major role in cell injuries which are phospholipases, proteases, and nucleases (Bhandari and Asnani, 1989). Cell membrane is damaged by proteases and phospholipases and nucleases increase the chances of genetic alteration of host cells leading to autoimmune response. MG also released hydrogen

peroxide in direct proximity to the host cell membrane may lead to oxidative stress (Cole *et al*., 1968).

Mycoplasma infections are known to be associated with damage to host cells and tissues due to reactive oxygen species generated by both the immune system of the host and the bacterium as its primary virulence factor (Rottem, 2003; Szczepanek *et al*., 2014; Xu *et al*., 2015). MGA0676 (putative lipoprotein) and cytokines play a major contributor role in manifestation of disease during mycoplasma infection by recruitment and activation of leucocytes and may play a significant role in the immunopathology observed during mycoplasma infection (Majumder, 2014; Xu *et al*., 2015).

2.3.4 Clinical Signs

All age groups of birds are susceptible to this disease, but young birds are more susceptible to infection than adults (Nunoya *et al*., 1995; Seifi and Shirzad, 2012). Under natural conditions, the incubation period may vary considerably from 3 to 38 weeks (Jordan, 1979). In flocks, infection occur through eggs, clinical signs might develop at the age of three to six weeks in some cases or, in other cases, only near the onset of egg production.

The clinical signs associated with MG infection in chickens include respiratory rales, nasal discharge, coughing, and occasionally conjunctivitis (Ley, 2003; Saif *et al*., 2003). MG infections vary from asymptomatic to severe depending on the infecting strain and other factors. More severe infections are seen when the birds are infected concurrently with Newcastle disease virus, infectious bronchitis virus, *Escherichia coli* or other pathogens. Uncomplicated infections frequently cause no clinical signs or mortality in chickens (Bradbury, 2001). Flocks may have serologic evidence of infection with no obvious clinical signs (Ley and Yoder, 1997). There is reduced egg production in laying flocks, but is usually maintained a lowered level (Branton and Deaton, 2005; Mohammed *et al*., 2005). Production losses between 10 and 20 % in layer cases (Bradbury, 2001).

Talukdar *et al.* (2017) studied was conducted to determine the prevalence of infectious diseases from 258 Sonali birds of Bogra Sadar Upazila. They found clinical signs of mycoplasmosis included tracheal rale, nasal discharge, coughing, facial edema and lacrimation and reduced feed consumption.

Kamrul *et al.* (2016) investigated the prevalence of poultry diseases in Gazipur district of Bangladesh. A total of 679 poultry birds (313 layers, 338 broilers and 28 cockrels) were brought for diagnosis. The diseases were diagnosed by clinical signs were nasal and ocular discharge, swollen head, rales, anorexia and poor weight gain.

Haque *et al.* (2015) study 455 birds for the prevalence of mycoplasmosis of chicken at kotwali Thana in Chittagong from 2010 to 2011. The disease was diagnosed based on clinical signs including nasal discharge, coughing and rales. Depression, conjunctivitis, swollen eye, eyelid edema and other more general signs were included such as in appetence, depression, weight loss and a drop in egg production.

2.3.5 Post mortem lesions

Rajkumar *et al.* (2018) revealed 33.0% and 11.65% prevalence of MS and MG was observed in samples from live birds of different states in India and in case of postmortem samples collected from CRD affected birds, 25.98% and 9.45% were positive for MS and MG respectively.

Susitha *et al.* (2017) were examined a total of 13,350 carcasses during a period from March, 2013 to February, 2014 and the incidence of CRD was found to be 11.5% by post mortem examination. They found among different age groups chicks, growers and adults, the incidence was 6.62, 18.52 and 9.25% respectively and the incidence of MG was observed highest during summer (16.99%) followed by winter (9.24%) and rainy season (6.24%).

A study was conducted to determine the prevalence of infectious diseases from 258 sick and dead Sonali bird of Bogra Sadar Upazila, based on history, clinical findings and postmortem lesions (Talukdar *et al*., 2017). In post-mortem findings, cloudy air sac, pericarditis, perihepatitis, congested and mucoid trachea were demonstrated. The prevalence of Newcastle disease, Coccidiosis, Colibacillosis and Mycoplasmosis were 11.24%, 13.95%, 14.72% and 12.79% respectively.

Kamrul *et al.* (2016) investigated the prevalence of poultry diseases in Gazipur district of Bangladesh. A total of 679 poultry birds (313 layers, 338 broilers and 28 cockrels) either dead or live were brought for diagnosis. The diseases were diagnosed on the basis of history, clinical signs and post-mortem findings. Gross lesion MC complex included

catarrhal exudates in nose and trachea, cloudy fibrinous air sacs, fibrinous pericarditis, severe congestion of lungs with extensive fibrosis were found. The diseases encountered of MG 14.7%, 7.1% and 14.28% in layers, broiler and cockerel respectively. According to age variation they also found 2% in 0-8wks, 12% in 8-20wks, and 32% in above 20wks in age groups.

Badruzzaman *et al.* (2015) diagnosed the prevalence of commercial chickens at Sylhet division of Bangladesh during the period from June 2013 to May 2015 at the Field Disease Investigation Laboratory (FDIL), Sylhet. A total of 2110 either dead or sick commercial chickens were diagnosed for the existing diseases. The diagnosis of different diseases was done based on the clinical history of the flock, age of affected birds, clinical signs and symptoms, gross and microscopic examinations. Among the diseases they found 11.66% prevalence of MG. According to the age groups, disease prevalence was recorded as age group 61- above days (6.6%), followed by 36-60 days (2.6%), 21-35 days (1.6%) and 0-8 days (0.8%) and the diseases frequently occurred in summer season (5.3 %) followed by winter (4.2%) and rainy season (2.1%).

Haque *et al.* (2015) studied prevalence of mycoplasmosis of chicken at kotwali thana in Chittagong was conducted from 2010 to 2011. A total of 455 either dead or sick birds were examined during the period. Diagnosis was based on history, clinical signs, serum plate agglutination (SPA) test and postmortem lesions. Diagnosis was confirmed by post-mortem examination of dead chickens. Lesions comprising catarrhal exudates in the nasal passages, infraorbital sinuses, trachea, and bronchi, caseous exudates in the air sacs, fibrinous or fibrinopurulent pericarditis, perihepatitis and congestion in lungs were observed. The overall prevalence of mycoplasmosis was found 15.38% in postmortem examination.

Sultana *et al.* (2012) study was designed to record prevalence of respiratory diseases in broiler and layer flocks in District, Lahore Pakistan during the period July, 2011 to June, 2012. Out of 109 layer flocks, the prevalence of infectious coryza, colibacillosis and CRD in layer was found to be 29-35%, 10.11% and 12.84% respectively. Out of 220 broiler flocks, the prevalence of infectious coryza, colibacillosis and CRD were found to be 32.72% 21.36% and 11.81% respectively. The prevalence of CRD in layer was observed to be more in winter (17.64%) followed by spring (15.78%) summer (9.67%) and autumn (8.00%).

Uddin *et al.* (2010) was conducted a study to determine the prevalence of diseases in various age groups and different season in different poultry farms of some selected areas at Narsingdi district of Bangladesh. The diseases were diagnosed based on clinical history, clinical signs and symptoms prior to death, lesions observed after postmortem examination of dead birds and isolation and identification of causal agents. A total 1263 dead and sick birds were examined and was found Chronic Respiratory Disease (CRD)/ Mycoplasmosis in 9.87%. In age variation, highest (5.37%) in 8-20 days and lowest (0.32%) in above 60 days and the poultry diseases occur mostly in winter season (3.7%) and summer (0.8%).

In different upazilas of Sylhet region a pathological investigation was carried out on poultry diseases from 1352 samples of dead and sicks birds during the period of November 2001 to October 2002 (Islam *et al*., 2003). Diagnosis of different disease conditions were made on the basis of the history, age of birds, clinical signs, gross and microscopic lesions. They found incidence of Mycoplasmosis was 5.32% in Sylhet region.The highest (3.92%) number of cases was recorded in the age group of 8-21 days and lowest (0.00%) in above 36 days group and the poultry diseases occur mostly in 1.5% in winter and 0.59% in summer season.

Giasuddin *et al.* (2002) investigated postmortem and serological investigations of poultry diseases in different farms of Bangladesh. A total numbers of 1653 either dead or sick birds were examined. The incidence of aflatoxicosis was highest (27.59%) followed by nutritional deficiency (12.40%), infectious bursal disease (11.80%), chronic respiratory disease (8.11%). In sero-evaluation, commercial and native birds were found 93-97, 80 - 55, 56-12, 60-73, 67-61 and 22-3% sero-positive for Newcastle disease, infectious bursal disease, pullorum, *M. galisepticum, M. synovie* and chicken infectious anemia respectively.

Talha *et al.* (2001) carried an experiment on 381 dead and sick birds from different upazillas of Mymensing during the period of July 1998 to October 1999. They diagnosed 11.55% MG infection based on the basis of the history, age, clinical signs, gross and microscopic lesions and cultural tests.

2.3.6 Diagnosis

Three major avian mycoplasma clean programs included National Poultry Improvement Program (NPIP) of United States of America (USA, 1997; USDA, 2004), National

Sanitary and Hygienic Control Program in France (Stipkovits and Kemp1996), and National Avian Sanitary Program as Programa Nacional de Sanidade avícola (PNSA) in Brazil (Villa, 1998) are practiced.

History, clinical signs, gross and microscopic examinations help the diagnosis of avian mycoplasmosis in naturally infected birds that are similar to the lesions described in experimentally infected birds (Qasem *et al*., 2015). Rapid and early diagnosis of mycoplasma infections is important to prevent the spread of infection and limit economic losses to the poultry industry. There are three approaches to diagnose MG that are isolation and identification of organism, identification of specific antibodies and species-specific nucleic acid detection using molecular tools like PCR (Dufour-Zavala *et al*., 2008; OIE, 2008; Qasem *et al*., 2015) and molecular techniques namely, PCR is suggestive. PCR is highly sensitive and use to monitor MG infection (Garcia *et al.*, 2005; Ley, 2013). Differentiation of isolates and tracing MG infections for epidemiological studies is usually done by molecular techniques such as random amplified polymorphic DNA (RAPD) (Fan *et al*., 1995; Geary *et al.*, 1994), restriction fragment length polymorphism analysis (RFLP) (Fan *et al.*, 1995b), gene targeted sequencing (Ferguson *et al*., 2005) or amplified fragment length polymorphism (AFLP) (Hong *et al.*, 2005). The scientists demonstrated that AFLP results correlated well with DNA sequence analysis and RAPD with AFLP analysis having a much higher discriminatory power and reproducibility. However, RAPD analysis is still widely accepted for strain differentiation of MG isolates (Fan *et al.*, 1995b).

2.3.6.1 Isolation and identification of MG by cultural method

The standard procedure for MG diagnosis is isolation and /or identification of the organism (Ley, 2003), the strongest evidence of infection available and collection of mycoplasma field strains allows study and comparison of properties of pathogenicity and antibiotic sensitivity (Kempf *et al.,* 1994). MG is a slow-growing, relatively fastidious organism that requires one or more weeks for growth and identification. Isolation of MG has often unsuccessful as a result of the culture overgrowth of saprophytic mycoplasmas that inhibit the upper respiratory tract, particular in older chickens (Garcia *et al*., 2005). Several laboratory to be used commercial liquid and agar media for MG isolation, are known such as Frey medium (Frey *et al*., 1968), SP-4 medium (Bradbury, 1998), PPLO medium (Kleven, 2008b).

From live birds, swabs may be taken from the choanal cleft, oropharynx, oesophagus, trachea, eyes, cloaca and phallus but in case of dead birds; samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs, exudates from the infraorbital sinuses and joint cavities. Samples may also be collected from dead-in-shell embryos or chickens or poullets that have broken the shell but failed to hatch. Samples can be taken from the inner surface of the vitelline membrane and from the oropharynx and air sacs of the embryo (Quinn *et al.,* 2002). The reason considers collecting the trachea for isolation because the upper respiratory tract is generally accepted as the portal of entry in natural infection of MG and the trachea also appears to be the preferential site of infection, although disease signs are usually manifested in other parts of the respiratory system (Levisohn and Kleven, 2000). In many cases the organisms are cleared from lesions but may persist in upper respiratory tract (Kleven, 1994). During the acute stages of infection (up to 60-90 days post infection), the population of organisms are found in the upper respiratory tract and the incidence of infection in the flock are high. In such cases 5-10 cultures from the trachea or choanal cleft are often sufficient. However, in chronic cases when infection with mycoplasma strains of low pathogenicity is suspected, 30-100 individuals should be cultured (Kleven, 1998a).

All samples should be examined as soon as possible after collection. If transportation is necessary, small pieces of tissue should be placed in mycoplasma broth or swabs should be vigorously agitated in 1-2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before the specimens are taken and then replaced in the swab holders for transportation. An ice pack or some other means of chilling should be included as MG die rapidly at room temperature. Serial dilutions of specimens in mycoplasma broth may be of value because the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit growth unless they are diluted out (OIE, 2012).

Thus organisms are fastidious; they require a protein based enriched medium (supplemented) with serum or serum factors, yeast extracts, glucose and bacterial and/or fungal inhibitors (Charles and Graham, 1989; Quinn *et al*., 2002). Horse or swine serum (inactivated at 56°C for 1 hour) should be used in media for the growth of MG. It is important that each new batch of medium should be tested with recently isolated MG cultures of low invitro passage because the ability of each batch of medium, for some components especially serum and yeast extract are responsible to vary the growth of

organism (Charles and Graham, 1989). Yeast extracts and other growth factor may be beneficial and is usually supplied by commercial yeast autolysate or by fresh yeast extract. Glucose is a common supplement which is fermented by MG and several other species. MG are resistant to penicillin (as they lack cell wall), which is added in the medium to inhibit (discourage) the growth of gram positive bacteria and the component thallium acetate for which MG are relatively resistant, helps to inhibit gram negative bacterial and fungal contamination (Gordon, 1979; Charles and Graham, 1989; OIE, 2012).

Some laboratories make serial dilutions of inoculum in broth to avoid the inhibitory effects of a large inoculum where tissue enzymes tend to break down glucose and cause a drop in pH or tissue inhibitors may be present. It is therefore, necessary to make serial dilutions of up to 10^{-3} for successful isolation (Charles and Graham, 1989; OIE, 2012).

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes of P^H. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should not be discarded as negative for at least 20 days. Broth medium should be examined daily for acidity, indicated by a change from red to orange or yellow in the indicator (Quinn *et al*., 2002).

The tiny, smooth, circular, translucent masses 0.1-1 mm in diameter with dense, elevated centers ("fried egg appearance") are suggestive indication of mycoplasma species (Ley, 2003; Kleven, 1998). They may shown more than 0.2-0.3 mm in diameter and frequently occur in ridges along the streak line, because closely adjacent colonies readily coalesce (Ley, 2003). They may not have the typical "fried egg" appearance and colonies may appear on the first passage but they are often more pigmented and fail to passage on mycoplasma media (OIE, 2004).

Bacterial colonies may appear on first passage, but they are often more pigmented and fail to passage on mycoplasma media. Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG and necessitate purification of the culture by cloning (OIE, 2012).

Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to MG. The homologous antiserum should have a considerably higher titer (OIE, 2012; Quinn *et al*., 2002).

Tiong *et al.* (1979) screened 240 batches of chickens with chronic respiratory syndrome for Mycoplasmas, 105 batches (43.8%) were positive for Mycoplasmosis. A total of 110 isolates of Mycoplasma was cultured, of which 9 (3.75%) isolates were identified as *M. gallisepticum.*

Branton *et al.* (1984) isolated MG from 15 (14.85%) of 101 tracheal swab samples and from 51 (47.23%) of 108 choanal cleft swab samples. This study indicates that swabs taken from the choanal cleft region yielded higher isolation rates and are more easily obtained than tracheal swabs.

Hanif and Najeeb (2007) isolated 5/53 (9%) breeder farms in PPLO media from 1324 tracheal swabs by conventional culture method in Lahore, Pakistan.

Khalda *et al.* (2013) recovered 7 (4.11%) isolates from 170 tracheal swabs in PPLO media in Sudan.

Rauf *et al.* (2013) collected tracheal swabs, lung and airsac from birds suffering from respiratory diseases, isolated *M. gallisepticum* using Frey's medium found from white leghorn laying birds, 27.6% (104 out of 380) samples were positive.

Jalaladdini *et al.* (2014) cultured tracheal swabs in PPLO broath were observed *Mycoplasma* colonies in 61 plates of 88 or 69%.

Ahmed *et al*. (2015) identified 22.83% MG from tracheal swab in PPLO cultural media of Niger State.

Jaffer *et al.* (2015) were evaluated the prevalence of MG from tracheal and choanal swabs in Kuwait poultry farms using PPLO media culture. Fifty chicken samples were tested gave only seven (14%) were positive with culture methods.

Ferguson-Noel and Williams, (2015) describe cultivation techniques are laborious, slow and expensive and require sterile conditions and problems are overgrowth of faster-growing mycoplasma species and other organisms or no growth in subcultures and can take up to four weeks, and even then, the result can be negative or be hampered by mixed infections.

2.3.6.1.1 Morpholoical charecteristics

Bukte Swati Ramrao (2015) stated by Giemsa stain MG organism is Gram negative, circular /coccoid shape annular forms i.e. circular and elliptical pleuropneumonia like organism was characterized chiefly by the lack or thinness of stainable material in the central region of the organism. The protoplasm appeared to be concentrated at the periphery of the circular PPLO particle-evenly distributed in the form of a ring, or unevenly distributed, yielding 'lopsided rings', 'signet rings' and monopolar forms.

2.3.6.1.2 Biochemical test

Rasool *et al.* (2017) were collected tracheal and cloacal swabs (n=500), samples from suspected chicken, were cultured on Modified Frey, s medium for isolation of MG. Preliminary identification of MG was done by culturing and biochemical characterization. In biochemical test they found glucose fermentation, arginine hydrolysis and tetrazolium dye reduction test.

Hassan *et al.* (2014) isolated MG colony in PPLO broth and agar and were subjected to biochemical reactions including glucose fermentation, arginine hydrolysis, tetrazolium reduction for preliminary MG identification in commercial and backyard poultry in different parts of Pakistan and Azad Jammu and Kashmir. A total of 142 isolates of *M. gallisepticum* from 573 flocks were identified.

2.3.6.2 Serological diagnostic tests

For many years, diagnosis of avian mycoplasmosis was based on serological assays to detect antibody production. Serological screening is routinely used as an indicator of MG infection include serum plate agglutination test (SPA), haemaglutination inhibition test (HI) and enzyme linked immunosorbant assay (ELISA) for MG (Kleven, 2008b; OIE, 2008; Khalifa *et al*., 2013). Serology by agglutination and ELISA methods are commonly used for surveillance of mycoplasma infection. HI test is used as a confirmatory test.

Serological procedures are available to aid for the diagnosis of MG when infection is suspected and are also useful for flock monitoring in the control programs (Gordon, 1979; Talha, 2003). A positive serologic test together with history and symptoms typical of the disease would constitute a presumptive diagnosis pending isolation and identification of the organism (Gordon, 1979). Traditionally, diagnosis of MG infection in the poultry

industry depended upon serological screening using SPA, ELISA and HI test, which historically are considered the most reliable tools for identifying subclinical infection in the flock (OIE, 2004; Moscoso *et al*., 2004; Barua *et al*., 2006; Purswell *et al*., 2012; Kaboli *et al*., 2013) however, they sometimes lack the required specificity and sensitivity (Carli, and Eyigor, 2003; Kaboli *et al*., 2013). Radio immunoassay, Micro Immunofluorescence and Immunoperoxidase assay (IP) are also the techniques used for diagnosis of mycoplasma infection (Butcher, 2002b; Parker *et al*., 2002; Talha, 2003).

2.3.6.2.1 Detection of MG antibody by SPA test

Among serological tests, the SPA test can be used as a tool for quick detection of MG infection (Sarker *et al*., 2005; Seifi and Shirzad, 2012). Sera are stored at 4°C but not frozen if the samples not tested immediately after collection. The test should be carried out at room temperature (20-25°C) within 72 hours of serum collection and the reagents should also be stored at room temperature. Infected birds may positive as early as 7-10 days after infection (Kleven, 1994; Guanson *et al.* (2000) found that antibodies against MG could detectable with strong SPA reactions at 2 and 3 weeks after infection. SPA is probably the most commonly used test using commercially stained antigens, which vary in sensitivity and specificity from different manufacturers and batch to batch. The test is quick, relatively inexpensive and highly sensitive (Charles and Graham, 1989; Talha, 2003; OIE, 2012).

Non specific reactors (false positive) however, may occur in some flocks infected with M. synoviae, or those recently been vaccinated with oil emulsion vaccines and/or tissue culture vaccines originated against various organisms (Talha, 2003; Abdulfath, 2004).

SPA is a screening test and is prone to false positive reactions. It is possible to reduce false positive reactions by heating serum at 56°C for 30 minutes or by diluting serum or producing SPA antigen in medium containing liposomes instead of serum (Charles and Graham, 1989; Butcher, 2002b). There is no international standard for interpreting SPA test result, but a high proportion of positive sera in a flock (10% or more) indicate MG infection especially if confirmed by HI or ELISA (Butcher, 2002b).

Mahfuzul *et al.* (2014) investigated MG during the period from April 2011 to March 2012. A total of 480 blood samples from chickens were collected from different upazilas of Bhola district. The sampling considered the types of chicken (backyard and commercial

layer). The MG infection was higher (62.5%) in backyard chickens as compared to those being reared in commercial farming systems (53.61%).

A study was conducted to determine the antibodies against MG and MS from 1500 day old broiler chicks hatched from imported and local fertile eggs in Libya using SPA test (Elgnay and Azwai, 2013). They detected 5.2% and 0% MG antibodies from imported and local fertile eggs respectively. They also revealed that 3.4% and 6.4% antibodies were found positive against MG and MS respectively.

Rachida *et al.* (2013) to aim of this study was to estimate the seroprevalence of MG and MS infection in twenty-seven laying hen farms across eight provinces of Eastern Algeria. All sera samples were examinated by SPA. Out of the 540 sera samples tested, one hundred ninty-two showed the presence of antibodies against MG, corresponding to an infection rate of 33.1%. One hundred were positive for MS, corresponding to an infection rate of 18.5%.

Feizi and Nazeri (2012) stated that RSA method was specific method has minimum error with maximum sensitivity for detection of MG antibody. They tested 300 sera by RSA test for detection of MG antibody in Iran and found 52 (17.3%) sera were MG positive.

Ayim *et al.* (2012) studied limited knowledge exists about MG pathogen in the poultry industry in Ghana. This study was carried out to investigate the preva-lence of MG infection in commercial layer chickens in the Ga East district of the Greater Accra region of Ghana from March-October, 2010. A total of seven hundred and nineteen (719) ap-parently healthy individual chicken sera were collected from forty-nine (49) layer flocks. The SPA test using Nobilis® MG antigen was used to test sera for the presence of antibodies. The overall sero-prevalence of MG was found to be 59.1%.

Mukhtar *et al.* (2012) were collected a total of 640 sera samples from 81 commercial layer flocks with complaint of respiratory distress for seroprevalence of MG of Faisalabad of Pakistan during a period of one year from January to December, 2010. On the basis of serum plate agglutination test, 40 flocks were found positive for MG indicating a share of 49.01% among the respiratory diseases.

For the detection of antibody against MG by RPA test, 148 blood samples were collected from 23 flocks of layer and broiler birds in the eastern region of Algeria between October

2008 and September 2010 (Heleili *et al*., 2011). They found 81.15% and 84.81% of MG positive in laying hens and broilers respectively.

Jalil *et al.* (2010) carried out a cross-sectional study on 96 commercial layer farms of six upazilas of Khulna district to know the seroprevalence of MG infection during the period from August 2009 to July 2010. A total of 1268 sera samples were collected from randomly selected layer birds of different age groups and tested by SPA test by using commercial MG antigen to detect the presence of antibodies against MG. The overall prevalence of MG infection was recorded as 67.4%.

A serological survey was made on the prevalence of antibodies against MG and Salmonella infections in Rajshahi and surrounding district from 605 sera during the period of July 2006 to June 2007 (Hossain *et al*., 2010) by SPA test. They recorded that (14.1%), (45.1%) and (11.2%) were Salmonella, MG and concurrent infections respectively.

575 sera samples were collected from 115 flocks for serological investigation of MG antibody in the greater Rajshahi district during the period of July 2006 to June 2007 using SPA test and overall 55.13% seroprevalence was recorded (Hossain *et al*., 2007).

Barua *et al.* (2006) determined the sero-prevalence of MG in chickens in two selected areas; Lohagara and Satkania Upazila of Chittagong district, 400 sera samples were conducted from July to October 2004 and study was based on SPA test and revealed prevalence of MG were 53% in broiler and 73% in layer at Lohagara, where as 46% in broiler and 60% in layer at Satkania Upazilla.

Three hundred and eighty two blood samples were collected for seroprevalence study of selected Model Breeder Poultry Farms of Fani district during the period of January to May 2004, using SPA test (Sarker *et al*., 2005) and demonstrated that overall seroprevalence was 58.9%. They found 62.80% positive result in Feni sadar and 53.45% in Chhagoalnaiya Thana.

To know the seroprevalence of *Salmonella* and MG infection, 364 sera were collected from six model breeder poultry farms located at kalapara Upazilla under Patuakhali district (Sikder *et al*., 2005). They found *Salmonella* (23.46%) and *Mycoplasma* infection (46.88%) using SPA test. They stated that MG infection was 71.42% at 18 wks and 61.11% at 42 wks. Prevalence of salmonella was recorded highest in rainy season

(25.00%) than the winter season (21.88). On the contrary, Mycoplasma infection was recorded highest in winter season (61.45%) than the rainy season (51.74%).

Thekisoe *et al.* (2003) a total of 177 free-ranging chickens from 19 Qwa-Qwa villages of South Africa were bled from wing veins over a period of 6 months (June-November 2000). Serological tests indicated that 5% of chickens tested had been exposed to Newcastle disease, 43% to infectious bronchitis and 63% to MG infection.

Giasuddin *et al.* (2002) investigated postmortem and serological investigations of poultry diseases in different farms of Bangladesh. In sero-evaluation, commercial and native birds were found 60-73% sero-positive for MG.

Chrysostome *et al.* (1995) study seroprevalences to three infectious agents were studied in rural poultry farms in three different ecological regions in Benin. Of 260 sera, 65% were seropositive for Newcastle disease virus, 10% for *Salmonella pullorum* and 62% for MG.

2.3.6.2.2 Detection of MG antibody by iELISA test

Jordan *et al.* (2018) were collected 41 serum samples from five backyard farms in Trinidad and Tobago of West Indies. Samples were tested for antibodies to seven priority pathogens of poultry by ELISA. Antibodies were detected in 65% of the sampled birds for Infectious bronchitis virus (IBV), 67.5% for Infectious bursal disease virus (IBDV), 10% for Newcastle disease virus (NDV), 0% for Avian influenza virus (AIV), 0% for West Nile virus (WNV), 20% and 47% for MG and MS. They reveal the presence and circulation of important pathogens of poultry in selected backyard farms in Trinidad.

Rajkumar *et al.* (2018) analyzed 635 sera by iELISA test in Indian poultry flocks and results showed that the seroprevalence of MS and MG was 52.1 and 32.6% respectively.

A seroprevalence study of MG and MS antibody were detected by iELISA test from 459 unvaccinated backyard chickens of 4 villages of Southern Mozambique ((Augusto *et al*., 2017). They recorded the overall seroprevalence of MG and MS antibodies were 48.8% and 84.5% respectively.

Detection of anti MG antibodies, 98 sera was collected in different age groups of birds from different unorganized farms of district Rewa (Madhya Pradesh), India. Overall the MG antibodies were found 21.40% samples were positive to MG antibodies by ELISA test

(Namrata *et al*., 2016). They concluded that ELISA was highly specific test to detect MG antibody.

Total 103 sera samples were collected from 6 commercial layer farms to detect MG antibody of Namakkal region of Tamil Nadu, India and tested by iELISA test (Shanmugasundaram *et al*., 2016) and overall 53.40% seroprevalence was found.

For seroprevalence study of indigenous chickens of Nigeria, 552 blood samples were randomly collected and investigated by iELISA test and recovered 91.83% of MG/MS antibody (Ahmed *et al*., 2015).

5589 serum samples were collected from one hundred chicken flocks of different production periods of commercial layer, broiler and layer breeder for identification of the potential risk factors of Mg in Chittagong area from September 2010 to October 2012 and identified 32% birds were seropositive by iELISA kit (Islam *et al*., 2015).

Zulfekar *et al.* (2015) conducted a comparison research work on iELISA and SPA for detection of MG antibody from 563 blood samples which were randomly collected from selected commercial layer chickens at laying stage during the period of July to December, 2013 in Bogra districts and revealed that overall prevalence of MG antibody were 64.47% and 56.13% in iELISA and SPA test respectively.

Reddy *et al.* (2014) diagnosis 1827 sera from a total of 86 flocks in different region of India by iElisa test. The serological data of three types of birds tested was revealed the overall prevalence of 54.39% in Commercial Layers, 39.61% in Broiler Parents, and 20.80% in Commercial Broilers.

Herrero *et al.* (2009) studied 1291 sera from backyard chickens of Paraguay of South America using commercial i ELISA test and overall 26% prevalence of MG antibody were detected.

A total of 2777 sera were referred from unvaccinated flocks of different areas surrounding Lahore District and tested by i-ELISA to detect the status of IgG antibodies produced due to MG/MS field exposure (Ahmed *et al*., 2008).

2.3.6.2.3 Detection of MG antibody by HI test

Hemagglutination inhibition test has been commonly used to confirm reactors detected by SPA or ELISA and considered to be highly specific (Ley, 2003). However, the HI test is time consuming, the reagents are not commercially available and the test may lack sensitivity (Kempf *et al*., 1994). Generally, HI titer of 1:40 to 1:80 or greater are considered positive, but results must be interpreted on a flock basis (Kleven, 1994). The HI test detected IgG antibodies which becomes positive 3 weeks post challenge (Stipkovits, 2001). Disadvantage of HI test is considered to be less sensitive than other serological tests; infected birds may not test positive until 3 weeks or longer after infection. In addition, there is antigenic variation among MG strains as measured by HI (Kleven, 1994). According to OIE (2004), high proportion of positive sera in a flock with higher than 10 % indicates MG infection, especially when the sera confirmed by HI test or ELISA. However inclusive results make it necessary to attempt to isolate the organism or demonstrate the presence of its DNA.

Luciano *et al.* (2011) tested 712 sera for detection of MG antibody by HI test. 30 samples (4.21%) were positive in HI.

2.3.6.2.4 Comparison among SPA, ELISA and HI test for detection of MG antibody

Vadivalagan *et al.* (2018) tested 1350 sera using iELISA and SPA test in two seasons from October 2008 to September 2010 to detect the presence of antibodies against MG in commercial layer farms at few districts of Tamil Nadu, India and found that, 33.6% and 26.0% were positive in SPA and ELISA test in winter and 22.0% and 16.2% were positive in SPA and ELISA test in summer season respectively.

Atique *et al.* (2017) stated breathing ailment in Balochistan of Pakistan in layer flocks was affecting frugally massive damages. The antibodies existence of MG were testified in sera trials (N=720) in 60 layer farms with breathing indications in Quetta district, Pakistan. Blood samples were confirmed for MG antibody presence 21% by SPA and 35% iELISA.

For detection of MG antibody, 180 sera were investigated in AI-Qassim region, Saudi Arabia by SPA and ELISA test (Ayman *et al.*, 2016) and identified 83 (46.11%) and 97 (53.88%) isolates respectively.

Tinne *et al.* (2016) investigated the prevalence of MG in commercial poultry (5220 layers, 1224 broilers and 1020 meat turkeys), 56 racing pigeons and 890 wild birds in Belgium. Eighty-seven layer farms were first tested with RPA. Out of these layer farms, six (6.9%) demonstrated a positive result. To exclude any possible false positive reactions, positive serum samples were retested with ELISA and only two layer farms (2.3%) remained positive after confirmation.

Zulfekar *et al.* (2015) studied a comparison research work on iELISA and SPA for detection of MG antibody from 563 blood samples which were randomly collected from selected commercial layer chickens at laying stage during the period of July to December, 2013 in Bogra districts and revealed that overall prevalence of MG antibody were 64.47% and 56.13% in iELISA and SPA test respectively.

Feizi *et al.* (2013) were investigated in 3 broiler breeder rural farms located around Tabriz city of Iran during 2011-2012 for the diagnosis of prevalence of MG. In each farm 30 serum samples were obtained and tested by RSA and ELISA and results indicated that 42.22% and 33.33% of samples were positive by RSA and ELISA. It can be concluded that RSA and ELISA serological tests should be only used as screening in monitoring programs to detect MG in poultry flocks.

Luciano *et al.* (2011) tested 2,781 sera for detection of MG antibody by SPA, ELISA and HI test. From 2,781 samples tested, 736 (26.46%) were positive in SPA. From 712 SPA positive sera, 30 samples (4.21%) were positive in HI and 150 samples (21.06%) were positive in ELISA. Copositivity between ELISA and HI was 90% and conegativity was 82.0%. Agreement between HI and ELISA was rejected by McNemar's test ($p<0.001$) and Kappa coefficient showeda weak correlation between the two techniques ($k= 0.25$; $0.21,>k < 0.40$).Weak statistical correlation was observed between all serological tests and they should only be used for initial screening for MS.

Lamyaa and Abd El-Samie (2012) identified 51.6% and 48.3% MG antibodies by SPA and ELISA test in Egypt.

The seroprevalence of MG antibodies in chickens and chicks were investigated by SPA and ELISA test in Egypt and found that 69.9% and 58.3% in chickens and 48.7% and 60% in chicks were seropositive respectively (Osman *et al*., 2009).

2.3.6.3 Molecular characterization of MG isolates

2.3.6.3.1 Molecular characterization of MG isolates by 16S rRNA gene, 185bp, and 732bp

Rasoulinezhad *et al.* (2017) was successfully amplified the 16s rRNA gene of MG by PCR reactions were 16.66% and 48.38%, in commercial and backyard turkey farms respectively of Iran and the amplicon size was 185 bp.

Zakeri (2016) were used mgc2 and 16S rRNA gene to investigate the prevalence of MG in poultry of Iran by PCR. They revealed the 16S rRNA and mgc2 PCR diagnostic primers are specific for MG in tests of all avian Mycoplasmas present in the chicken trachea and are sensitive enough to readily detect MG in tracheal swabs from field outbreaks.

Hassan *et al.* (2014) studied molecular epidemiology of MG in commercial and backyard poultry was conducted in different parts of Pakistan and Azad Jammu and Kashmir. Molecular identification of the isolates was made with the application of PCR. A total of 142 isolates of MG from 573 flocks were identified and 732 bp products were obtained after PCR.

Rauf *et al.* (2013) were collected trachea, lung and air sac (n=380) from thirty eight commercial layer farms in triplicate suffering from respiratory diseases in and around district Lahore, Pakistan. DNA extracted from organs of infected birds was amplified by PCR using species specific primers of MG targeting 16SrRNA gene. Overall 27.6% and 68.94% samples were found as positive for MG in culture and PCR. They confirmed presence of 185 base pairs DNA band was considered positive for MG.

Hess *et al.* (2007) revealed that MG 16S rRNA gene based PCR had higher analytical sensitivity than other PCR methods.

Garcia *et al.* (2005) compared the 16S rRNA, mgc2, lipoprotein and gapA surface protein genes for detection MG and found that mgc2 and 16S rRNA methods had similar and the best detection limits. 16S rRNA.

Lauerman (1998) used 16S rRNA PCR method for confirmation of MG and MS infection in chickens.

2.3.6.3.2 Molecular characterization of MG isolates from direct tissue

Tomar *et al.* (2017) were collected 92 pooled tissue samples including trachea, lungs and air sacs from 92 different poultry flocks in Haryana (India) which were affected with respiratory infections and were undergone mycoplasmological examination by PCR and found 27% MG was positive. The present study suggests that the PCR assays performed for MG provide a simple, quick and precise tool to specifically detect these organisms from the field samples which are always found to be complicated by other pathogens.

Hossam *et al.* (2016) was used PCR by different approaches in comparison to the culture method. The study included 385 birds, from which (1,155) samples of trachea, lung tissue and air sacs were collected from different farms of Layers, broilers and Breeders flocks located in seven governorates in Egypt. Three detection methods of MG revealed that, the highest recovery rate of MG was achieved by PCR on 48 hours incubation PPLO broth (70.9%), followed by direct PCR on infected tissue (65.45%).

Sing *et al.* (2013) used polymerase chain reaction (PCR) for detection of MG infection in poultry using tissues directly. A total of 51 samples (trachea and airsacs) were collected from clinically suspected birds originating from district Hisar, India. PCR was carried out using specie specific primers for MG and identified 18 samples (35.3%). They concluded that tissues may be used for rapid screening and detection of MG in poultry.

During the period of four months, November 2008-February 2009, 48 commercial broiler farms (located in Lahore district) with respiratory symptoms were examined (Aamir *et al*., 2011). A detailed study of clinical signs and gross lesions was recorded on examination of suspected birds of each flock. Furthermore, tissues samples of trachea and lungs were collected from each flock and were kept at -80°C until they were tested through PCR. The polymerase chain reaction (PCR) detected MG infection in birds from 22/48 (46%) farms.

To investigate the prevalence of MG infection in poultry industry of Iran 324 swab samples were collected from chaharmahal Va Bakhtiari Province of Iran (Bagheri *et al*., 2011). Total DNA was extracted and 16s rRNA region was amplified by PCR using specific primers. A number of 96 (29.63%) samples out of 324 were positive on the basis of PCR analysis.

2.3.6.3.3 Molecular characterization of MG from culture isolates

Atique *et al.* (2017) were processed the 1112 lung tissue samples for isolation of MG by culturing in PPLO broth and agar and were analysed to identify the MG occurrence was 19% yielded a PCR product of 730 bp.

2.3.6.3.4 Comparison between culture and molecular techniques for isolation and characterization of MG

Hossam *et al.* (2016) studied 385 layers, broilers and breeders commercial poultry flocks in Egypt. They revealed the highest (65.45%) recovery rate of MG was achieved by direct PCR on infected tissue and the lowest (17.66%) recovery rate was from conventional cultivation method indicating that PCR is more sensitive.

Gondal *et al.* (2015) were collected 388 tracheal swabs and tissue samples from MG suspected poultry birds of 20 poultry flocks (Layer, broiler and breeder) in Khyber Pakhtoon khwa and Punjab provinces of Pakistan and characterized 27.3% and 49.74% by culture and PCR method.

Jafar *et al.* (2015) collected fifty samples (blood, swabs from trachea and choanal cleft) from different layer and broiler flocks for identify the prevalence of MG infection in Kuwait poultry farms and using serology, molecular and culture method and stated that 48%, 58% and 14 % were MG positive by ELISA, PCR and culture method respectively. They found both the PCR and ELISA methods are superior to culture method for detection of avian mycoplasmosis.

To detection MG infection of Kerman province of Iran, 88 tracheal swabs were collected and tested by culture and nested PCR (Jalaladdini *et al.,* 2014). They found 69% positive colonies on PPLO agar plates and 100% by nested PCR through GapA and 16srRNA genes. Result showed that PCR test was more sensitive than culture and this protocol can use as a remarkable way to diagnose MG infection in birds.

Rauf *et al.* (2013) were compared molecular diagnostic technique (PCR) with conventional culture isolation from White leghorn laying birds (n=380) suffering from respiratory diseases for MG identification from field cases in Pakistan. They found 27.6% in culture and 68.94% in PCR.

Osman *et al.* (2009) compared the sensitivity of PCR and culture method for investigate of MG and found 47% in PCR and 10.7% in culture method.

2.3.6.3.5 Comparison of MG loads in different tissues in cultural and molecular techniques

Gondal *et al.* (2015) were collected 388 (tracheal swabs and tissue) samples from MG suspected poultry birds of 20 poultry flocks (Layer, broiler and breeder) in Khyber Pakhtoon khwa and Punjab provinces of Pakistan and characterized by culture and PCR method. They revealed overall 27.3% by culture and 49.74% by PCR. They also isolate MG organism 39.3%, 15.9%, 27.4% and 25% by culture and 68.18%, 42.47%, 31.85% and 50% by PCR from tracheal swabs, tracheal tissues, lung tissues and air sacs respectively using universal and MG specific primer. They also declared higher percentage of MG confirmation through PCR is from medicated birds and PCR is rapid, sensitive and accurate method for diagnosis of MG from suspected cases and culture method is laborious and time consuming and failed to detect mycoplasma species from medicated birds and is less sensitive than that of PCR.

Jafar *et al.* (2015) collected fifty samples (blood, swabs from trachea and choanal cleft) from different layer and broiler flocks for identify the prevalence of MG infection in Kuwait poultry farms and using molecular and culture method and stated that. The choanal cleft gave more positive (60%) than the tracheal swab (56.6%) by PCR method whereas tracheal swab gave more positive (20%) than choanal cleft (5%) when adopted with culture method.

Rauf *et al.* (2013) detected MG from trachea (80.51%), lungs (72.2%) and air sacs (74.19%) by PCR and trachea (39.28%), lungs (15.92%), air sac (27.43%) by culture from white leghorn layer flocks in Pakistan.

Osman *et al.* (2009) also revealed 33.3%, 25.5% and 13.7% were culture positive when tracheal swab, trachea and lung tissue were adopted.

2.3.6.4 DNA sequences of *M. gallisepticum*

All molecular genetic methods for distinguishing organism subtypes are based on differences in the DNA sequence. Logically, DNA sequencing would appear to be the best approach to differentiating subtypes. DNA sequencing generally begins with PCR amplification of a sample DNA directed at genetic regions of interest, followed by sequencing reactions of the PCR products. DNA sequencing must be directed at a small region of the bacterial genome. DNA sequencing examines a very small portion of the sites that can potentially vary between strains. The variability within the selected sequence must be sufficient to differentiate different strains of a particular species.

PCR or PCR related methods are based on DNA polymorphism. The molecular biology of bacterial pathogen may be examined by sequence analysis (Enright *et al.*, 1999). The sequence analysis of housekeeping genes is a powerful convenient typing method and is suitable for a great variety of pathogens, leading to the understanding of inclusive epidemiology and inhabitant's structure of the infectious diseases (Maiden *et al.*, 1998).

Rasoulinezhad *et al.* (2016) performed PCR of 600 swab samples collected from 18 commercial and 31 backyard turkey flocks for detecting 16S rRNA gene in the samples and DNA sequence of mgc2 gene of positive sample and found that 48.38% of backyard and 16.66% of commercial farms were positive for MG. The molecular analysis indicated high sequence similarity between some Iranian turkeys isolates with Indian and Pakistanian MG isolates.

Lecis *et al.* (2016) reported the identification of 23 novel mycoplasma sequences during the monitoring of 62 birds of prey on admission to wildlife centers in Sardinia, Italy. Molecular investigation performed on pharyngeal swabs revealed 26 birds positive to *Mycoplasma* (42%). Sequence analysis based on 16S rRNA, 16S-23S rRNA intergenic spacer, and RNA polymerase β subunit (*rpoB*) gene highlighted cluster assignment and phylogenetic relationships among the identified types, classified within the hominis group.

Serena *et al*. (2013) characterised various *M. gallisepticum* genotypes within the region and highlight the unique differences between two genotypes found in South Africa and Zimbabwe. PCR targeting a partial region of the mgc2 gene was used to screen various poultry farms in South Africa and Zimbabwe for *M. gallisepticum*. Samples were characterised using multilocus gene-targeted sequencing. Portions of the surface protein

encoding pvpA, gapA and mgc2 genes and the uncharacterised surface lipoprotein gene designated MGA_0319 were sequenced and analysed. Nucleotide sequences were compared to vaccine and reference strains as well as to strains from different countries. The South African genotype contained unique mgc2 and pvpA gene regions, while the Zimbabwean genotype proved to be even more distinct with unique gapA, mgc2 and pvpA gene regions. In addition, BLAST results showed high similarities in the partial mgc2 gene region between the South African and Zimbabwean genotypes and the 'atypical' Israeli RV-2 strain, suggesting a link in its epidemiology.

2.3.7 Differential Diagnosis

In poultry, the differential diagnosis includes respiratory diseases such as Infectious bronchitis, mild Newcastle disease and avian influenza, *Haemophilus paragallinarum*, *Pasteurella multocida* and *Mycoplasma synoviae* infections should also be ruled out. In turkeys, other pathogens to consider include avian pneumovirus, *Pasteurella multocida*, and Chlamydia and *M. synoviae*. Mixed infections with *M. gallisepticum* and other organisms can occur. (Gross *et al*., 1990; David H. Ley, 2013).

2.3.8 Economic Importance

MG infections cause great economic losses in the poultry industry due to decreased hatchability and egg production, reduced quality of day-old chicks, reduced growth rate, increased costs of eradication measures involving site cleaning and depopulation, and increased drug and vaccination costs (Kaboli *et al*., 2013; Khalifa *et al*., 2013).

MG is the most economically significant mycoplasmal pathogen of poultry. MG infections can cause significant economic losses on poultry farms from chronic respiratory disease reduced feed efficiency, decreased growth and egg production. The carcasses of birds sent to slaughter may also be downgraded. MG infections are notifiable to the World Organization for Animal Health (OIE). This organism has been eradicated from most commercial chicken and turkey breeding flocks in the United States; however, it remains endemic in many other poultry operations (Carpenter *et al*., 1981; Ley and Yoder, 1997). The most common economic impacts of MG are decreased egg production in layers (Mohammed *et al*., 1987; Levisohn and Kleven, 2000a; Bradbury, 2007). MG is believed to cost the worldwide poultry industry over $780 million every year. In the United States it is believed to cost over $120 million on egg production alone. Infection can lead to the

culling of an entire flock to prevent further spread (Ley and Yoder, 1997). Also, chickens have been documented to lose about 16 eggs over their laying cycle of 45 weeks. This adds up to be a loss of about $140 million annually in the United States alone (Mohammed *et al*., 1987). The impact of MG on egg production was studied, where MG-infected flock produced 12 fewer eggs per hen than uninfected flock. MG F-strain-vaccinated hens produced 6 eggs more than unvaccinated infected hens. Losses in commercial layer flocks in Southern California were estimated to be approximately 127 million eggs due to MG. Egg production losses in association with costs of MG control programs were approximately $7 million (Mohammed *et al.,* 1987).

CHAPTER 3

MATERIALS AND METHODS

The study and entire laboratory works were conducted from February 2014 to January 2018 in the Molecular Biology and Bacteriology laboratories of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur and in the Laboratory of National Institute of Biotechnology (NIB) Savar, Dhaka, Bangladesh.

3.1 Materials used

3.1.1 Study area

The commercial layer poultry flocks which were observed and obtained samples from northern five Districts of Bangladesh namely Rangpur, Dinajpur, Thakurgaon, Panchagorh and Joypurhat. A total of 10 (ten) farms were selected where 2 (two) farms were from each district for this entire study. These districts were selected based on the location accessibility; poultry farm population and location closeness to the Microbiology laboratory of the HSTU, Dinajpur.

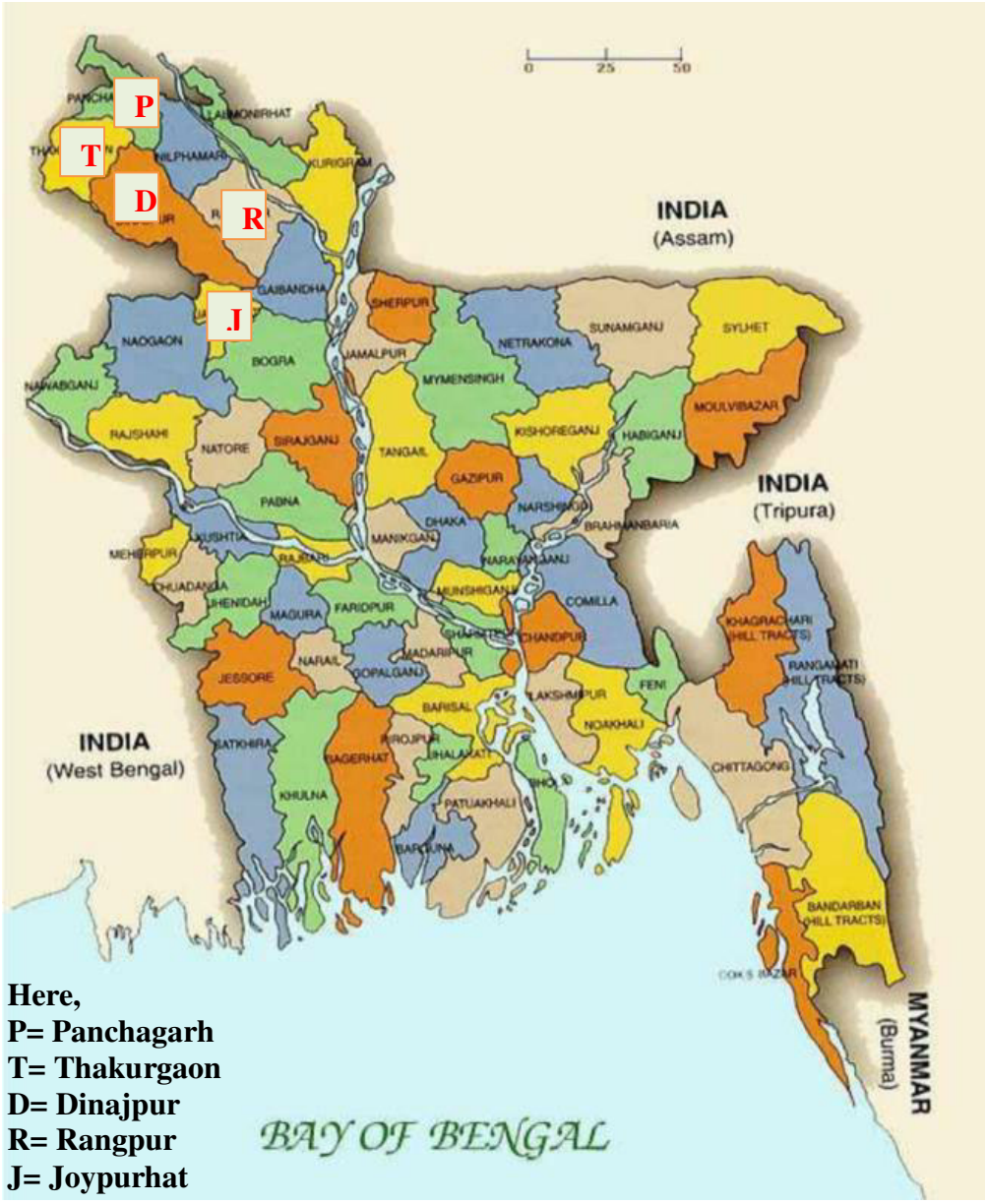


Figure 3: Map showing study area

3.1.2 Flock selection

In the commercial poultry production system, samples from commercial layer chickens were obtained from the ten selected commercial poultry farms in northern part of Bangladesh. Names of farms on which samples were collected have been omitted due to client confidentiality privileges which were coded arbitrarily as farm-A, farm-B, farm-C, farm-D, farm-E, farm-F, farm-G, farm-H, farm-I and farm- J.

Each flock of birds was divided into 4 groups based on their ages- Group-1 (0-8 weeks), Group-2 (9-20 weeks), Group-3 (21-40 weeks), Group-4 (above 40 weeks). Eight flocks of

commercial layer of Isa Brown and two flocks of Sonali cross breed were reared separately in cage system in the five selected districts of Bangladesh. A brief information about the flocks–

Table 2: List of farms with location and bird population

Sl.No.	Location			Flock size	Breed	Sex
	District	Upazilla	Farm identity			
1.	Rangpur	Rangpur sadar	Farm-A	1100	Isa brown	Female
2.		Rangpur sadar	Farm-B	1750		
3.	Dinajpur	Dinajpur sadar	Farm-C	2900		
4.		Birol	Farm-D	2850		
5.	Thakurgaon	Pirgonj	Farm-E	1450		
6.		Pirgonj	Farm-F	1050		
7.	Panchagarh	Panchagarh Sadar	Farm-G	1820		
8.		Botha	Farm-H	1580		
9.	Joypurhat	Joypurhat sadar	Farm-I	2230	Sonali	
10.		Pachbibi	Farm-J	1620		
Total				**18,350**		

3.1.3 Flock maintenance

The bio-security measures in all of the 10 (ten) farms were strict, with good management practice of proper ventilation and good hygienic status which was of standard one. The birds were fed of commercial feed containing no antibiotic used against mycoplasmosis. All the

flocks of birds were dewormed and vaccinated as per schedule but the flocks were not vaccinated against Mycoplasma.

3.1.4 Criteria for detection of MG antibody and organism

Serological prevalence of Mycoplasma antibody and the isolation, identification and characterization of organism were made on the basis of history, signs and symptoms prior to death of birds, post mortem lesions of dead birds, supported by cultural, serological and molecular technique using standard methods for Mycoplasma identification (OIE, 2012).

3.1.5 Equipment

Different kinds of equipment were used during the experiment which were: Digital Analytical Balance, Autoclave, Microwave oven, Hot Air Oven, Vortexer, Centrifuge and Micro centrifuge, Electrophoresis Apparatus, Gel documentation system, Refrigerator, Freezer (-20^{o}C, -80^{o}C), Water bath, Bacteriological Incubator, CO_2 Incubator, Compound Microscope, Inverted Microscope, UV spectrophotometer Nanodrop spectrophotometer, Electronic pH meter, Candle jar, Biosafety cabinet, Laminar air flow, Thermal cycler 96 well, Thermal cycler (G storm), Computer, Gas stove, Clean bench, Stop watch, Thermometer.

3.1.6 Plastic wares, glass wares and other appliances

During laboratory work different kinds of plastic wares , glass wares and other appliances were used such as: Sample collection vials, Measuring cylinder, Glass bottles, Test tubes (with and without Durham's fermentation tube and stopper), Centrifuge tubes, PCR tubes, Falcon tubes, Test tube stand, Conical flask, Pipette, Slides, Cover slips, Syringe with needle, Micropipettes (100 -1000 µl, 20 – 200 µl, 1 -10 µl, 0.1 – 2.5 µl), Multi-channel pipette (100µl, 300µl), Micropipette tips, Tube stands for 1.5 ml PCR clean tubes, Eppendrof tubes (1.5ml, 0.5 ml and 0.2ml), Stirrer sticks, Scissors, Scalpel, Blade with handle, Hand gloves, Mask, Bacteriological loop, Petri dishes, Hanging drop slide, Glass rod spreader and Ice box.

3.1.7 Reagents and chemicals

3.1.7.1 Culture media

PPLO agar, PPLO broth, Yeast extracts, Glucose, Arginine, Thallium acetate, Phenol red, Ampicillin, Penicillin, Horse serum were used for isolation of MG organism.

3.1.7.2 Positive sample

Mycoplasma gallisepticum 6/85 strain vaccine (Mycovac, Intervet).

3.1.7.3 Mycoplasma antigen

SPAFAS MG plate antigen manufactured by Charles River Laboratories (Inc. Wilmington, MA, USA, 01887, U.S. Vet. License no.344, Catalog No.551301) was used for detection of Mycoplasma specific antibody by SPA test.

3.1.7.4 Enzyme linked immunosorbent assay (ELISA) kit

BioChek MG ELISA Kit, Lot No. CK114, Manufactured by BioChek UK Ltd.Co.TW4 5P Hounslow, UK, composed of wash buffer solution, conjugate reagent, substrate reagent and stop solution were used for antibody detection of MG organism.

3.1.7.5 PCR reaction component

3.1.7.5.1 DNA extraction

Kit method: Pure Link^{TM} Genomic DNA mini kit comprised of: Lysis / Binding Buffer, Digestion Buffer, Wash Buffer 1 and 2, Elusion Buffer), Pure Link^{TM} Genomic DNA Purification Kit.

Chemical method: TE buffer, SDS, proteinase K, Sodium acetate, phenol, chloroform, isoamyl alcohol, isopropanol, ethanol.

Hit lysis method: PBS, PCR grade water.

DNA pucification: Pure Link^{TM} Genomic DNA purification mini kit.

3.1.7.5.2 Polymerase chain reaction (PCR) reagents: PCR Master Mix, 1 X Tris Acetate EDTA (TAE), Ultra Pure^{TM} Agarose, Ethidium bromide, Loading Dye, DNA Marker

(50bp and 100 bp DNA ladder), Phosphate buffered saline (PBS) solution, Nuclease free water.

3.1.7.6 Oligonucleotide primers

Two sets of primer for *M.gallisepticum* were synthesized by Macrogen, Singapur.

Table 3: MG primer set with sequence and product size

Sl. No	Primer name	Primer sequence	Product size	Reference
1.	MG14F	5'- GAG-**C**TA-AT**C**-TGT-AAA-GTT-GGT-**C**-3'	185 bp	OIE 2004
	MG13R	5'-G**C**T-T**CC**-TTG-**C**GG-TTA-G**C**A-A**C** -3'		
2.	MGGha_F	5'-GGA-TCC-CAT-CTC-GAC-CAC-GAG-AAA-A-3'	732 bp	Nascimento *et al*., 1991
	MGGha_R	5'-CCT-TCA-ATC-AGT-GAG-TAA-CTG-ATG-A-3'		

3.1.8 Data collection/ Questionnaire for interview

To establish the correlation of farms, study the prevalence and risk factors of the disease some information were collected from ten selected farms of the study area which are: about qualitative variables (present of maternal antibodies against MG at one-day-old of age, type of breed, antibiotic consumed during previous three months, any vaccination against MG in their parent stocks and also them) and quantitative variables (age of chickens, production of egg rate, flock size, number of flock, house number, mortality and morbidity rate).

3.1.9 Samples

Table 4: Collection of blood from suspected birds based on age, season and location for antibody detection of *M. gallisepticum*

District	Farm identity	Sample	Groups				Seasons		Total
			G-1	G-2	G-3	G-4	Summer	Winter	
Rangpur	Farm-A	Blood	23	23	23	23	46	46	92
	Farm-B	Blood	23	23	23	23	46	46	92
Dinajpur	Farm-C	Blood	23	23	23	23	46	46	92
	Farm-D	Blood	23	23	23	23	46	46	92
Thakurgaon	Farm-E	Blood	23	23	23	23	46	46	92
	Farm-F	Blood	23	23	23	23	46	46	92
Panchagarh	Farm-G	Blood	23	23	23	23	46	46	92
	Farm-H	Blood	23	23	23	23	46	46	92
Joypurhat	Farm-I	Blood	23	23	23	23	46	46	92
	Farm-J	Blood	23	23	23	23	46	46	92
Total			**230**	**230**	**230**	**230**	**460**	**460**	**920**

Group-1 (0-8 weeks), Group-2 (9-20 weeks), Group-3 (21-40 weeks), Group-4 (above 40 weeks).

3.1.9.1 Serum

A total of 920 blood samples were collected which comprised of 184 sera from each group of selected poultry flocks for detection of MG antibody.

Serum preparation: A total of 920 blood samples were aseptically and randomly collected from the wing vein at different ages of sick birds which had respiratory distress. From each bird 3 ml of blood was collected using 5ml sterile disposable syringe and needles without any anticoagulant as described by Kelly and Alworth, 2013. Blood was allowed to clot in the syringe and kept for 2 hours at room temperature. Blood samples were carried out to the laboratory of department of Microbiology, HSTU, Dinajpur. Blood

containing syringe were kept in the refrigerator at 4°C for 3 hours. The syringes were left horizontally and then vertically for the serum to ooze out. A clean straw colour serum was seen around the clotted. After that serum liquid portion of each sample was decanted into centrifuge tube, centrifuge at 2500 rpm for 5 minutes to have clear serum and then serum were transferred into sterile eppendrof tube and some portion of each serum was preserved at 4°C for SPA test and some portion of each serum kept at -20°C for HI and ELISA test till further analysis.

3.1.9.2 Suspected samples

Tracheal swabs (n=920) were also collected at the time of blood collection from suspected birds and 52 lungs and 52 air sacs were also collected for isolation and molecular characterization of etiological agent from postmortem examination at different ages.

Table 5: Samples collected for isolation and identification of etiological agent from different suspected tissues based on age, season and location differences by culture method

Location	Groups				Samples collected from			Season		Total samples collected
	G-1	G-2	G-3	G-4	TS	Lung	AS	Summer	Winter	
Rangpur	9	9	9	9	12	12	12	18	18	36
Dinajpur	7	7	7	9	10	10	10	15	15	30
Thakurgaon	7	7	7	9	10	10	10	15	15	30
Panchagarh	7	7	7	9	10	10	10	15	15	30
Joypurhat	7	7	7	9	10	10	10	15	15	30
Total	**37**	**37**	**37**	**45**	**52**	**52**	**52**	**78**	**78**	**156**

TS= Tracheal swab, AS = Air sac

Group-1 (0-8 weeks), Group-2 (9-20 weeks), Group-3 (21-40 weeks), Group-4 (above 40 weeks).

3.2 Methods

3.2.1 Layout of the experimental design at a glance

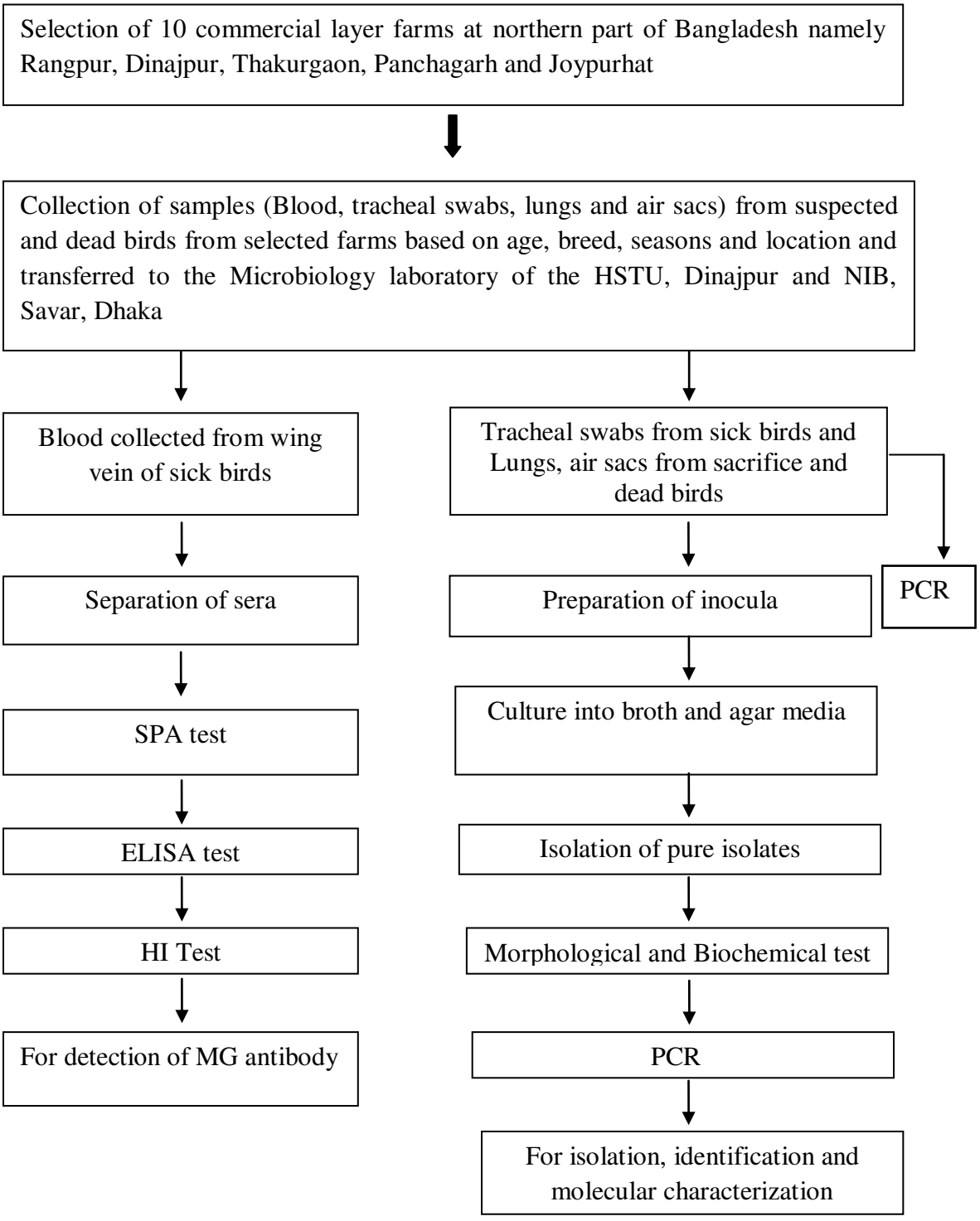


Figure 4: Schematic illustration of the experimental layout at a glance

3.2.1.1 Detection of MG antibody by SPA test

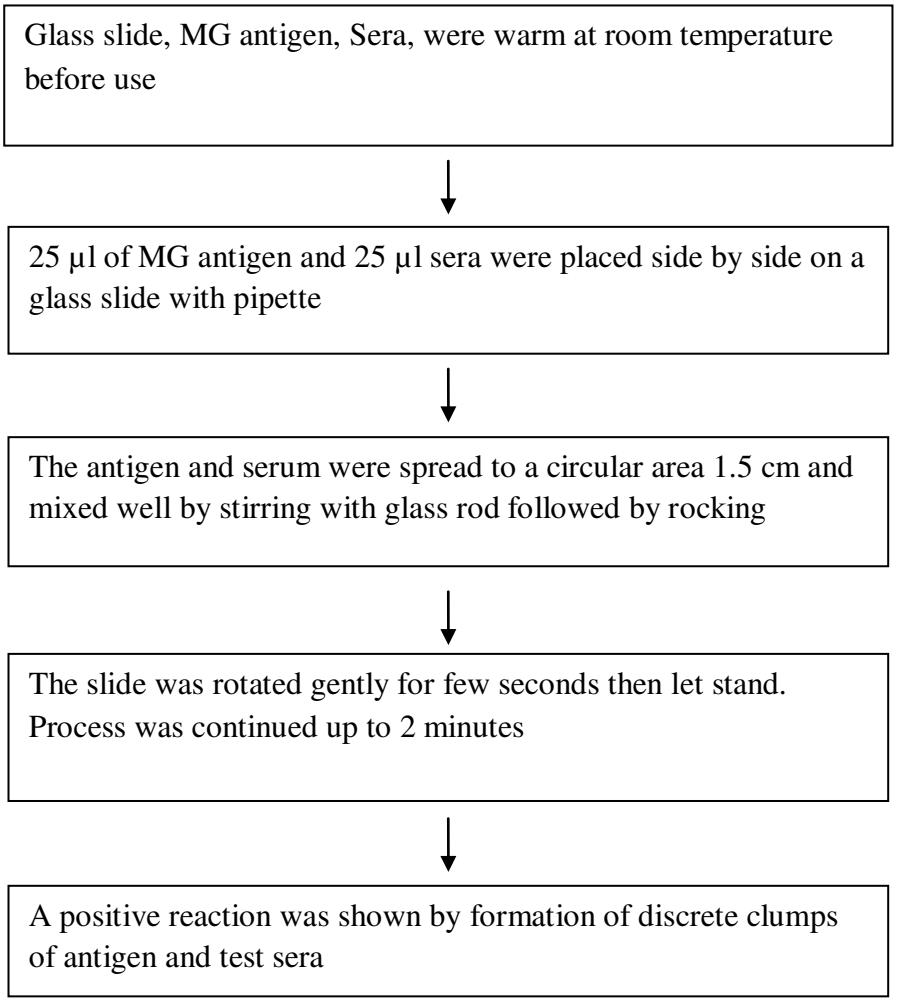


Figure 5: Detection of MG antibody by SPA test

3.2.1.2 Detection of MG antibody by iELISA test

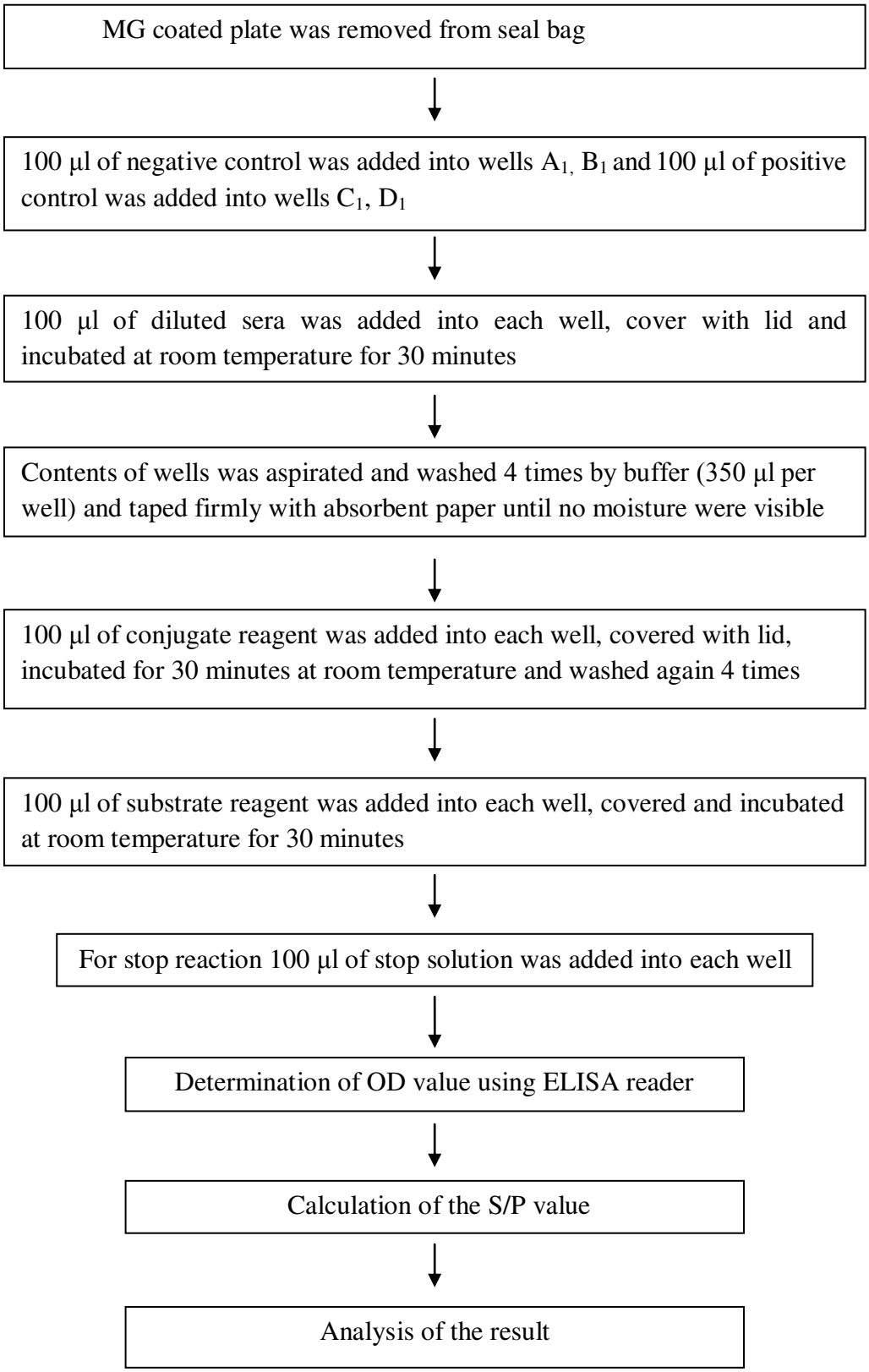


Figure 6: Schematic illustration of the iELISA procedur

3.2.1.3 Detection of MG antibody by HI test

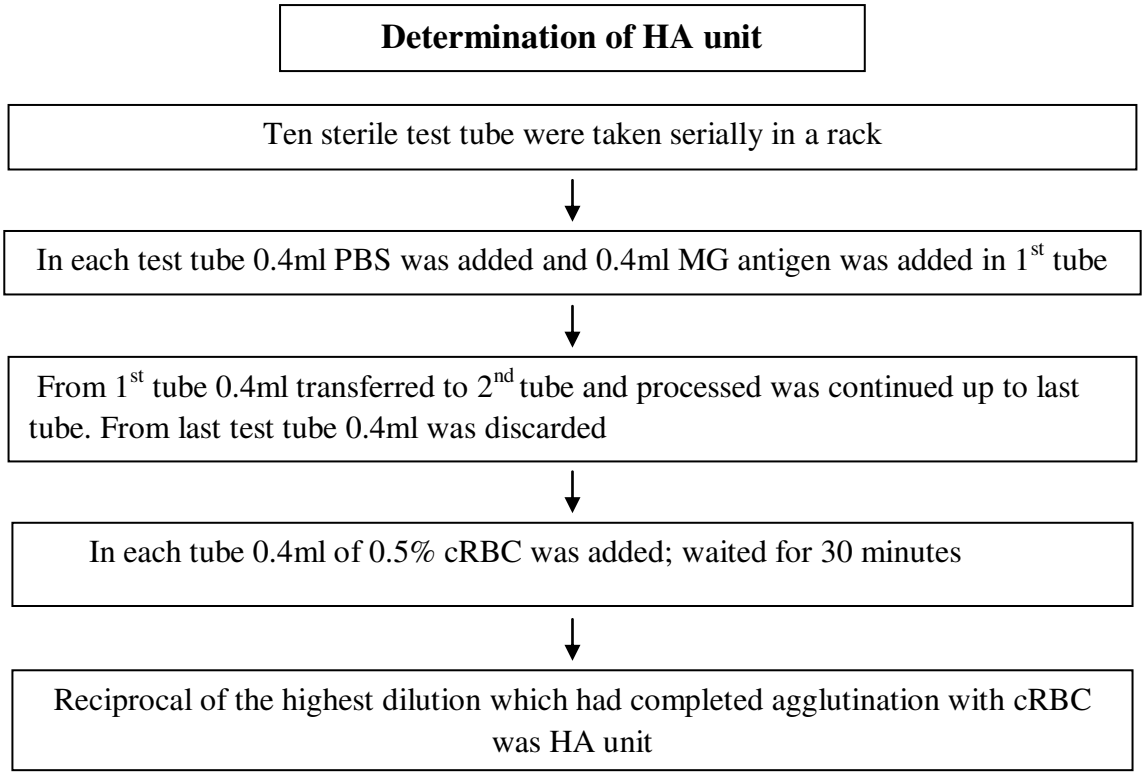


Figure 7: Determination of HA unit

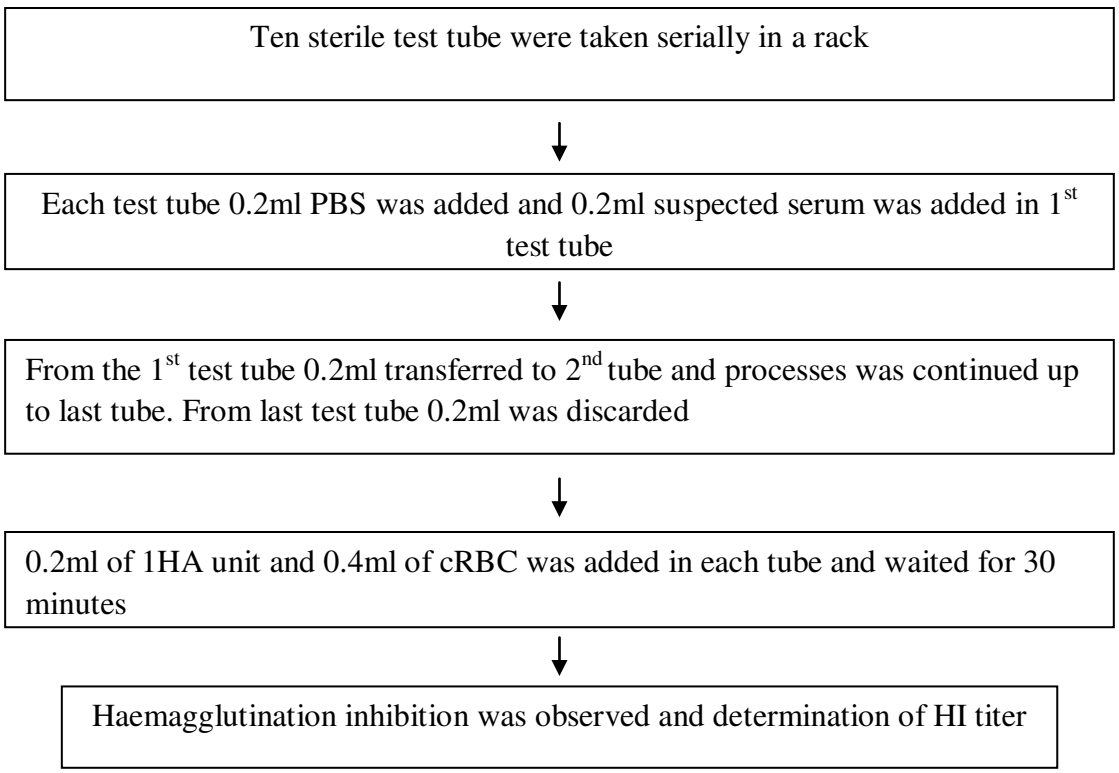


Figure 8: Procedure of HI test

3.2.1.4 Isolation of MG isolates by cultural characteristics

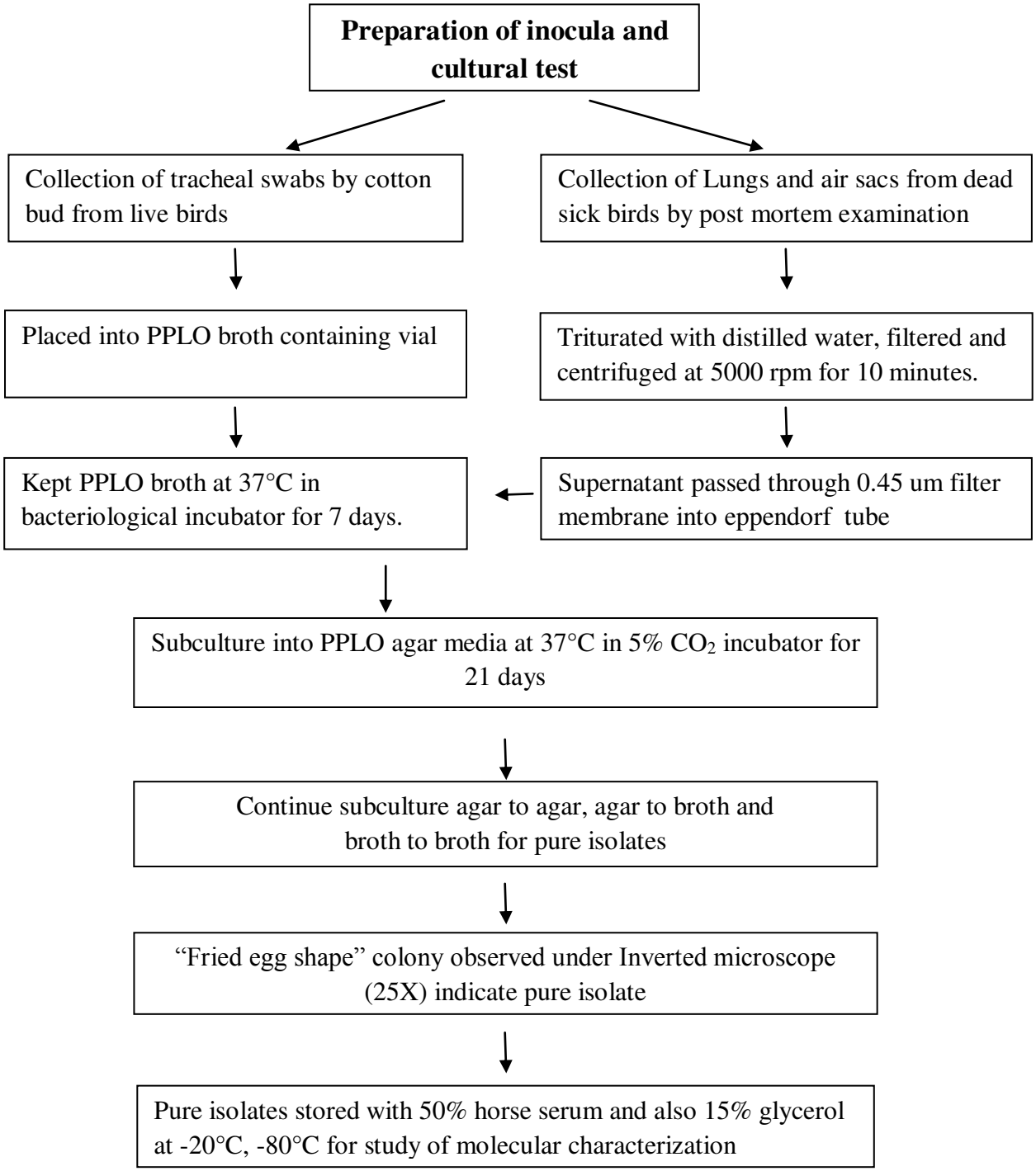


Figure 9: Isolation of MG isolate by cultural test

3.2.1.5 Molecular characterization of MG

3.2.1.5.1 DNA extraction

3.2.1.5.1.1 DNA extraction by Heat lysis method

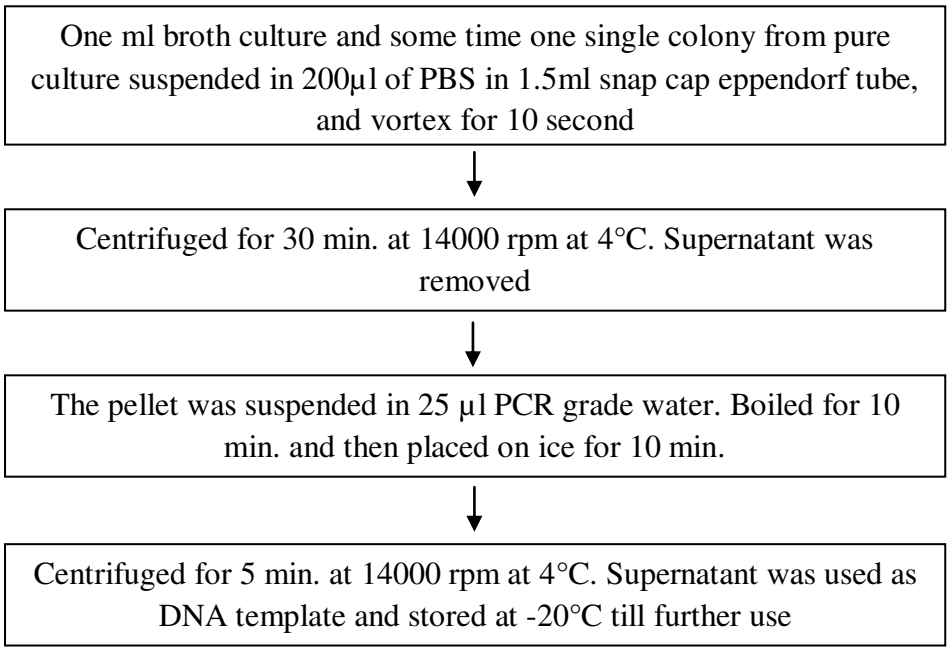
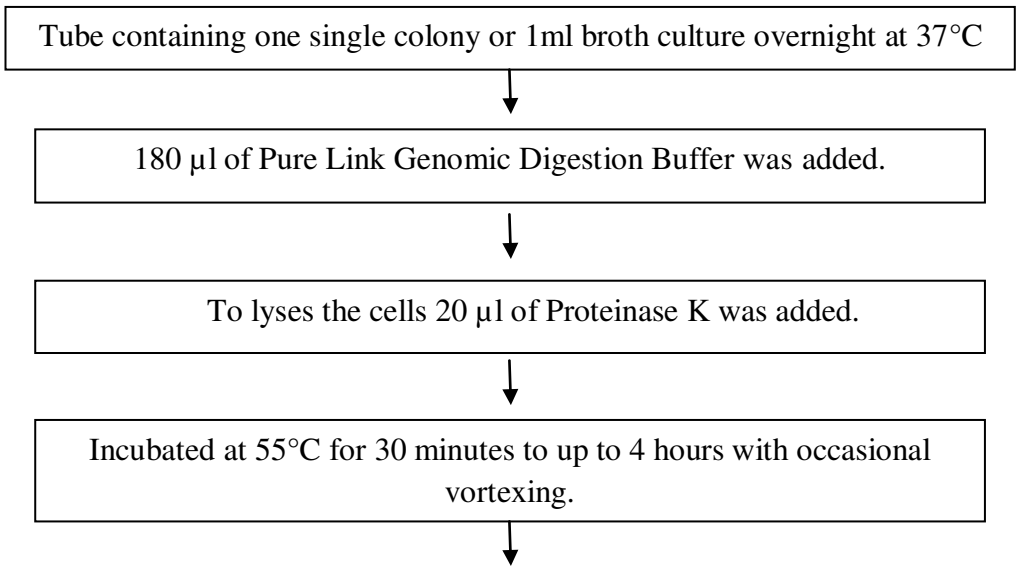


Figure 10: DNA extraction by boiling method

3.2.1.5.1.2 DNA extraction by kit method (Pure Link ™ Genomic DNA mini kit)



3.2.1.5.1.2 DNA extraction by kit method (Continued)

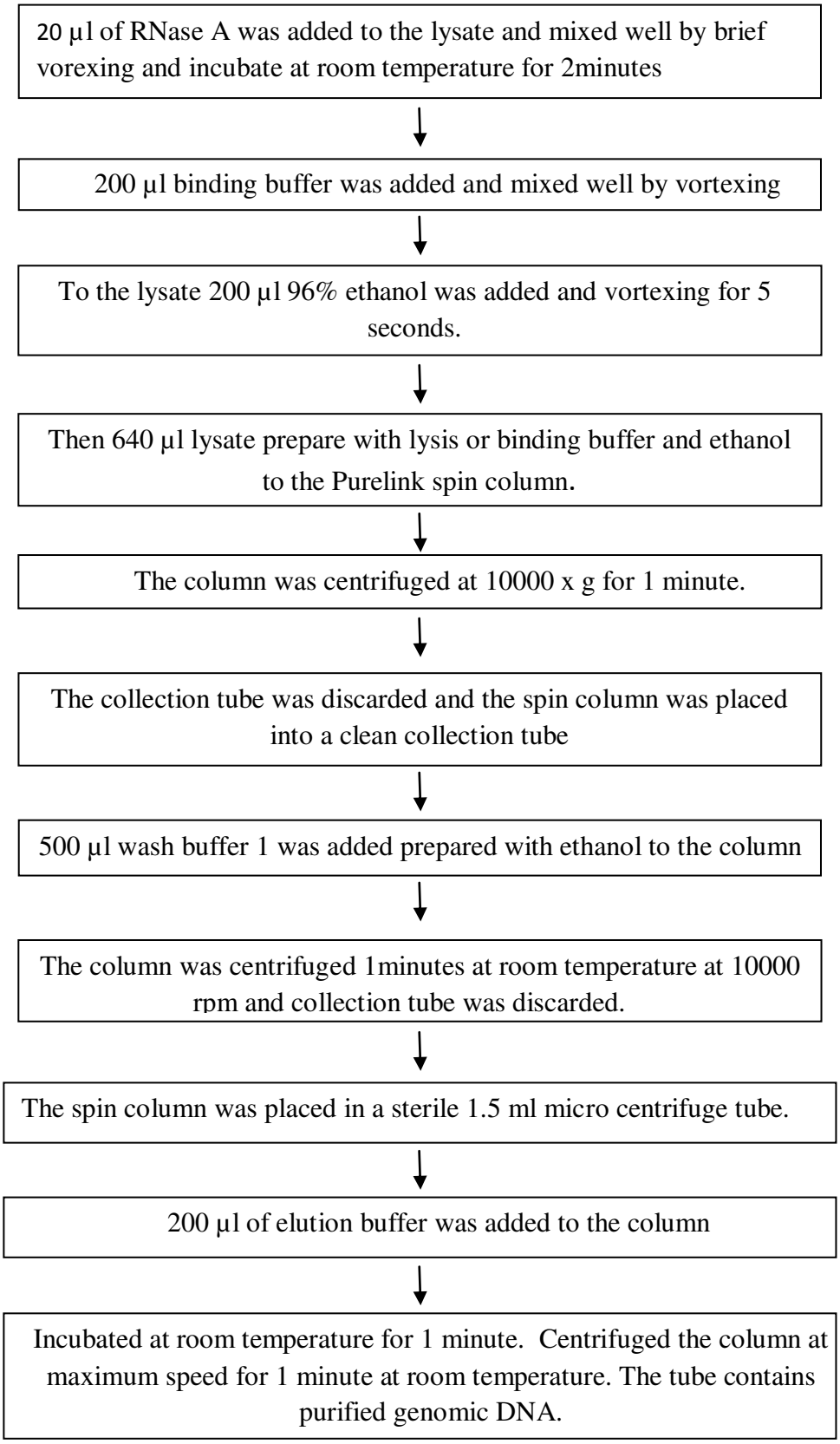


Figure 11: DNA extraction by kit method

3.2.1.5.1.3 DNA extraction by chemical method

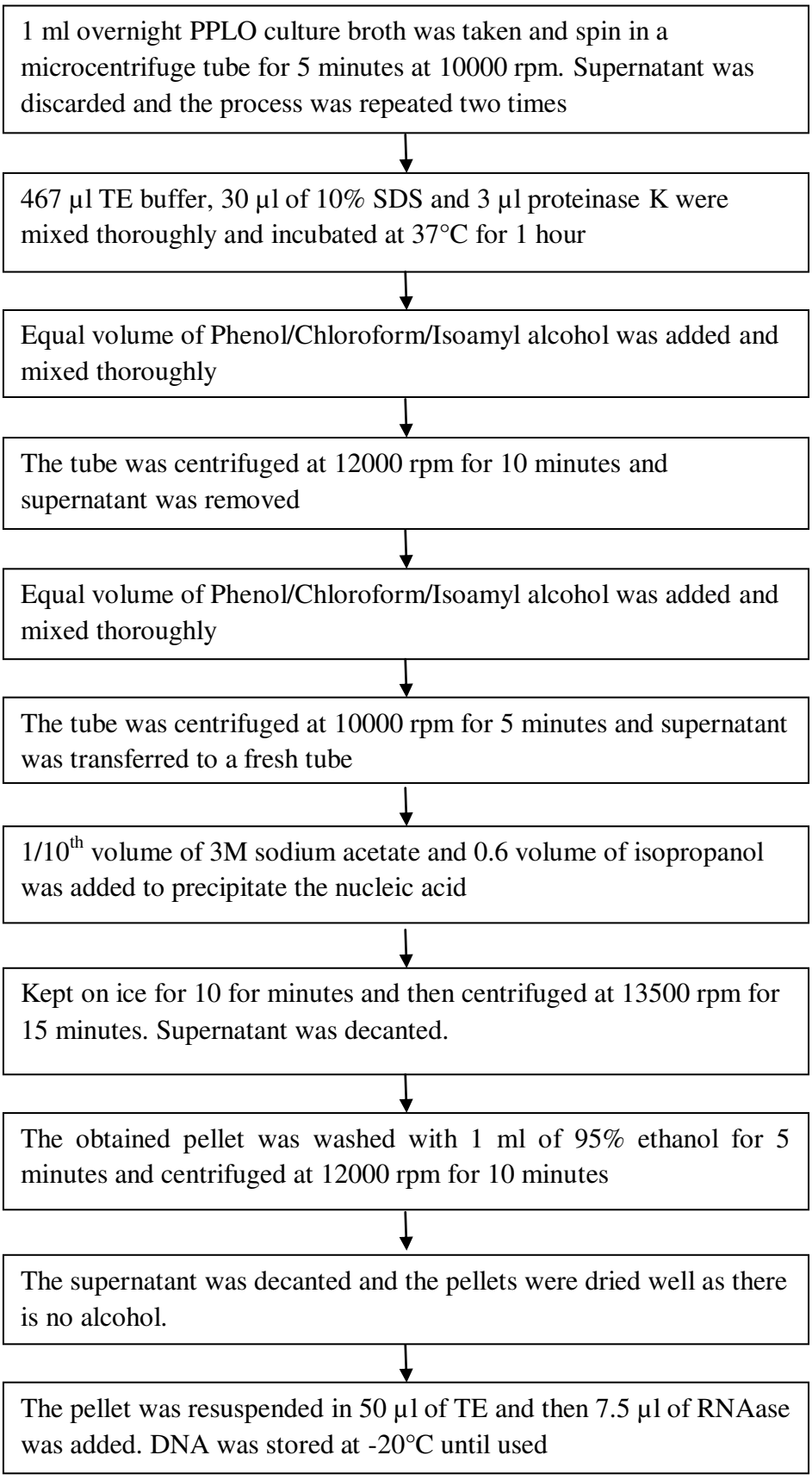


Figure 12: DNA extraction by chemical method

3.2.1.5.2 Molecular characterization of MG by PCR

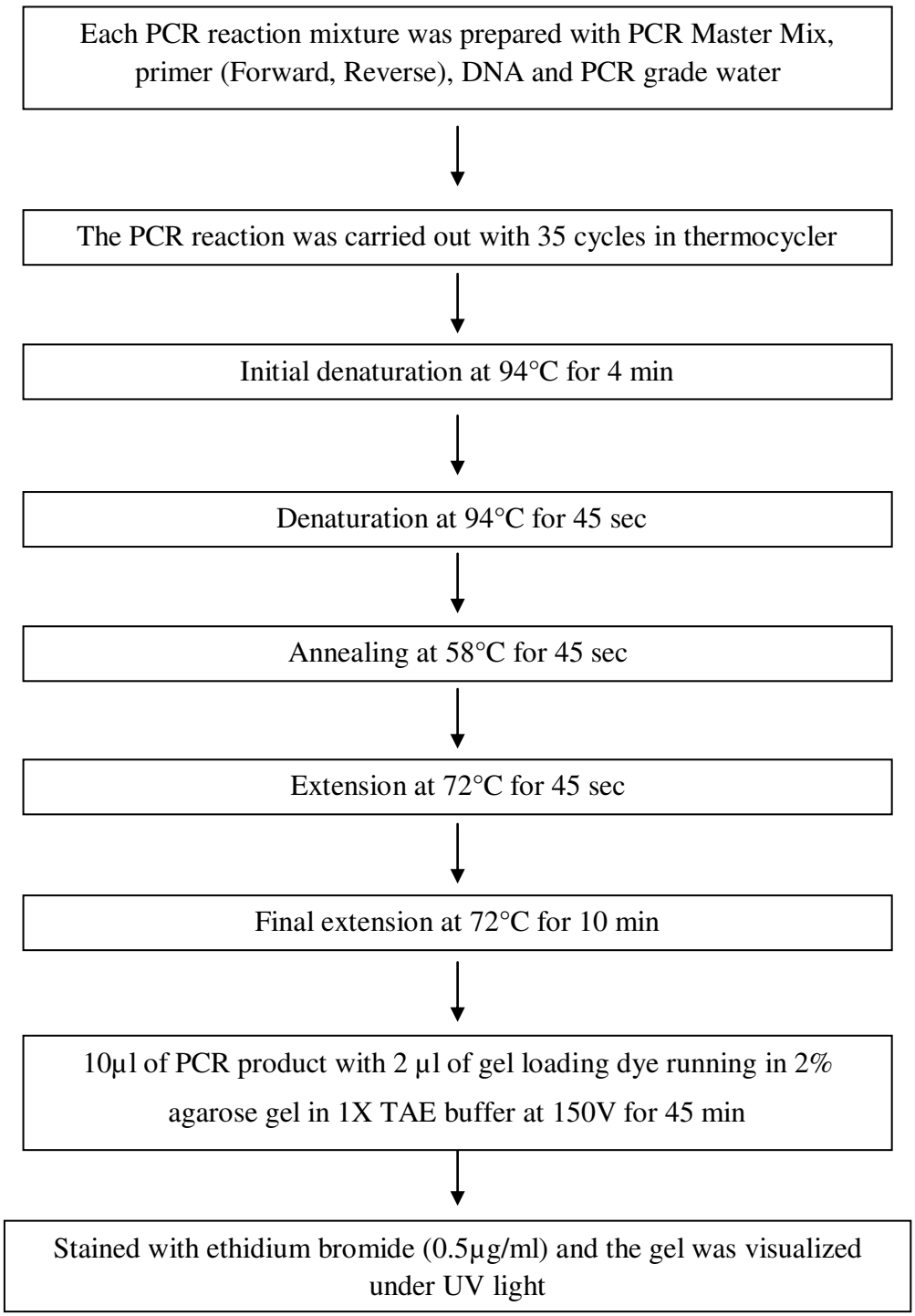


Figure 13: Molecular identification by PCR

3.2.2 Detection of MG antibody by Serum Plate Agglutination (SPA) test

3.2.2.1 Test procedure

The SPA test was conducted with crystal violet-stained MG antigen (Charles River Laboratories). All components (glass plate, MG antigen, sera sample, positive control and negative control) were to warm at room temperature before use. The antigen was shacked well to mix. Antigen was always undiluted. All sera samples were inactivated by heating 56°C for 30 minutes to destroy nonspecific inhibitory substance. Two equal aliquots of antigen and serum were added to the plate. The test was carried out in a standard glass plate, to which 25 µl of MG antigen and 25 µl of fresh serum were added side by side with pipette and spread to a circular area of approximately 1.5 cm diameter and mixed well by stirring with glass rod, followed by rocking. Gently rotated the plate for few seconds, then let stand. After one minute the plate rotated again, allowed to stand and reactions read when the 2 minutes was reached. A positive reaction showed formation of discrete clumps of stained material, normally starting at the edge of the mixture. Negative reactions show little change in the opaque serum antigen mixture after 2 minutes. Positive reactions retested by SPA on 1:8 dilution in 0.85% NaCl saline solution or 1:5 and 1:10 with 0.5M phosphate-buffered saline (PBS), pH 7.2. Those sera which were positive in the first SPA test but showed negative reaction in the second SPA test were consider suspected. The flocks with more than 10% positive reactions were considered positive serologically based on the suggestion of Kleven and Bradbury (2008). Sera were considered positive when clots were observed in dilutions up to 1:10.

SPA titers greater or equal to 1:10 are considered positive, 1:5 are suspicious, and titers lower than 1:5 are considered negative. Negative reaction was indicated by absence of agglutination reaction. Care was taken so that the natural granulation of the antigen (due to the presence of whole cells) was not taken as a positive reaction. The agglutination reactions were scored according to the following scheme.

_	=	No agglutination, no background clearing
+	=	Small clumps, no background clearing
++	=	Medium sized clumps, almost complete background clearing
+++	=	Large clumps, almost complete background clearing
++++	=	Very large clumps, mostly in the periphery, complete background clearing

N. B Only the agglutination score ++ or greater were recorded as a positive.

3.2.2.2 Data analysis

Data were entered in MS Excel spread sheet and exported to SPSS soft ware, Version 22 for analysis. Prevalence data were analyzed using chi-square (χ^2) for calculation of correlation among different variables.

3.2.3 Detection of MG antibody by Enzyme linked immunosorbent assay (ELISA) test:

3.2.3.1 Reagents

MG coated plate: Inactivated antigen on microtitre plates

- Conjugate Reagent: Anti-chicken, Alkaline phosphates in this buffer with protein stabilizers, inert red dye and sodium azide preservative (0.1% w/v).
- Substrate Tablets: PNNP (p-Nitrophenyle Phosphate) tablets to dissolve with substrate buffer.
- Substrate Buffer Reagent: Diethanolamine buffer with enzyme co-factors.
- Stop Solution: Sodium Hydroxide in Diethanolamine buffer.
- Sample Diluents Reagent: Phosphate buffer with protein stabilizers and sodium azide preservative (0.1 % w/v).
- Wash Buffer Sachets: Powdered phosphate buffered saline with tween.
- Negative control: Specific pathogen free serum in phosphate buffer with protein stabilizers and sodium azide preservative (0.1 % w/v)
- Positive control: Antibodies specific to MG in in phosphate buffer protein stabilizers and sodium azide preservative (0.1 % w/v).

3.2.3.2 Preparation of reagents and chemicals

Each serum sample was diluted in individual eppendorf tube which was at 0.002-fold (1/500) dilution i.e. adding 1ul serum to 500 µl sample diluents. Each diluted serum in the dilution rack and the corresponding wells of the antigen-coated plate was recorded. Before use, preparation of solution was carried out according to the methods provided by BioChek MG antibody Test Kit-CK 114. Dilution was not necessary of both positive and negative control which was ready into the supply kit.

3.2.3.2.1 Preparation of wash solution

Prior to preparation of wash solution, the concentrate wash solution was allowed to reach at room temperature. The wash concentrate (1 sachet) was diluted with 1000 ml sterilized distilled water) according to the methods provided by BioChek MG antibody Test Kit-CK 114. To ensure a homogenous solution, the concentrate was mixed in a bottle by inversion to dissolve any precipitated salts.

3.2.3.2.2 Preparation of substrate reagent

For the preparation of substrate reagent 1 tablet added into 6 ml substrate buffer into a clean container and wait up to 10 minutes for proper mixing. The prepared reagent should be use on day and if necessary should be store at 4°C in dark condition up to one week.

3.2.3.3 Test procedure

Sera that were positive in SPA test were analyzed for the detection of antibodies against MG using a commercially available ELISA antibody Test Kit according to the manufacturer's instructions which were briefly as follows:

- From the sealed bag MG coated plate was removed.
- Wells A1 and B1 were filled by the added of 100 µl of negative control reagents.
- Wells C1 and D1 were filled by the added of 100 µl of positive control reagents.
- 100 µl of diluted serum samples were added into the appropriate wells. Plate was covered with lid and incubated at room temperature for 30 minutes.
- Contents from wells were aspirated and washed 4 times with wash buffer (350 µl per well) solution. Plate was inverted and firmly taps by absorbent paper until no moisture was visible.
- 100 µl of Conjugate reagent was added into each appropriate well. Plate was covered with lid and incubated at room temperature for 30 minutes.
- Repeated wash procedure as in 5.
- Then each appropriate well were filled with 100 µl of Substrate reagent. Plate was covered with lid and incubated at room temperature for 15 minutes.
- To stop reaction, 100 µl of stop solution was added to each well.

- The microtitre plate reader was blanked on air and the plate placed on ELISA reader and recorded the absorbance of controls and the samples by the reading at 405 nm.

The samples and controls OD values were read using an automated microplate reader at 405 nm for each sample; the sample-to-positive (S/P) ratios were calculated from OD values by the formula.

3.2.3.4 Validity specifications

The result of the test should be valid when the mean negative control absorbance should be below 0.30 and the difference between the mean positive control and the mean negative control optical densities (OD) should be greater than 0.15 according to the BioChek MG antibody Test kit- CK 114.

Variance in laboratory temperature will lead to lower or higher absorbance values. Test sample values will be relative to the control values and the test will still be valid.

The MG positive control had been carefully standardized to represent significant amounts of antibody to MG in chicken serum. The relative amounts of antibodies in chicken samples could then be calculated by reference to the positive control. This relationship is expressed as S/P ratio (Sample to Positive Ratio)

3.2.3.4.1 Calculation of the negative control mean OD (NCx^-) (well A1 and B1)

$$NCx^- = \frac{A1\ (405) + B1\ (405)}{2}$$

Here, A1 = OD value of negative control -1, B1 = OD value of negative control -2

3.2.3.4.2 Calculation of the positive control mean OD (PCx^-) (well C1 and D1)

$$PCx^- = \frac{C1\ (405) + D1\ (405)}{2}$$

Here, C1 = OD value of positive control -1, D1 = OD value of positive control -2

3.2.3.4.3 Interpretation of results

If the S/P ratio was less than 0.50, the sample was classified as negative for MG antibodies.

If the S/P ratio was greater than 0.50, the sample was classified as positive for MG antibodies.

3.2.3.4.4 Calculation of S/P ratio of unknown samples

$$S/P = \frac{\text{Mean of test sample} - \text{Mean of negative control}}{\text{Mean positive control} - \text{Mean of negative control}}$$

Example of sample calculation:

Mean of test sample = 0.82
Mean positive control = 0.55
Mean of negative control = 0.23

$$S/P = \frac{0.82 - 0.23}{0.55 - 0.23}$$

$$S/P = \frac{0.59}{0.32}$$

$$= 1.84$$

This sample is positive for MG antibodies because 1.84 is greater than 0.5

3.2.3.5 Statistical analysis

Data were entered in MS Excel spread sheet and exported to SPSS soft ware, Version 22 for analysis. Prevalence data were analyzed using chi-square (χ^2) for calculation of correlation among different variables.

3.2.4 Detection of MG antibody by Haemagglutination Inhibition (HI) test

3.2.4.1 Preparation of 0.5 % chicken RBC (cRBC): For detection of MG antibody by HI test.

From healthy bird 4.5 ml blood was collected in a 10ml plastic syringe containing o.5ml sodium citrate anticoagulant solution. Equal amount of PBS was added and centrifuged in

a graduated centrifuge tube at 1500 rpm for 5minutes. The supernatant was removed including buffy coat and process was repeated for three times. During the last turn, the cells were maintained in PBS for 15 minutes and then centrifuged at 2000 rpm for 10 minutes to obtain packed cell. The sediment blood cells were used for preparation of 0.5% RBC suspension. Then 99.5 ml PBS was taken in a beaker and 0.5 ml sediment blood cells was added to prepare 0.5% chicken RBC and stored at 4°C for 7 days.

3.2.4.2 Determination of 1HA unit

For determination of 1HA unit 2 fold dilution was made:

- At first ten sterilized test tubes were set serially and o.4 ml PBS was taken in each tube.
- Then o.4 ml MG antigen suspension was added in 1st tube and mixed properly to make two fold dilutions.
- From the first tube, 0.4 ml of solution was transferred to the 2nd tube. Then, 0.4ml solution was transferred from to 2nd to 3rd tube
- The same procedure was continued up to last tube.
- From the last tube, 0.4 ml of solution was discarded.
- After that, 0.4 ml of 0.5% chicken RBC was added in each tube and kept at room temperature for 30 minutes to observe Haemagglutination.
- Reciprocal of the highest dilution of antigen which had completed the agglutination reaction with chicken RBC was considered as 1HA unit.

3.2.4.3 Test Procedure

- Ten sterilized test tubes were taken serially for carry on the experiment
- 0.2 ml of PBS was placed in each tube and then 0.2 ml of prepared sera was added in 1st tube to make two fold dilutions.
- Then 0.2 ml of solution was transferred from the 1st tube to 2nd tube. This process was continued up to the last tube and then 0.2 ml solution was discarded from the last tube.
- After that, 0.2 ml of HA unit solution and 0.4 ml of chicken RBC were added in each tube and was kept for an hour at room temperature.
- Then the tubes were observed for haemagglutination inhibition reactions

3.2.4.4 Observation

In HI test, titers equal or greater than 1:80 was considered positive, between 1:20 and 1:40 was suspicious, and below 1:20 was considered negative (Fiorentin *et al*., 2003, Feberwee *et al.*, 2005 and Courtney and Cornell (1990).

3.2.5 Isolation and identification of MG by Culture method

For the isolation and preliminary identification the standard procedures were followed as detailed OIE (2012). During these procedures PPLO broth and agar were constituted according to the formulation of Frey *et al.* (1968).

3.2.5.1 Preparation

3.2.5.1.1 Fresh yeast

250 g commercial dried yeast was dissolved in distilled water till 1000 ml total volume. This suspension was boiled in a boiling water bath for 30 min. It was then cooled and centrifuged at 3000 g for 30 min. The supernatant was taken, sterilized by filtration through 0.45 µm filter paper, aliquoted into 50 ml and stored at -20°C till use.

3.2.5.1.2 Glucose

10% Glucose solution was prepared by dissolving 10 g glucose in distilled water till 100 ml total volume. The solution was sterilized by filtration through 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

3.2.5.1.3 Arginine

10% Arginine solution was prepared by dissolving 10 g arginine in distilled water till 100 ml total volume. The solution was sterilized by filtration through 0.45 µm filter, aliquoted into10 ml, and stored at -20°C till use.

3.2.5.1.4 Ampicillin

1% Ampicillin solution was prepared by dissolving 1 g ampicillin in distilled water till 100 ml total volume. The solution was filtrated through a 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

3.2.5.1.5 Penicillin G sodium

A solution of Penicillin G sodium 100,000 IU/ml was prepared by dissolving 10,000,000 Unites of Penicillin G sodium in distilled water till 100 ml total volume to give a solution of 100,000 units per ml. The solution was filtered through a 0.45 µm filter, aliquoted into 10 ml and was stored at -20°C till use.

3.2.5.1.6 Thallium acetate

5% Thallium acetate solution was prepared by dissolving 5 g Thallium acetate in distilled water till 100 ml total volume. The solution was filtrated through a 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

3.2.5.1.7 Phenol red

0.1% Phenol red solution was prepared by dissolving 0.1 g of phenol red powder in 2.8 ml of 0.1 M NaOH. This mixture was made up to 100 ml with double distilled water to make 0.1% solution and autoclaved at 121°C for 20 minutes, aliquoted into 10 ml, and then stored at -20°C.

3.2.5.1.8 Preparation of inocula

1) Tracheal swab: 920 tracheal swabs were randomly collected from trachea by sterile cotton buds during the period of blood collection from the affected birds at different ages and showed the identical symptoms of rales, swollen face and eyes with lacrimation, cyanotic comb and wattles, nasal discharge. Sterile cotton bud was vigorously rubbed the mucosa of the trachea and inoculated into 3 ml PPLO broth containing 15% horse serum in vials and transported immediately to the laboratory of Department of Microbiology, HSTU, Dinajpur with cool box and stored at -20°C until used.

At the time of use the tracheal swab were diluted by five consecutive 10-fold dilutions (4.5 ml PBS in each test tube and 0.5 ml sample mixed first tube, mixed thoroughly. Then 0.5ml was transferred into 2nd tube and process was continued up to 5^{th} (last) tube and 0.5ml aspirited from 5th tube.

2) Tissues: Tissues (lung and air sacs) were collected from sick and dead birds through postmortem examination in 25 ml buffered glycerol (50% glycerol) in falcon tube. All samples were kept at - 20°C until they were tested through culture and PCR.

Tissues were triturated directly without any enrichment in pestle and mortar and mixed with distilled water. Then homogenous tissue suspension was passed through sterile filter paper into the test tube and centrifuged at 5000 rpm for 20 minutes. The supernatant was passed through of 0.45µm filter membrane into eppendrof tube which was ready for culture or DNA extraction.

3.2.5.1.9 PPLO broth

Table 6: Composition of PPLO broth

Name of composition	**Quantity**
Part A	
PPLO broth powder	14.7 g
Distilled water	700 ml
Part B	
Horse serum (heat inactivated)	150 ml
Fresh yeast extracts (25% solution)	100 ml
Glucose solution (10% solution)	10 ml
Arginine solution (10% solution)	10 ml
Penicillin (100 000 IU/ml)	10 ml
Ampicillin (1% solution)	10 ml
Thallium acetate (5% solution)	10 ml
Phenol red (0.1 % solution)	20 ml

- Part A was autoclaved at 121°C for 20 minutes and then kept at 50°C in water bath.
- Components of part B were aseptically mixed together and added to part A.
- P^H was then adjusted to 7.4 with 1 M NaOH. Prepared 5 ml broth was distributed in sterile 10 ml plastic tubes and kept at 4°C for up to 6 months.

Culture in PPLO Broth: Based on the standard cultural isolation procedures (Quinn, *et al*; 2002; OIE, 2012). From each diluted sample 300 µl was mixed with 5ml of PPLO broth in separate tube. The caps of liquid medium contain tube were tightly sealed before incubation at 37°C to avoid spurious changes in P^H. The samples were incubated at 37°C for seven to ten days. The broth was checked daily. Positive broth tubes were showing uniform growth turbidity and color of the broth was changed from red to orange or yellow

in the indicator. Positive broth was sub-cultured in PPLO broth and PPLO agar. For obtaining a pure isolate broth culture were filtered through 0.25 µm pore size syringe filter.

3.2.5.1.10 Agar

Table 7: Composition of PPLO agar

Name of composition	**Quantity**
Part A	
PPLO agar powder	10 g
Distilled water	700 ml
Part B	
Horse serum (heat inactivated)	150 ml
Fresh yeast extracts (25% solution)	100 ml
10% Glucose solution (10% solution)	10 ml
10% Arginine solution (10% solution)	10 ml
Penicillin (100 000 IU/ml)	10 ml
Ampicillin (1% solution)	10 ml
Thallium acetate (5% solution)	10 ml

- Part A were autoclaved at 121 °C for 20 minutes and kept at 50°C in water bath.
- Components of part B were aseptically mixed together and warmed to 50°C in water bath and was then added to part A.
- Prepared agar medium was distributed in sterile 4 cm petri dishes and kept at 4°C for up to 6 months.

3.2.5.2 Culture in PPLO agar

From the positive PPLO broth, the inoculum was inoculated into PPLO agar and incubated at 37°C with 5% CO_2 for twenty one days and observed under Inverted microscope (25X) at every three days interval. The plates were showing no growth up to 15 days of humid condition, indicated negative.

Round with central nipple and translucent flat periphery, like fried egg shape colonies were marked under microscope indicated as prominent and visible characteristics of

Mycoplasma. Then colony were marked using lab marker pens and were incised along with agar by special scalpel and sub-cultured on to Mycoplasma agar by pushing the block method and also sub-cultured in to Mycoplasma broth. The agar was incubated at 37°C in 5% CO2 incubator and the broth at 37°C in bacteriological incubator for 7 days. For obtaining a pure culture of Mycoplasma isolates same procedures were contained for three to five times. The pure isolated colonies were inoculated stock suspension in 50% horse serum and slowly frozen at -80°C for molecular characterization.

3.2.6 Characterization of MG by Biochemical test

Fermentation of glucose was done as preliminary biochemical identification of Mycoplasma. This biochemical test was conducted in PPLO broth culture tubes of about 5ml in to which 0.1ml of glucose was added to one of the tubes and colony was put in the tube and incubated at 37°C.

3.2.7 Morphological characterization by staining

Staining of colonies suggestive of *Mycoplasma* spp. was performed as per method described by as below Quinn *et al*., 2004.

Giemsa staining

All the isolates were subjected to microscopic examination of morphology using Giemsa stain. The methanol-fixed smear was stained with Giemsa stain for a period of 30 to 40 min. in a coplin jar. The stained smear was air dried by gentle blotting between absorbent filter papers. The dried smear was observed under 1000 X power under oil immersion.

3.2.8 Characterization of MG isolates by PCR technique

3.2.8.1 DNA extraction by heat method

DNA template for the PCR assay was prepared by heat lysis method proposed by OIE, 2012. For total genomic DNA extraction, one micro liter of positive broth culture or a loopful of positive colony were removed from the surface of agar plate (Fresh culture) and suspended in 200 µl sterile PBS in 1.5 ml snap-cap eppendorf tube and vigorous mixing with vortexure. The suspension was centrifuged for 30 minutes at 14,000 rpm at 4°C (Fig 30). The supernatant was carefully removed with a Pasteur pipette and the pellet was suspended in 25 µl PCR grade water. The tube and the contents were boiled for 10

minutes and then placed on ice for 10 minutes before centrifugation at 14,000g for 5 minutes. The supernatant was used as DNA template. The total DNA was measured at 260 nm optical density as per method described by Sambrook and Russel (2001). The extracted DNA was kept at -20°C till further use.

- One ml broth culture and some time one single colony from pure culture suspended in 200µl of PBS in 1.5ml snap cap eppendorf tube and vortex for 10 second.
- The suspension was centrifuged for 30 min. at 14000 rpm at 4°C.Supernatant was removed.
- The pellet was suspended in 25 µl PCR grade water. The suspension was boiled for 10 min. and then placed on ice for 10 min.
- Then the suspension was centrifuged for 5 min. at 14000 rpm at 4°C.Supernatant was used as DNA template and stored at -20°C till further use.

3.2.8.2 DNA extraction by chemical method

The following steps from addition of proteinase K, Buffer AL, absolute alcohol, washing, elution and freezing of eluted DNA were done from Mycoplasma culture.

- Inoculated a 25 ml of liquid culture with the bacterial stain of interest. Grow in conditions appropriate for that stain until the culture is saturated.
- 1.0 ml of the overnight culture in a microcentrifuge tube was spine for 5 minutes at 10000 rpm.
- The pellet was suspended in 25 µl PCR grade water. Boiled for 10 min. and then placed on ice for 10 min.
- The supernatant was discarded.
- This step was repeated. Drained well onto a kimwipe.
- The pellet was resuspended in 467 µl TE buffer by repeated pipetting. 30 µl of 10% SDS and 3 µl ml of 20 mg/ml proteinase K were added to give a final concentration of 100 mg/ml proteinase K in 0.5% SDS. Mixed thoroughly and incubated 30 min. to 1 hr at 37 °C.
- 500µl of Phenol/chloroform/Isoamyl alcohol was added. Thoroughly mixed but

very gently to avoid shearing the DNA, by inverting the tube until the phases was completely mixed.

- The tubes were centrifuged at 12000 rpm for 10 minutes.
- Aqueous was removed, viscous supernatant (450 µl) was transferred to a fresh microcentrifuge tube, leaving the interface behind. Equal volume of phenol/chloroform/Isoamyl alcohol was added, and spin in a microcentrifuge at 10000 rpm for 5 minutes.
- The supernatant was transferred to a fresh tube (400 µl).
- 1/10th volume of 3 M sodium acetate was added and mixed.
- 0.6 volumes of isopropanol were added to precipitate the nucleic acids, keeped on the ice for 10 minutes.
- Centrifuged at 13500 rpm for 15 minutes.
- The supernatant was decanted.
- The obtained pellet was washed with 1 ml of 95% ethanol for 5 minutes. Then centrifuged at 12000 rpm for 10 minutes.
- The supernatant was decanted.
- The pellets were dried well as there is no alcohol.
- The pellet was resuspended in 50 µl of TE and then 7.5 µl of RNase. DNA was stored at 4°C for short term and at -20°C for long term.

3.2.8.3 DNA extraction by kit method

Genomic DNA extraction was performed with the use of the Genomic DNA extraction kit (Pure Link^{TM} Genomic DNA mini kit) as per the following protocol:

- 1ml of overnight broth culture by centrifugation.
- 180 µl of Pure Link Genomic Digestion Buffer was added.
- Then 20 µl of Proteinase K was added to lyse the cells.
- Incubated at 55ºC for 30 minutes to 4 hours with occasional vortexing.

- 20 µl of RNase A was added to the lysate, mixed well by brief vortexing and incubated at room temperature for 2 minutes.
- 200 µl binding buffer was added and mixed well by vortexing to obtain a homogenous solution.
- 200 µl 96% ethanol was added to the lysate and mixed well by vortexing for 5 seconds.
- Then the 640 µl lysate was prepared prepare with binding buffer and ethanol to the spin column.
- The column was centrifuged at 10000 x g for 1 minute.
- The collection tube was discarded and the spin column was placed into a clean collection tube supplied with the kit.
- 500 µl wash buffer 1 was added with ethanol to the column.
- The Column was centrifuged at room temperature at 10000 x g for 1 minute.
- The collection tube was discarded and the spin column was placed into a clean collection tube supplied with the kit.
- 500 µl wash buffer 2 was added with ethanol to the column.
- The column was centrifuged at maximum speed for 3 minutes at room temperature and collection tube was discarded.
- The spin column was placed in a sterile 1.5 ml micro centrifuge tube.
- 200 µl of purelink genomic elution buffer was added to the column.
- Incubated at room temperature for 1 minute. The column was centrifuged at maximum speed for 1 minute at room temperature. The tube contains purified genomic DNA.
- The purified DNA were stored at - 20ºC for future use or used for the desired downstream application.

3.2.8.4 Quantification of DNA

Quantification of extracted DNA was done spectrophotometrically at 260 nm and 280 nm using UVspectro-photometer and Nanodrop spectro-photometer (Thermo Scientific, USA) for determination of DNA concentration and purity. To determine quantity of DNA, 1 µl of DNA sample was loaded in the Nanodrop Spectrophotometer. With the help of Nano 2000 software the quantity and purity of DNA was evaluated.

3.2.8.5 Polymerase chain reaction (PCR) amplification

The PCR protocols adapted targeting 16SrRNA gene of *M. gallisepticum*. The PCR primers were obtained from Macrogen, Singapore (Table 3). Positive sample was used 6/85 strain vaccine (Mycovac, Intervet). The PCR protocol was supposed to generate 185bp and 732bp amplicons suggestive for infectivity due to *M.Gallisepticum*. The reaction was carried out in 25 µl volume (Table 9) in the PCR tubes. The promega PCR Master Mix was used to amplify genomic DNA in a programmable thermocycler (G Stom). A total of 35 cycles of PCR amplification was carried out (Table 9) and the amplicons were electrophoresis in 2% gel agar. The images were captured by using a gel documentation system.

3.2.8.5.1 Amplification reaction for PCR

Amplification of the 16SrRNA gene was performed using positive and negative controls and 25 µl volume of the reaction mixture was dispensed into each PCR tube. The tubes were then placed in a thermal cycler (G-Stom thermal cycler). Following optimum running conditions as described by Wang *et al.* (1997) in the digital program of thermal cycler were adopted which included.

Table 8: Composition of PCR reaction mixture and thermal profile (Rauf *et al*., 2013) with primer set-1(OIE 2004)

Component	Quantity (µl)	Thermal profile				
		Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
		1 cycle	35 cycle			1 cycle
Master Mix	12	94 °C 4 min.	94 °C 45 sec	58 °C 45 sec	72 °C 45 sec	72 °C 10 min.
Forward primer (10 pmol/µl)	1					
Reverse primer (10 pmol/µl)	1					
Template DNA	2					
Nuclease-free Water	9					
Total volume	25					

3.2.8.5.2 Electrophoresis and visualization of PCR product

DNA was analyzed on a agarose slab gel (w/v) with ethidium bromide (0.5 µg/ ml) in TAE buffer (1X Tris acetate EDTA containing 40 mM Tris, 20mM acetic acid, 0.5 M Tris ethylene diamine tetraacetic acid). Preparation of 2% agarose gel for electrophorosis and visualization of PCR product were

- 2% agarose solution was prepared in 1 X TAE buffer by melting the gel powder in a microwave oven for proper melting.
- 5 µl of ethidium bromide (0.5 µg/ ml of gel) was added to 100 ml melted agarose solution.

- Melted agarose solution was poured onto casting tray (Fig. 41) carefully to avoid bumping and allowed to solidify.
- The comb was removed from the gel and hardened gel with tray was transferred to the electrophoresis tank containing sufficient TAE buffer to submerge the gel.
- A total of 10 µl of PCR product was mixed with 2 µl of 6X Gel loading dye and the sample was loaded to the appropriate well of the gel carefully.
- 2 µl of 100bp DNA ladder was loaded to the appropriate well for accurately determining the DNA size marker of PCR product.
- Started the switch of the electrophoresis apparatus and was run at 150 volt, for 45 minutes.

After the end of the gel running amplified PCR product was analyzed under UV light (Uvitec, Cambridge, UK) and photographed in a gel documentation system for record keeping.

Table 9: Composition of PCR reaction mixture and thermal profile (Rauf *et al*., 2013) with primer set-2 (Nascimento *et al*., 1991)

Composition	**Total volume (25µl)**	**Thermal profile**				
		1 cycle	**35 cycle**			**1 cycle**
		Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
Master Mix	12.5	94°C 5 min.	94°C 1 min.	55°C 1 min.	72°C 2 min.	72°C 10 min.
Forward primer (100 pmol/µl)	.5					
Reverse primer (100 pmol/µl)	.5					
Template DNA	2					
Nuclease-free Water	9.5					

3.2.8.5.3 Electrophoresis and visualization of PCR product by 2 No primer set (Nascimento *et al*., 1991)

DNA was analyzed on a agarose slab gel (w/v) with ethidium bromide (0.5 µg/ ml) in TAE buffer (1X Tris acetate EDTA containing 40 mM Tris, 20mM acetic acid, 0.5 M Tris ethylene diamine tetraacetic acid). Preparation of 1.5% agarose gel for electrophorosis and visualization of PCR product were

- 1.5% agarose solution was prepared in 1 X TAE buffer by melting the gel powder in a microwave oven for proper melting.
- 3 µl of ethidium bromide (0.5 µg/ ml of gel) was added to 50 ml melted agarose solution.
- Melted agarose solution was poured onto casting tray (Fig. 41) carefully to avoid bumping and allowed to solidify.
- The comb was removed from the gel and hardened gel with tray was transferred to the electrophoresis tank containing sufficient TAE buffer to submerge the gel.
- A total of 10 µl of PCR product was mixed with 2 µl of 6X Gel loading dye and the sample was loaded to the appropriate well of the gel carefully.
- 2 µl of 50bp DNA ladder was loaded to the appropriate well for accurately determining the DNA size marker of PCR product.
- Started the switch of the electrophoresis apparatus and was run at 100 volt, for 45 minutes.

After the end of the gel running amplified PCR product was analyzed under UV light (Uvitec, Cambridge, UK) and photographed in a gel documentation system for record keeping.

3.2.9 DNA Sequencing

3.2.9.1 Purification of PCR product

For PCR fluid purification, Purelink Quick Gel Purification and PCR Purification Combo kit, Invitrogen by Life Technologies was used according to manufacturer's

recommendations. Briefly, before starting 96% ethanol to wash buffer (w_1) and isopropanol to binding buffer (B_1) was added according to the levels of the bottle.

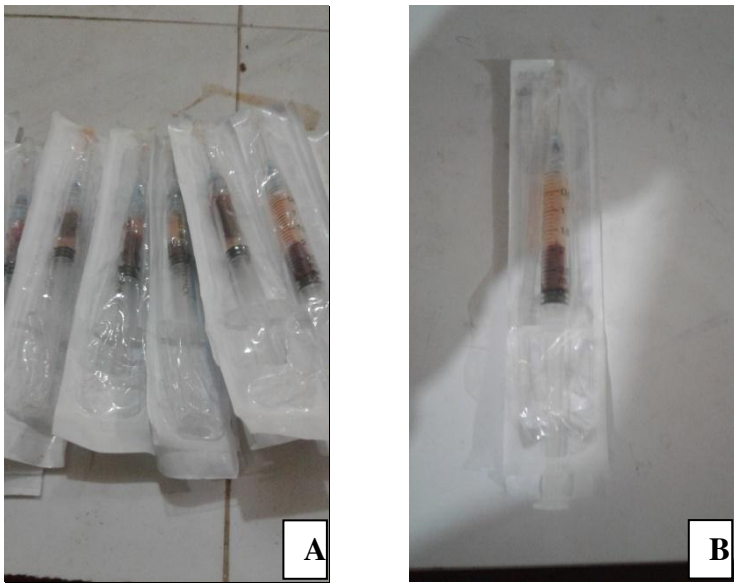
1. Combine: 4 volumes of binding buffer (B_1) and 1volume of isopropanol were added with 100 ul of PCR product.
2. Load: The sample was pipetted into Purelink clean-up spin column in a wash tube and centrifuged the column at 10,000g for 1 minute. The flow-through was discarded.
3. Wash: The column was re-inserted into the wash tube. 650ul wash buffer (b_1) was added with ethanol and centrifuged the column at 10,000g for 1 minute.
4. Remove the ethanol: The flow-through was discarded and the column was placed in the same wash tube. The column was centrifuged at 10,000g for 3 minutes.
5. Elute: The column was placed into a clean 1.7ml elution tube and added 50ul elution buffer to the column and incubated the column at room temperature for 1 minute. The column was centrifuged at 10,000g for 1 minutes.
6. Store: The elution contains the purified PCR product. Store the purified DNA at 4°C for immediate use and at -20°C for long term use.

Purified DNA of the PCR products were sent for sequencing to National Institute of Biotechnology (NIB), Savar, Dhaka. Nucleotide sequences of the 16s RNA gene were identified through direct sequencing from both directions using primer as shown in Table 3.

3.2.9.2 Sequence Analysis

The web-based Pair wise BLAST (Basic Local Alignment Search Tool) search programs under NCBI (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST) was used for the primary determination and comparison among different strains of the raw sequence data. The FASTA format was used in the achievement of database search. Bio Edit Sequence Alignment Editor Program (Hall, 1999) based on Clustal W Multiple alignment (Thompson *et al.*, 1994) was used in the assembling and analysing of sequence data to ensure sequence authenticity.

Figure 14: Collection of tracheal swab in PPLO broth



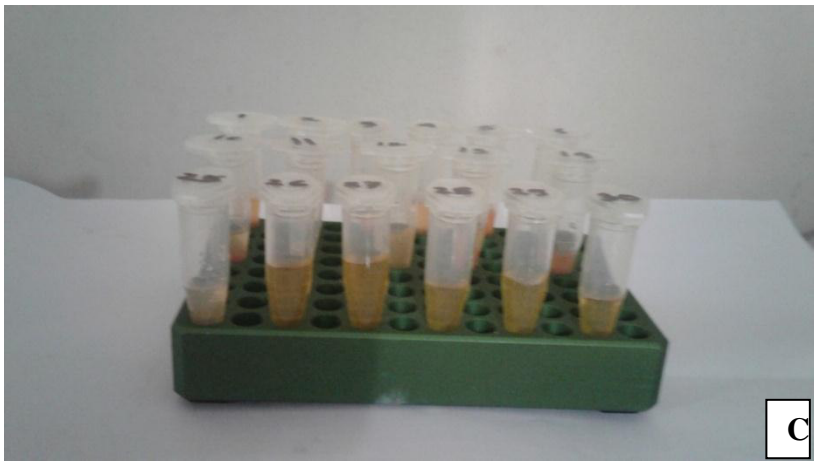


Figure 15: Collection of blood from birds (A) and separation of sera for serological test (B and C)

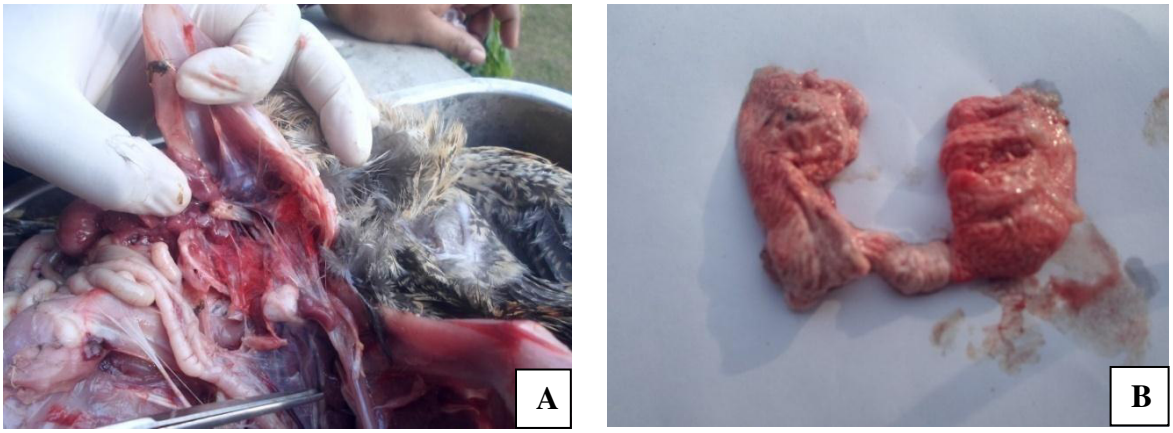


Figure 16: Collection of visceral samples (A) and lung (B) from post mortem of birds.

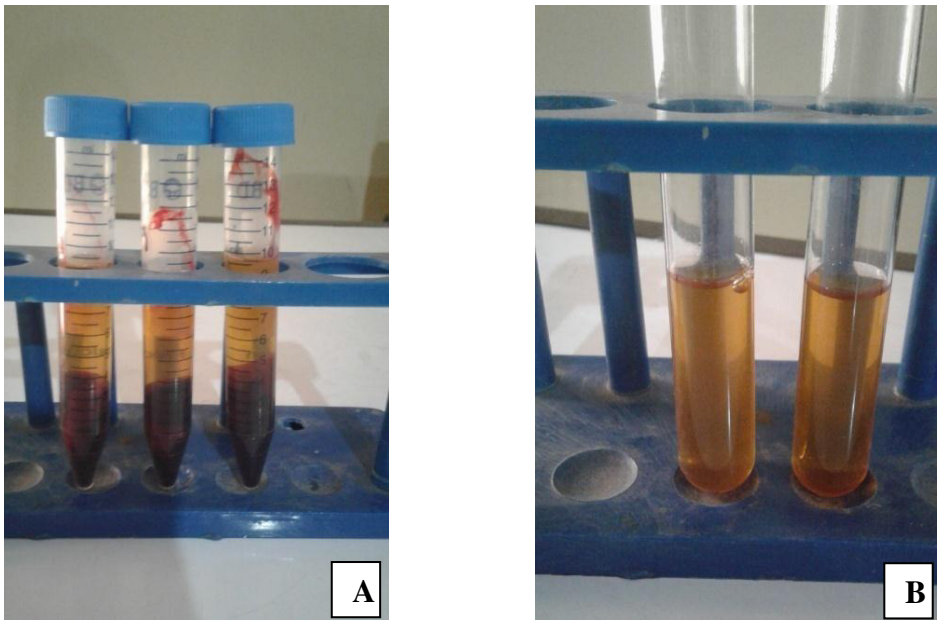


Figure 17: Separation (A) and collection of serum (B) from horse

Plate 1: Boiling for DNA extraction

Plate 2: Cooling on ice

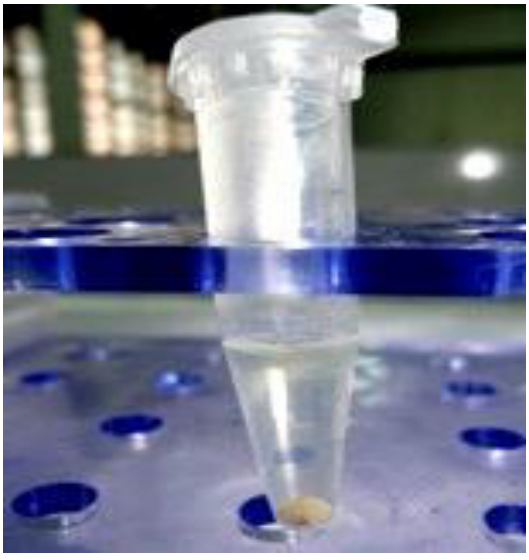


Plate 3: Extracted DNA

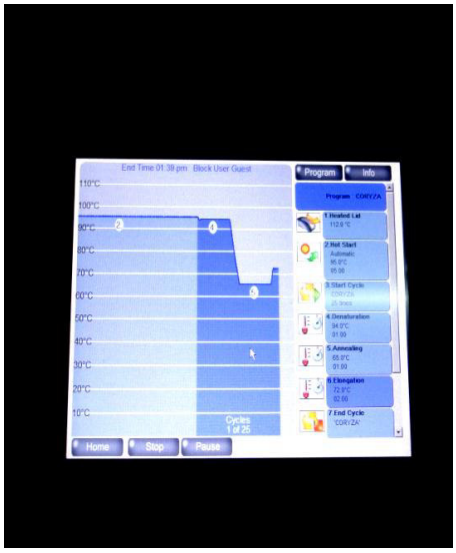


Plate 4: PCR reaction cyle

Figure 18: DNA extraction by PCI

Figure 19: Centrifugation of DNA

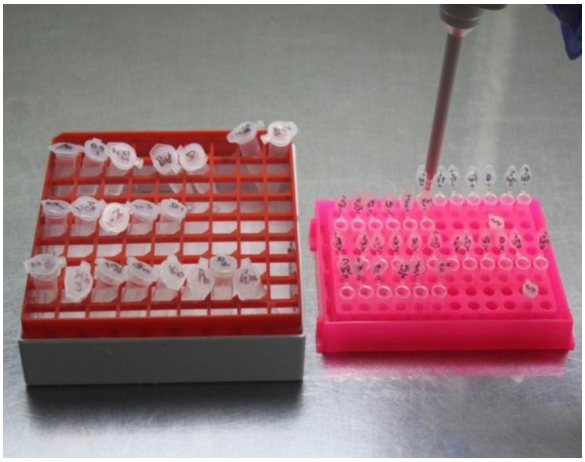


Figure 20: PCR reaction product

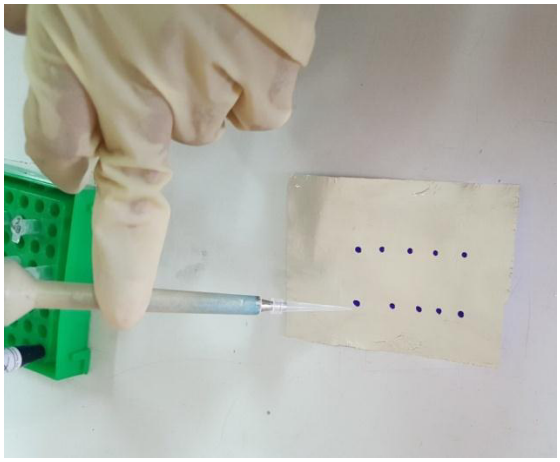


Figure 21: Loaging dye mixture with PCR product

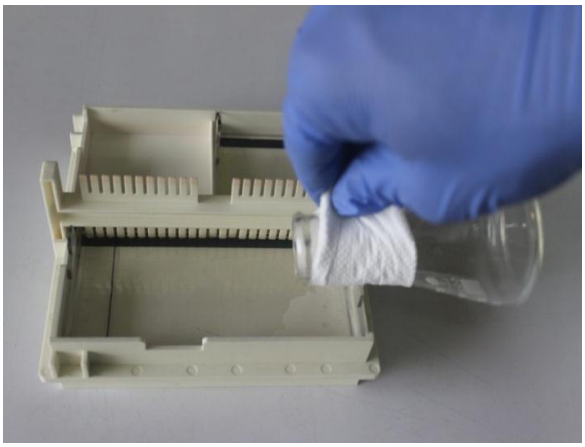


Figure 22: Preparation of gel

Figure 23: Loading of gel

CHAPTER 4

RESULTS

The present study was conducted on 10 randomly selected commercial layer poultry farms of 5 northern districts of Bangladesh. For proper isolation, identification and molecular characterization of etiological agent of MG, 920 blood samples and tracheal swabs, air sac and lung were collected from suspected to be infected birds which had respiratory problems based on age, breed, season and location of birds and were examined by observing typical sign and symptom, post mortem examination, serological, cultural and molecular test. The results of all laboratory work are revealed.

4.1 Results of epidemiological investigations

4.1.1 Observation on epidemic behavior of the *M. gallisepticum* in layer chicken of as per structured questionnaire and clinical signs and symptoms

The epidemic behavior of the causative agents of MG was studied based on age, breed, season, symptom and structured questionnaire. During the study period a total of 18,350 layer chickens were observed at four age groups. The clinical signs of etiological agent were observed which were nasal discharge, coughing, swelling of the facial skin and eyelids, increased lacrimation (Plate 5) and reduced feed consumption.The overall 11.54% incidence of MG was recorded. As per location variation the incidence of MG was recorded highest 12.73% in Joypurhat and lowest 9.97% in Panchagarh (Table 10) comparing with other districts. In age variation highest 5.11% incidence of MG was recorded in group-4 in comparing lowest (1.22%) in group-1 (Table 10). It is also recorded highest 12.73% incidence occur in Sonali breed compare to Isabrown 11.84%.

Table 10: Observation on epidemic behavior of suspected *Mycoplasma gallisepticum* in layer chickens based on age, location, season and breed

Location		Total Birds /Flock	Total No. of birds affected in a flock with respiratory disorders				Affected (%)	Location (%)	Season	
District	F		G-1	G-2	G-3	G-4			Summer (Affected/Total)	Winter (Affected/ Total)
Rangpur	F-1	1100	15	42	52	65	174 (15.81)	11.40	530/ 9,175 **(2.88%)**	1587/ 9,175 **(8.65%)**
	F-2	1750	14	36	43	58	151 (8.63)			
Dinajpur	F-3	2900	50	70	87	167	374 (12.90)	11.60		
	F-4	2850	39	56	72	126	293 (10.28)			
Thakurgaon	F-5	1450	21	33	48	75	177 (12.20)	11.84		
	F-6	1050	11	21	35	52	119 (11.33)			
Panchagarh	F-7	1820	17	29	44	75	165 (9.07)	9.97		
	F-8	1580	12	25	46	91	174 (11.01)			
Joypurhat	F-9	2230	27	51	77	135	290 (13.00)	12.73		
	F-10	1620	19	32	55	94	200 (12.35)			
Total		**18,350**	**225 (1.22)**	**395 (2.15)**	**559 (3.05)**	**938 (5.11)**	**2117 (11.54)**	**11.54**		
χ^2-value			**541.95**				**13.82**			
P-value			**0.001***				**0.008***			

F=Farm, G-1= Group-1 (0-8 wks), G-2= Group-2 (9-20 wks), G-3= Group-3 (21-40 wks), G-4= Group-4 (Above 40 wks)

*= Significant at 5% level of probability (P<0.05)

4.2 Determination of MG antibody by serological test

4.2.1 Determination of MG antibody by SPA test

Diagnosis of the MG antibody a total of 920 blood samples were collected from 10 selected flocks of different northern districts of Bangladesh. From each flock 92 blood samples were collected (23 blood samples from each group), sera were prepared and MG antibody investigated by SPA test of which 526 sera were seropositive. The overall incidence was detected as 57.17%. The incidence in Rangpur, Dinajpur, Thakurgaon, Panchagarh and Joypurhat were found 51.09%, 61.96%, 55.43%, 57.61% and 59.78% respectively. The highest incidence was found in Dinajpur (61.96%) and the lowest in Rangpur (51.09%) in comparison to other districts (Table 12).

Table 11: Prevalence of MG antibody by SPA test considering age, season and location

Location	Age (wks)																						Total Prevalence	
	0-8						9-20						21-40						Above 40				P	S
	Summer		Winter		Total		Summer		Winter		Total		Summer		Winter		Total		Summer		Winter		Total			
	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P		
Rangpur	23	8	23	10	46	18	23	9	23	13	46	22	23	11	23	14	46	25	23	13	23	16	46	29	94	184
Dinajpur	23	10	23	14	46	24	23	12	23	15	46	27	23	14	23	16	46	30	23	15	23	18	46	33	114	184
Thakurgaon	23	10	23	12	46	22	23	13	23	13	46	26	23	12	23	14	46	26	23	13	23	15	46	28	102	184
Panchagarh	23	10	23	13	46	23	23	14	23	15	46	29	23	11	23	15	46	26	23	14	23	14	46	28	106	184
Joypurhat	23	12	23	12	46	24	23	11	23	15	46	26	23	14	23	15	46	29	23	15	23	16	46	31	110	184
Total	**115**	**50**	**115**	**61**	**230**	**111**	**115**	**59**	**115**	**71**	**230**	**130**	**115**	**62**	**115**	**74**	**230**	**136**	**115**	**70**	**115**	**70**	**230**	**149**	**526**	**920**
P/S	**111/230 (48.26)**						**130/230 (56.52)**						**136/230 (59.30)**						**149/230 (64.78)**				**526/920 (57.17)**	
χ^2-value	**13.30**																						**5.26**	
P-value	**0.004***																						**0.26**	

S = Sample P = Positive

*= Significant at 5% level of probability (P<0.05)

Table 12: Prevalence of MG antibody in different locations by SPA test

Study area	Tested sera	Positive sera	Prevalence (%)	χ^2	P – value
Rangpur	184	94	51.09	5.256	0.26 (NS)
Dinajpur	184	114	61.96		
Thakurgaon	184	102	55.43		
Panchagorh	184	106	57.61		
Joypurhat	184	110	59.78		
Total	920	526	57.17		

NS means Not Significant

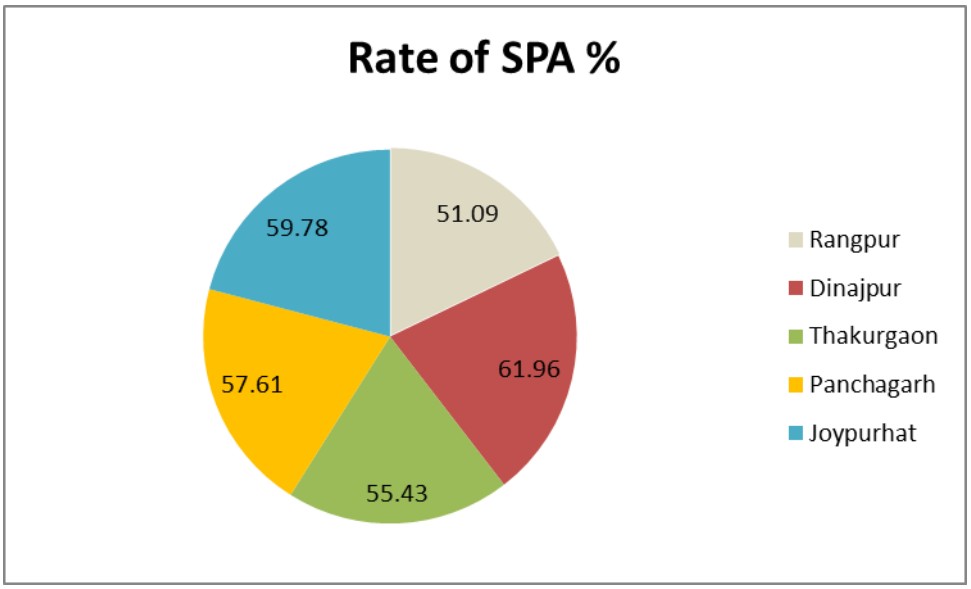


Figure 24: Prevalence of MG antibody in different locations by SPA test

The chickens were divided into four groups according to age. From each age group 23 sera were collected and determined the prevalence of MG antibody. The maximum prevalence was found in group-4 (64.78%) followed by group-3 (59.30%), group-2 (56.52 %) and group-1 (48.26%) respectively (Table 13).

Table 13: Seroprevalence of MG in chickens according to age by SPA test

Groups	Tested sera	Positive sera	Positive percentage (%)	χ^2	P - value
G – 1	230	111	48.26	13.30	0.004*
G – 2	230	130	56.52		
G – 3	230	136	59.30		
G – 4	230	149	64.78		
Total	920	526	57.17		

G - 1= 0-8wks, G - 2= 9-20wks, G - 3= 21-40wks, G - 4=Above 40wks

*= Significant at 5% level of probability (P<0.05)

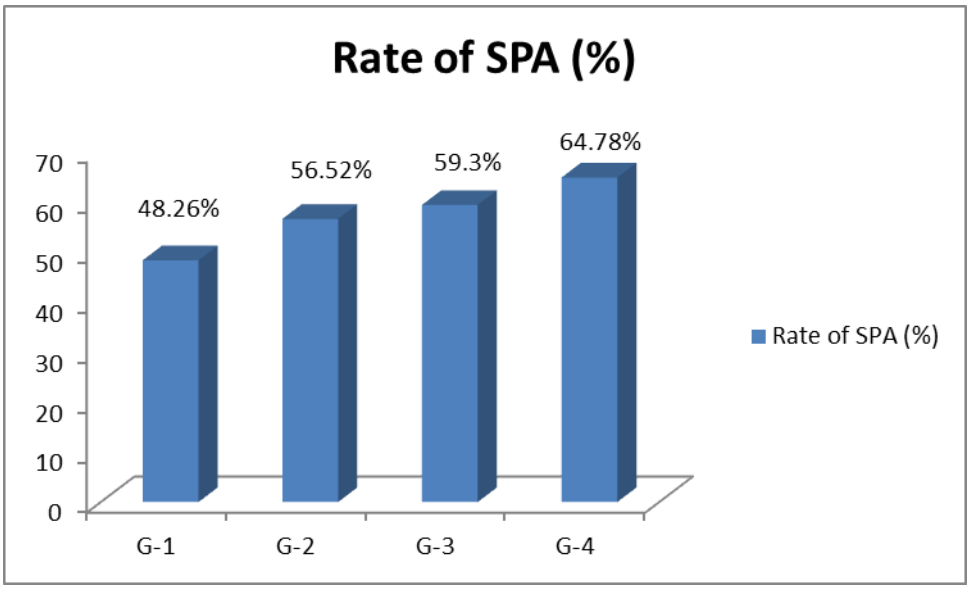


Figure 25: Seroprevalence of MG in chickens according to age by SPA test

For seasonal investigation of MG antibody of this study, 920 sera were collected from birds in two seasons. The numbers of samples were equal in both seasons. In winter season 460 sera and in summer 460 sera samples were collected. The prevalence was found 61.96% in winter and 52.39% in summer season by SPA test respectively (Table 14). It is illustrated that the highest prevalence was found in winter compared to summer season.

Table 14: Seasonal seroprevalence of *M. gallisepticum* antibody in chickens by SPA test

Season	Tested sera	Positive sera	Prevalence (%)	χ^2	P –value
Winter	460	285	61.96	8.594	0.003*
Summer	460	241	52.39		
Total	920	526	57.17		

*= Significant at 5% level of probability (P<0.05)

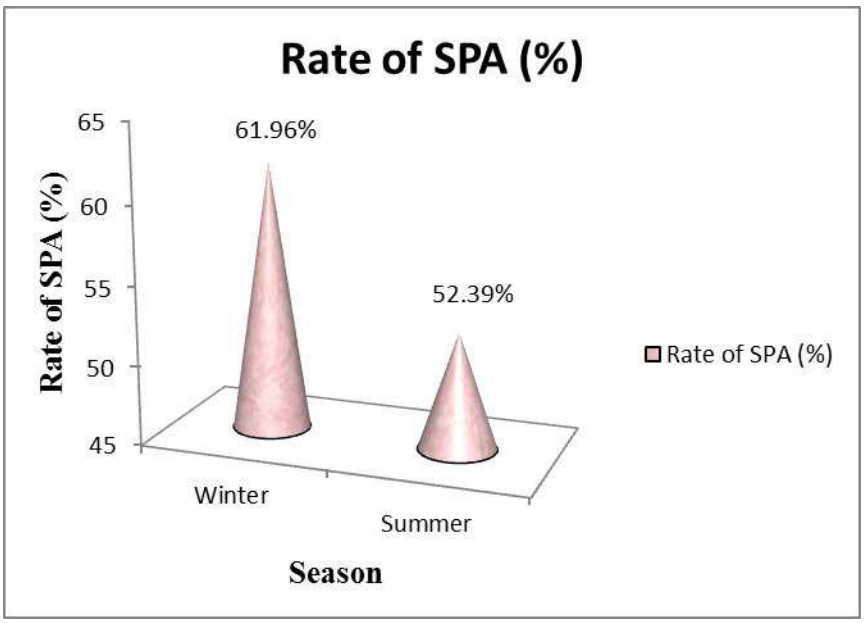


Figure 26: Seasonal seroprevalence of *M. gallisepticum* antibody in chickens by SPA test

Results showed that the prevalence of MG antibodies between two commercial layer breeds, sonali was shown higher (59.78%) and lower (56.52%) prevalence in Isa brown (Table 15).

Table 15: Seroprevalence of MG antibody in different breeds by SPA test

Breeds	Tested sera	Positive sera	Prevalence (%)	χ^2	P -value
Sonali	184	110	59.78	0.639	0.424 (NS)
Isa Brown	736	416	56.52		
Total	920	526	57.17		

NS means Not Significant

The prevalence of MG antibody showed higher in large flocks. The highest (61.96%) prevalence was found in flock size 2501- above birds and lowest (51.44%) prevalence was found in flock size 1000-1500 birds (Table 16).

Table 16: Seroprevalence of MG antibody in relation to flock size by SPA test.

Flock size (No. of birds/flock)	Tested sera	Positive sera	Prevalence (%)	χ^2	P-value
1000-1500	276	142	51.44	5.903	0.116 (NS)
1501-2000	368	215	58.42		
2001-2500	92	55	59.78		
2501-above	184	114	61.96		
Total	920	526	57.17		

NS means Not Significant

4.2.2 Determination of MG antibody screening by ELISA test

For diagnosis of MG antibody 920 sera tested by SPA test. SPA positive 526 sera again tested for detection of MG antibody through ELISA test and found 164 sera were seropositive. The overall prevalence was found 31.18%.The highest (52.35%) prevalence was found in group-4 and the lowest (17.12%) was in group-1 (Table 17).

Table 17: Screening of seropositive MG antibody by ELISA test

Groups	Tested sera	Positive sera	Prevalence (%)	χ^2	P – value
Group-1	111	19	17.12	46.782	0.00*
Group-2	130	29	22.31		
Group-3	136	38	27.94		
Group-4	149	78	52.35		
Total	526	164	31.18		

*= Significant at 5% level of probability (P<0.05)

4.2.3 Screening of MG antibody by HI test

In this test 80 representative samples from 164 ELISA positive sera were screened. The overall prevalence was found 18.75%.The highest (40%) prevalence was found in group-4 and the lowest (10%) was in group-1 (Table 18).

Table 18: Screening of seropositive MG antibody by HI test

Groups	Tested sera	Positive sera	Prevalence (%)	χ^2	P-value
Group-1	20	2	10	8.123	0.044*
Group-2	20	2	10		
Group-3	20	3	15		
Group-4	20	8	40		
Total	80	15	18.75		

*= Significant at 5% level of probability (P<0.05)

4.3 Post mortem examination

11.55% bird was suspected as a MG from sacrificed and dead birds. The identical lesions were catarrhal inflammation of sinuses, trachea and bronchi, congested and hepatized lung, cloudy and thickened air sacs contained mucous and caseous exudates, besides hyper plastic lymphoid follicles on the walls and hyperemic and mucoid trachea were observed. Fibrinous perihepatitis and pericarditis were also observed in suspected birds (Fig. 27).

4.4 Prevalence of *M.gallisepticum* isolates in different organs by cultural test

Mg was isolated after culturing in PPLO broth and agar. The PPLO broth colour was changed from red to orange and yellow with clear solution after 3 to 7 days of incubation and subcultured immediately for pure isolates. Sometimes the changing of colour from red to yellow with turbidity within 2 days of incubation was found which was indicated the medium was contaminated.

These results are based on visualization of typical 'fried egg' appearance of individual colonies (Plate 7) by observing agar plates under 10X objective lens of Stereo Microscope.

For isolation of MG in cultural method a total of 156 tissues (52 tracheal swabs from representative live birds and 52 lungs, 52 air sacs from post mortem examination) were

culture and overall results revealed only 9 tissues (5.77%) were shown 'fried egg' colony. The highest 7 (13.46%) positive isolates were detected from tracheal swabs and 1 (1.92%) positive isolate recovered from lung and 1 (1.92%) from air sacs. Results were significant (Table 20).

According to location basis the highest prevalence of MG were found in Dinajpur and Joypurhat and followed by Rangpur, Panchagarh. There were no significant variation according to location.

According to age variation highest 5 (3.20%) positive isolates recovered from group-4 (above 40wks) and followed by 2 isolates (1.28%) from group-3 (21-40wks), 1 (0.64%) isolate from group-2 (9-20wks) and 1 (0.64%) isolate from group-1 (0-8wks). It was also found 3 (1.92%) and 6 (3.85%) positive isolate in summer and winter season respectively (Table 21). Results were no significant in age and season.

Table 19: Identification of etiological agent load in different suspected tissues based on age, season and area differences by culture method

Area	Age (Groups)								Samples						Season				Total collected Samples	Total positive samples (%)
	G -1		G -2		G -3		G -4		TS		L		AS		Summer		Winter			
	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P		
Rangpur	9	0	9	0	9	1	9	1	12	2	12	0	12	0	18	1	18	1	36	**9 (5.77%)**
Dinajpur	7	0	7	0	7	1	9	2	10	2	10	0	10	1	15	1	15	2	30	
Thakurgaon	7	0	7	0	7	0	9	0	10	0	10	0	10	0	15	0	15	0	30	
Panchagarh	7	0	7	0	7	0	9	1	10	1	10	0	10	0	15	0	15	1	30	
Joypurhat	7	1	7	1	7	0	9	1	10	2	10	1	10	1	15	1	15	2	30	
Total	**37**	**1**	**37**	**1**	**37**	**2**	**45**	**5**	**52**	**7**	**52**	**1**	**52**	**1**	**78**	**3**	**78**	**6**	**156**	
χ^2-value	**3.651**								**8.490**						**1.061**					
P-value	**0.302**								**0.014***						**0.303**					

TS = Tracheal swab, L = Lung, AS = Air sac, T = Tested sample, P = Positive sample

G-1=Group-1 (0-8 wks), G-2=Group-2 (9-20 wks), G-3=Group-3 (21-40 wks), G-4=Group-4 (above 40 wks)

*= Significant at 5% level of probability (P<0.05)

Table 20: Tissue distribution pattern by culturing of MG from different tissue samples

Tissue used for cultural test	Culture			χ^2	P - value
	Tested Samples	Positive Samples	Percent of Positive Samples		
Tracheal swabs	52	7	13.46	8.490	0.014*
Lung	52	1	1.92		
Air sacs	52	1	1.92		
Total	156	9	5.77		

*= Significant at 5% level of probability (P<0.05)

4.5 Morphological and Biochemical characterization

In my findings 9 positive isolates were perform annular forms i.e. circular and elliptical pleuropneumonia like organism was characterized chiefly by the lack or thinness of stainable material in the central region of the organism. The protoplasm appeared to be concentrated at the periphery of the circular PPLO particle-evenly distributed in the form of a ring, or unevenly distributed, signet rings and monopolar forms and tubes containing glucose with positive colony agar were examined daily for biochemical characterization and showed that glucose was fermented indicated by color change (red to yellow).

4.6 Molecular detection of MG isolates

Conventional PCR method was done by 16s rRNA gene and two sets of MG primer were used in this study and successfully amplified 185 bp and 732 bp amplicons. In the present study, 156 tissues (Tracheal swab, lung and air sacs) were cultured in PPLO broth and agar. DNA was extracted from 9 positive culture isolates and directs from 48 different tissues (tracheal swabs, Lung and air sac without culture).

From PCR result, 7 (14.58%) positive bands showed successfully in 185bp which were 6 bands from direct 48 different tissues and 1 band from positive culture isolates (Fig. 28). It is also revealed that 6 (12.5%) positive bands showed successfully in 732bp from direct 48 different tissues and no band from positive culture isolates (Fig. 29).

It is illustrated that 25% prevalence of MG was found from tracheal swab and 6.25% from lung and air sacs in PCR from direct tracheal swabs in both primers.

4.7 Comparative prevalence of MG in Culture and PCR

In my findings, 156 tissues were cultured and only 5.77% tissues were found as a "fried egg" shaped colony which were suspected to MG and 12.5% prevalence of MG were found in PCR from 48 direct tissues (Table 21).

Table 21: Comparative study with Culture and PCR

Tested samples	**Culture**				**PCR (without culture)**			
	Tested samples	**Positive samples**	**%**	**Overall (%)**	**Tested samples**	**Positive samples**	**%**	**Overall (%)**
Tracheal swabs	52	7	13.46	5.77	16	4	25	12.5
Lung	52	1	1.92		16	1	6.25	
Air sacs	52	1	1.92		16	1	6.25	
Total	156	9	5.77		48	6	12.5	
χ^2	3.945							
P – value	0.047*							

*= Significant at 5% level of probability (P<0.05)

4.8 Comparative prevalence of MG isolates

Different test were performed for isolation, identification and characterization of MG from field samples and found 5.77%, 57.17%, 31.18%, 18.75% and 12.5% by culture, SPA, iELISA, HI and PCR test respectively (Table 22).

Table 22: Comparative prevalence of MG isolates in different test

	SPA	iELISA	HI	Culture	PCR
No.of tested sera	920	526	80	156	48
No.of positive sera	526	164	15	9	6
Positive percent (%)	57.17	31.18	18.75	5.77	12.5
χ^2	**231.696**				
P – value	0.000*				

*= Significant at 5% level of probability (P<0.05)

4.9 Sequencing of positive DNA

Sequences of *16S* rRNA gene from MG strains found in other countries were downloaded from GenBank. Sequences were edited to have the same start and end nucleotide sequences and were then aligned with respective sequences of the present study.

The 16S rRNA gene of uncultured MG sequences of the present study showed 99 to 100% per cent similarity with the sequence of the duck isolate from South Africa (AccessionNo. LN811535.1), chicken isolate of Spain (KC995374.1) and USA (Accession No.NR025912.1 and MF196178.1) as demonstrated by blast (NCBI).

Plate 5: Swollen face with nasal and ocular discharge and open mouth breathing

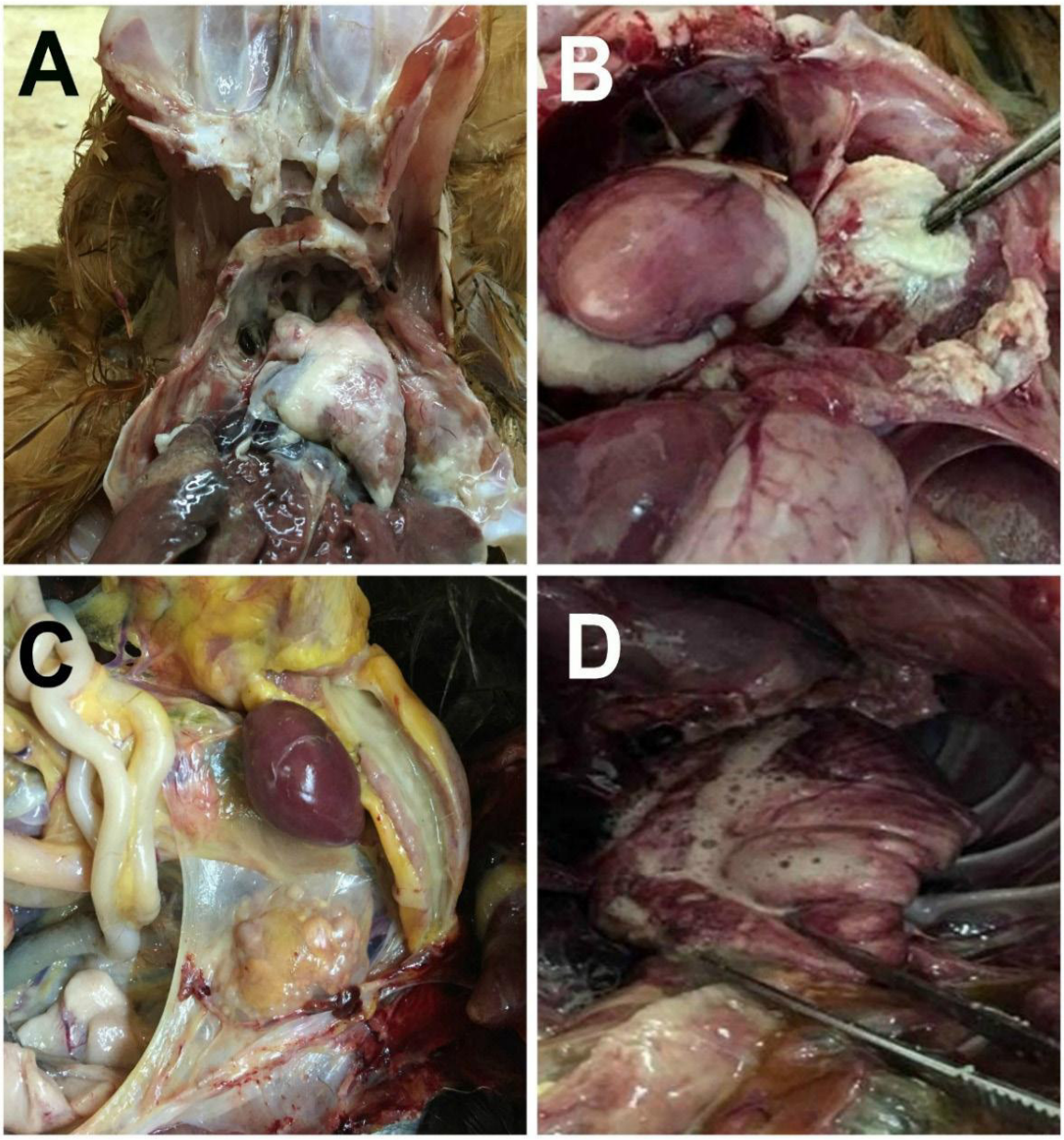


Figure 27: Postmortem findings (A: Heart covered with fibrinopurulent covering, while liver was congested and perihepatitis. B and C: Caseous materials were found in the bronchi and pneumonic areas in the lungs were observed. Air sacculitis was observed in air sacs which are covered with caseous exudates complicated infections. D: Lungs were dark red color appearance and showed congestion, Frothy exudate was present in some cases.)

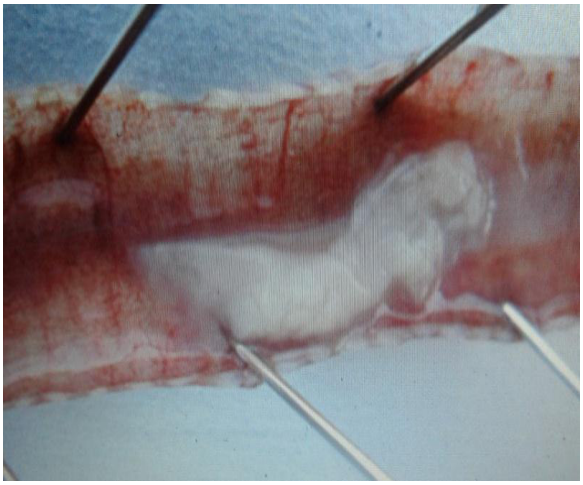


Plate 6: Cloudy material and congested trachea

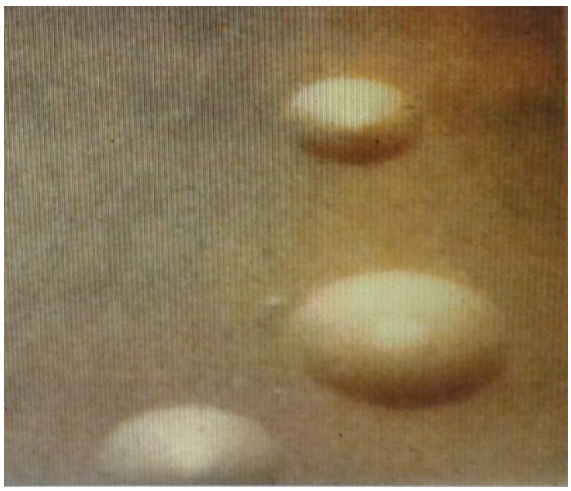


Plate 7: “Fried egg colony” in PPLO agar

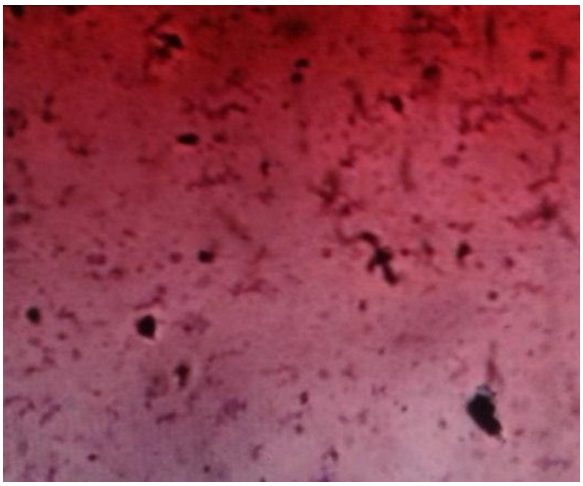


Plate 8: Giemsa staining

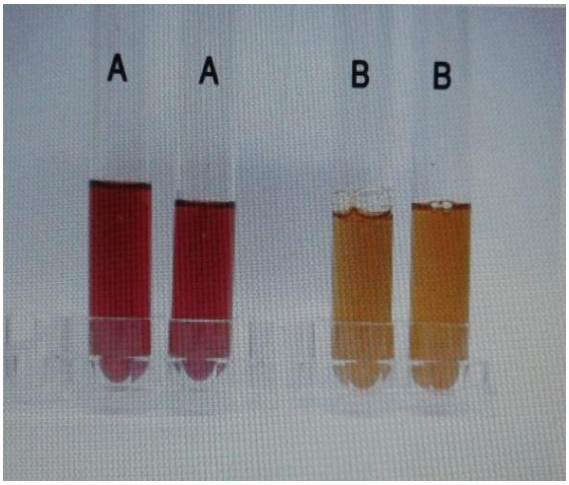


Plate 9: Broth media before (A) and after (B) growth of MG

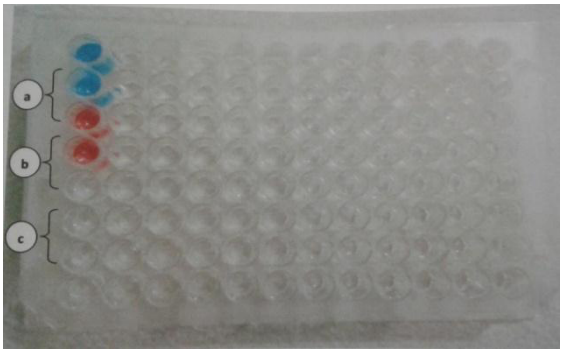


Plate 10: Diluted serum, negative control (a), positive control (b) and reference control (c) taken on MG coated plate

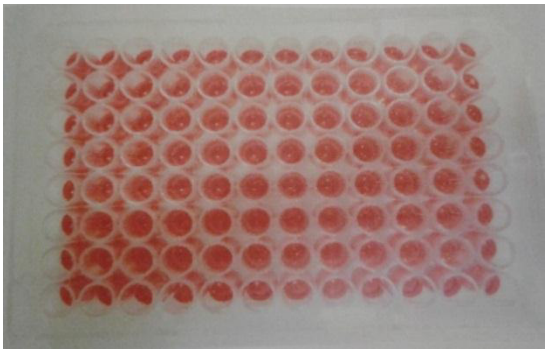


Plate 11: Conjugate reagent containing MG coated plate

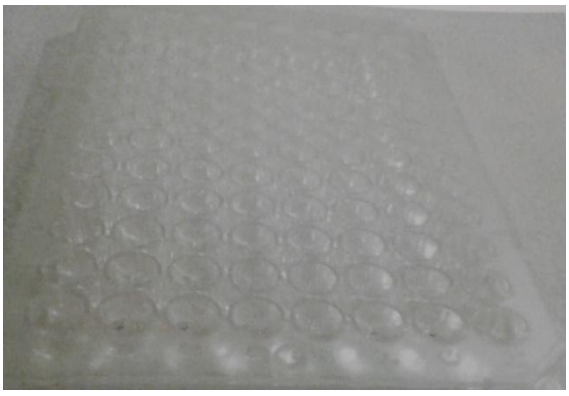


Plate 12: Colorless substrate reagent containing MG coated plate

Plate 13: Yellow colour develops in positive case

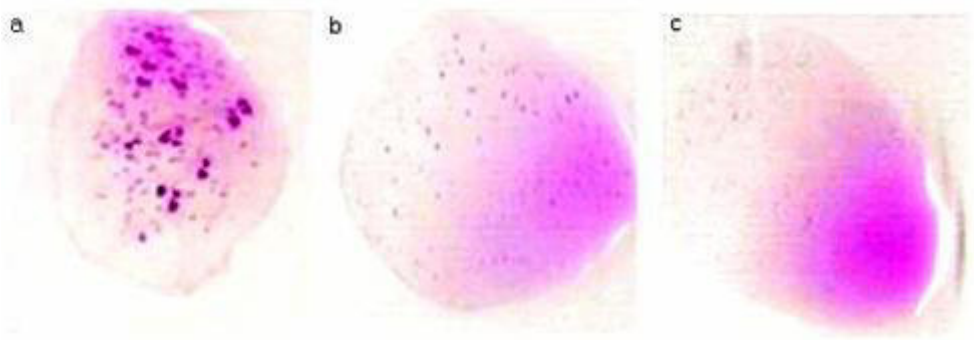


Plate 14: Rapid slide agglutination test. (a) Positive agglutination, (b) suspect and (c) negative agglutination.

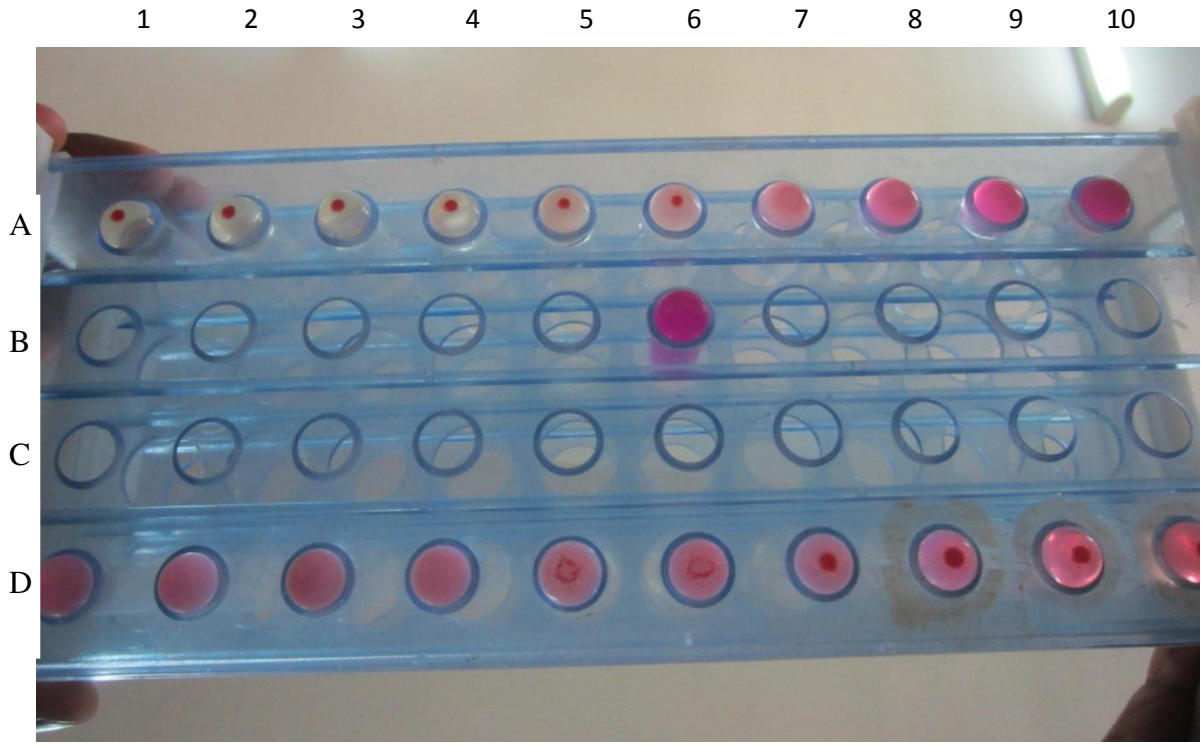


Plate 15: Determination of HI titer

A= HI, D= HA

Lane A7-A10= Complete Haemagglutination Inhibition

Lane D7-D10= Complete Haemagglutination

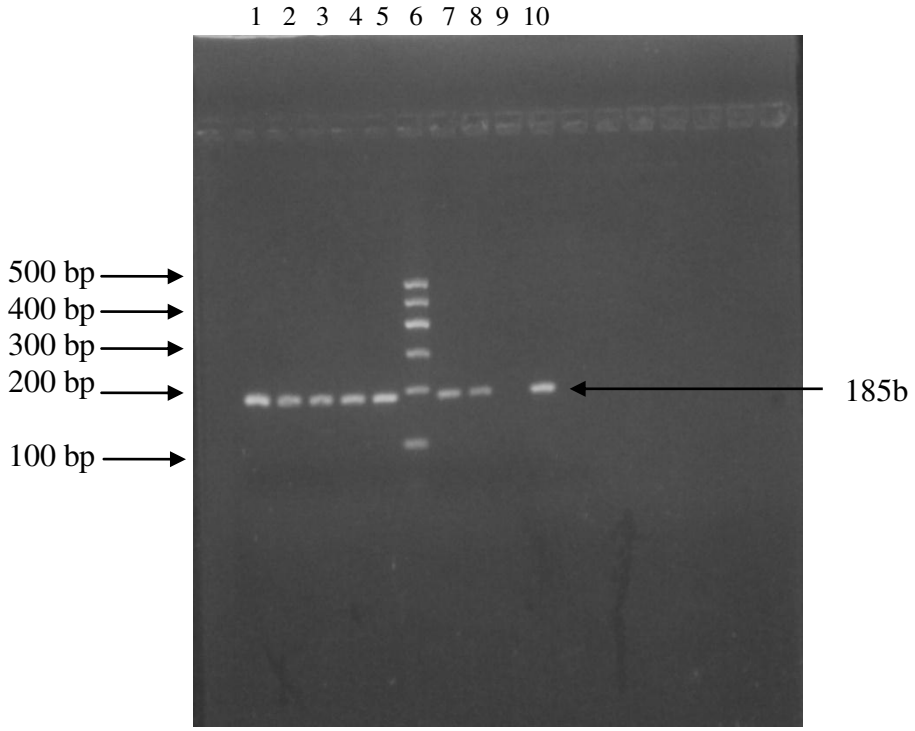


Figure 28: Electrophoresis analysis (2% agarose gel) of PCR product of 185bp: 1-4 (Tracheal swab, without culture), Lane 5(Tracheal swab, from culture isolates), Lane 6 = 100 bp DNA marker, 7 and 8 (Air sac and lung without culture) and Lane 9 = Negative control, Lane 10 = Positive control.

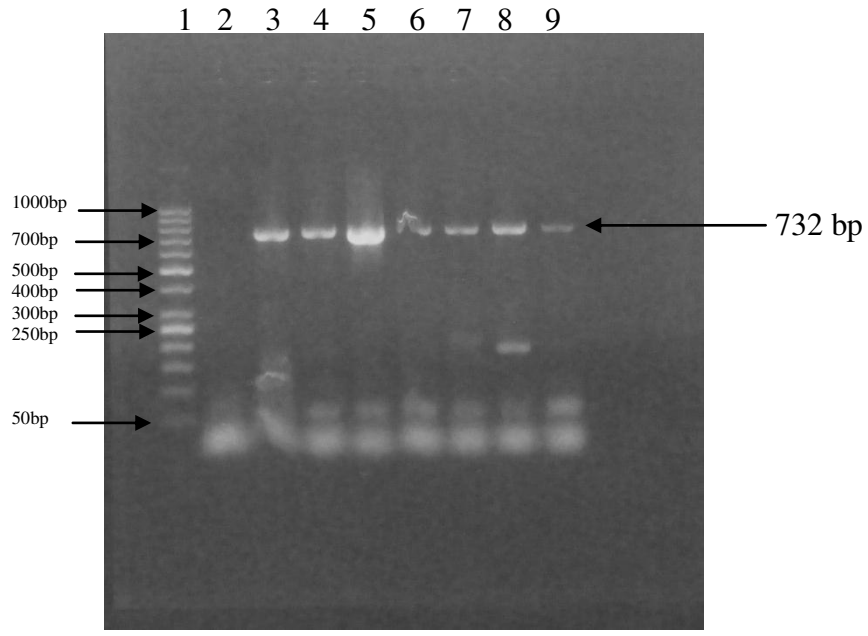


Figure 29: Electrophoresis analysis (1.5% agarose gels) of PCR products of 732bp, Lane 1 = 50 bp DNA marker, Lane 2 = Negative control, Lane 3 = Positive control, Lanes 4,5,6,7 (Tracheal swab, without culture), 8, 9 (Air sac, lung without culture).

```
LN811535.1        GAGCTAATCTGTAAAGT
KC995374.1        GAGCTAATCTGTAAAGT
NR_025912.1       GAGCTAATCTGTAAAGT
185bp             GAGCTAATCTGTAAAGT
MF196178.1        GAGCTAATCTGTAAAGT
                  *****************

LN811535.1        TGGTCTCAGTTCGGATTGAGGGCTGCAATTCGCCCTCATGAAGTCGGAATCACTAGTAAT
KC995374.1        TGGTCTCAGTTCGGATTGAGGGCTGCAATTCGCCCTCATGAAGTCGGAATCACTAGTAAT
NR_025912.1       TGGTCTCAGTTCGGATTGAGGGCTGCAATTCGCCCTCATGAAGTCGGAATCACTAGTAAT
185bp             TGGTCTCAGTTCGGATTGAGGGCTGCAATTCGCCCTCATGAAGTCGGAATCACTAGTAAT
MF196178.1        TGGTCTCAGTTCGGATTGAGGGCTGCAATTCGCCCTCATGAAGTCGGAATCACTAGTAAT
                  ************************************************************

LN811535.1        CGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAA
KC995374.1        CGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAA
NR_025912.1       CGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAA
185bp             CGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAA
MF196178.1        CGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAA
                  ************************************************************

LN811535.1        CTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGCGCATGTCTAGG
KC995374.1        CTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGCGCATGTCTAGG
NR_025912.1       CTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGCGCATGTCTAGG
185bp             CTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGCAAACGGTTTTA
MF196178.1        CTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAAGGAAGCGCATGTCTAGG
                  *************************************************..* *  *: .
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Figure 30: Result of DNA sequenceing

CHAPTER 5

DISCUSSION

The poultry meat alone contributes 37% of the total meat production in Bangladesh (Begum *et al*., 2011) and poultry contributes significantly to the welfare of the people both at household and national level. The poultry industry represents one way of accomplishing several national goals under a single banner. Employment, poverty alleviation and improved nutrition are all potential benefits from continued support and encouragement of poultry development. Despite its high growth rate, poultry sector is facing a lot of problems in terms of infectious diseases; among them respiratory tract infections has its major role.

Therefore, rapid, accurate, precise and sensitive detection of such respiratory pathogens which include *Mycoplasma* spp. as one of the important pathogen is essential for control of the outbreaks.

The study entitled as serological and molecular charactarization of *Mycoplasma gallisepticum* organism from layer poultry in northern part of Bangladesh was selected with the main objectives covering study of MG antibody using serological (SPA, iELISA and HI) test and isolation and characterization by cultural and molecular techniques.

MG is the most pathogenic and economically significant throughout the world (Raviv and Ley, 2013). Cell shape being spherical, pear shaped, spiral and filamentous forms. The size of spherical or coccoid form ranges from 0.25 to 0.45μm. They are extremely pleomorphic, small prokaryotic, have a single trilaminar membrane. MG infections are known as “chronic respiratory disease” (CRD) of chickens. Respiratory rales, coughing, nasal discharge, swollen sinuses, suborbital swelling and conjunctivitis are the clinical manifestations of chickens caused by MG (Ley 2003, Saif *et al*., 2003). MG is spread only short distances by the air borne route. Reduced feed consumption and loss of body weight are the most characteristic signs of naturally-occurring MG disease in adult flocks (Kleven and Noel, 2008; Ley, 2008). MG is transmitted vertically within some eggs (transovarian) from infected breeders to progeny (Kleven, 1998; Bradbury, 2001; OIE, 2008) and play a significant role in the epidemiology (Armour and Ferguson-Noel, 2015; Raviv and Ley, 2013). Horizontally via infectious aerosols and through contamination of feed, water, and

the environment and by human activity on fomites (shoes, equipment, etc) (Nascimento *et al*., 2005; Pitcher and Nicholas, 2005; Dhondt *et al*., 2007 and Razin and Hayflick, 2010).

The infected birds showed high morbidity, low mortality (5%), weight loss (22-30%), feed conversion ratio (11-20%), egg production and hatchability and increased condemnation of carcass at processing plants (10-20%) (Steinlage *et al*., 2003; Evans *et al*., 2005; Nascimento *et al*., 2005; Shankar, 2008). In breeders and layers, the disease causes tremendous drop in egg production and an increase in embryo mortality (Ley, 2003; Branton and Deaton, 2005; Mohammed *et al*., 2005). Production losses between 10 and 20% in layer cases (Bradbury, 2001). In severe cases, mortality may go as high as 30% (Donald *et al*., 1990; Ley 2003).

This disease causes vast economical losses of poultry industry not only Bangladesh, also in many other country of the world. In Bangladesh information is rare about isolation and characterization of MG diagnosis which is constant threat for rising poultry industry and it is control by antimicrobial drugs and also by the imported vaccine. The uses of drugs which results drug resistant. To prevent the economic losses of poultry industry by MG control, it is necessary to isolate and characterize of etiological agent.

The success of MG control programs depends on accurate and fast techniques for diagnosis. The present study covering for MG diagnosis were various techniques using necropsy to observe gross and microscopic lesions. There were three approaches to diagnosis of MG which were; isolation and identification of the organism using cultural, morphological, biochemical test, serology to determine the specific antibodies including SPA, ELISA and HI tests and molecular characterization by PCR test in layer poultry in northern part of Bangladesh.

In the present study total 18,350 birds were observed in ten selected farm at the northern part of Bangladesh based on age, breed, location four different ages. For epidemiological investigation, information received from farmers by using a set questionnaire and also observed clinical signs and symptoms and post mortem examination. In association with the epidemiological investigation, the overall incidence rate was 11.54%. The birds were observed nasal discharge, coughing, swelling of the facial skin and eyelids, increased lacrimation and reduced feed consumption, dull, depressed with ruffled feather, open mouth breathing. This present findings supported by Ley and yoder, (1997); Ley (2003); Saif *et al.* (2003); Haque *et al.* (2015); Kamrul *et al.* (2016); Talukdar *et al.* (2017).

Reduced feed consumption and loss of body weight were found in flocks. These findings also supported by Kleven and Noel, (2008); Ley (2008).

There was reduced egg production in laying flocks supported by Bradbury, (2001); Branton and Deaton (2005); Mohammed *et al.* (2005).

During present study the overall prevalence of MG in layer was 11.54% based on post mortem examination. This finding was similar to the observations of earlier workers, who reported 11.55% in Mymensingh sadar (Talha *et al*., 2001), 11.66% in Sylhet division (Badruzzaman *et al*., 2015) and 11.5% in India (Susitha *et al.*, 2017) and partially lower than 12.79% in Bogar sadar (Talukdar *et al*., 2017), 12.84% in Lahore, Pakistan (Sultana *et al*., 2012),

But prevalence of the present findings is higher than previous study, reported 8.11% in Bangladesh (Giasuddin *et al*., 2002), 5.32% in Sylhet (Islam *et al*., 2003), 9.87% in Norsindhi (Uddin *et al*., 2010), 9.45% in India (Rajkumar *et al*., 2018). It may be due to geographical distribution and cold environmental condition. MY Findings is lower, 15.38% in Chittagong (Haque *et al*., 2015), 14.7% in Gazipur (Kamrul *et al*., 2016). It may be due to proper housing, feeding and hygienic environment.

In my post-mortem findings there was serious involvement of trachea, lungs, air sacs, heart and liver which was supported by Saif *et al.* (2003) and showed cloudy and air sacculitis in the air sac, pericarditis, congested and mucoid trachea and lung revealed dark red color and congested. The color of the liver was slightly changed to pale and fibrinopurulent covering perihepatitis was observed.The results were close agreement with Haque *et al.* (2015); Kamrul *et al.* (2016); Talukdar *et al.* (2017).

In present finding, epidemiological investigation as per location variation the incidence of MG was recorded highest (12.73%) in Joypurhat and lowest (9.97%) in Panchagarh comparing with other districts and age wise prevalence was increased with the increase of age. Age wise variation highest (11.54%) incidence in above 40 wks in comparison lowest (1.22%) in 0-8 wks. This is supported by Kamrul *et al.* (2016) who reported, highest (32%) in above 20wks and lowest (2%) in 0-8wks in Gazipur district. Badruzzaman *et al.* (2015) also supported and revealed highest (6.6%) in above 60 days and lowest (0.8%) in 8-20 days.

There were some contradictory reports by Islam *et al.* (2003) who reported highest (3.92%) in 8-21 days, lowest (0.00%) in above 36 days. Susitha *et al.* (2017) found the incidence was 6.62, 18.52 and 9.25% in chicks, growers and adults respectively. Uddin *et al.* (2010) reported highest (5.37%) in 8-20 days and lowest (0.32%) in above 60 days. It is due vertical transmission.

Seasonal investigation in my epidemiological report was 8.65% in winter and 2.88% in summer season and supported by Islam *et al.* (2003) and Uddin *et al.* (2010).

It is also recorded highest 12.73% incidence occur in Sonali breed compared to Isabrown 11.84%. It is supported by Talukdar *et al.* (2017) who observed epidemiological prevalence of MG was 12.79% in Sonali breed.

In the present study serological evidence of MG in the respiratory distress was recorded from 920 sera using Serum Plate Agglutination (SPA) test and overall seroprevalence of MG was found 57.17%. This finding is in line with some other previous researchers who reported 57.15% in 6 divisions of Bangladesh (Prodhan, 2000), 58.90% in Fani (Sarker *et al*., 2005), 55.13% in Rajshahi (Hossain *et al*., 2007), 59.1% in Ghana (Ayim *et al*., 2012), 55.83% in Bhola (Mahfuzul *et al*., 2014), 56.13% in Bogra (Ali *et al*., 2015).

The result is lower than the previous researchers who reported 63%, 81.15% and 61.11%, 62%, 67.4% 73% and 60% in South Africa, Batna Districts of Estern Algeria, Benin, Khulna, Lohagara and Satkania Upazilla in Chittagong district by Thekisoe *et al.* (2003), Heleili *et al.* (2011) and Nouzha *et al.* (2013), Chrysostome *et al.* (1995), Jalil and Islam, (2010), Barua *et al.* (2006). This variation might be due to in nature of poultry farming, operational practices and good biosecurity measures (Prodhan, 2002; Dulali, 2003).

The findings were higher than previous reporters who reported 49.38%, 46.88%, 30%, 17.3%, 51.6%, 3.4%, 48%, 12.4%, 27% and 47.17% in Pakistan, Dhaka and Gazipur, Patuakhali, Paraguay, Azerbaijan, Libya, Turkey, Kotwali thana of Chittagong and Chittagong by Mukhter *et al.* (2012); Sikder *et al.* (2005); Herrero *et al.* (2009); Feizi *et al.* (2012); Lamya *et al.* (2012); Elegnay *et al.* (2013); Serpil *et al.* (2015); Saleque *et al.* (2003); Hauqe *et al.* (2015) and Islam *et al.* (2015). The higher prevalence might be due to the replacement of breeding stock with the progeny of the same flock. However, intensive nature of poultry farming provided opportunity for recycling of the pathogens due to population density (Prodhan, 2002).The other factors that contribute MG infection This

may be due to poor ventilation, contamination of litters and no restriction on the movement of the technical personnel, visitors and such other persons as well as other bio-security measures (Dulali, 2003).

The highest incidence rate (61.96%) was found in Dinajpur and followed by 59.78% in Joypurhat, 57.61% in panchagorh, 55.43% in Thakurgaon and 51.09% in Rangpur district respectively.the lowest (51.09%) at Rangpur in comparison to other districts.

In my findings, highest (64.8%) incidence was found above 40 weeks followed by 59.30% at 21-40 weeks, 56.52% at 8-20 weeks and 48.26% at 0-8 weeks (Table--) and results supported by Rajkumar *et al.* (2018), Islam *et al.* (2015), Ayim *et al.* (2012), Heleili *et al.* (2011) and Jalil *et al.* (2010) who reported MG infection was significantly higher in older birds than younger birds.

However, there were some contrary reports of earlier by Mahfuzul *et al.* (2014), Mukhtar *et al.* (2012), Uddin *et al.* (2010), Hossain *et al*. (2007), Sarkar *et al*. (2005), Sikder *et al.* (2005) who reported the prevalence was found to have decreased with the increase of age. Highest infection in the young chickens is probably due to the vertical transmission of the organisms.

We use SPA test based on the suggestion of Kleven and Bradbury, (2008), SPA test is quick, relatively inexpensive and sensitive and can be widely used as an initial screening test for flock monitoring and serodiagnosis. Comparison with the other serological tests, SPA is more sensitive than Elisa and HI, but less specific than them (Ley, 2008). However, the SPA test is prone to false positive results and non-specific reactions (Avakian *et al*., 1993; Osman *et al*., 2009).

In this finding result showed the highest (61.96%) prevalence of MG infection in winter season as compared to summer season (52.39%) and results were agreement with Zulfekar *et al.* (2015); Mahfuzul *et al.* (2014); Jalil *et al.* (2010); Hossain *et al.* (2010); Hossain *et al.* (2007); Sarker *et al.* (2005) and Sikder *et al.* (2005) in Bangladesh and in abroad with Vadivalagan *et al.* (2018) in Tamil Nado, India, Rachida *et al.* (2013) and Heleili *et al.* (2011) in Eastern Algeria and Mukter *et al.* (2012) in Faisalabad of Pakistan, who reported higher prevalence of MG infection occur during winter than in summer season. This seasonal variation in infection might be due to the sudden change in temperature and cold stress on the birds.

In my research the prevalence of MG antibody was found higher (59.78%) in Sonali breed than 56.52% in Isa brown breed. It is supported by Zulfekar *et al.* (2015).

In the present study the prevalence of MG antibody according to flock size was higher in larger flock size. The highest (61.96%) prevalence rate was recorded in flocks having high bird density (above 2500 birds) and lowest prevalence was shown in flock size 1000-1500 that was 51.44 % which is strongly supported by the previous investigations of Zulfekar *et al.* (2015); Ayim *et al.* (2012); Mukhtar *et al.* (2012); Hossain *et al.* (2010) and Hossain *et al.* (2007).

Highest infection rate in larger flocks might be correlated with poor ventilation, contamination of litter and no restriction on the movement of technical staff and visitors from one farm to the other as well as lack of basic bio-security measures.

SPA test is a simple, quick and inexpensive test for the screening of MG antibodies in serum. The test has efficient sensitivity but of low specificity (Vadivalagan *et al.,* 2016).

For detection of MG antibodies, 526 SPA positive sera were tested by iELISA test and found 164 sera were seropositive with the incidence rate of 31.18% (Table --) and are highly significant. The present findings were in close agreement with the previous results of 32% in Chittagong (Islam *et al*., 2015), 32.6% in India (Rajkumar *et al*., 2018).

In my findings result is slightly higher than others 26% of backyard chickens in Paraguay (Herrero *et al*., 2009), 21.40% in district Rewa, Madhya Pradesh (Namrata *et al*., 2016), 20% of backyard chickens in West Indies (Jordan *et al*., 2018). A lower level of detection is expected in backyard compared to intensively kept poultry, as multiple investigations have reported that smaller less densely populated flocks have lower levels of infection than larger more densely populated commercial flocks (Zulfekar *et al*., 2015).

The results of this study is lower than other previous researchers reported in Poland, Jordan, Italy, Egypt, India, Nigeria, Saudi Arabia, Tamil Nadu, Mozambique prevalence of MG 65.2%, 73.5%, 60%, 45.1%, 48.3%, 54.4%, 91.83% 53.88%, 53.40%, 48.8% by iELISA respectively in commercial layers by (Alina *et al*., 2000; Saad and Dirgham, 2008; Osman *et al*.2009; Lamyaa *et al*., 2012; Reddy, 2014; Ahmed *et al*., 2015; Ayman *et al*., 2016; Shanmugasundaram *et al*., 2016; Augusto *et al*., 2017).This may be due to quarantine and health strategy and elevation of farmer's knowledge about biosecurity.

In this study MG specific antibodies were detected 57.17% and 31.18% by SPA and iELISA test respectively which were consistent by the previous investigations of Lamyaa *et al.* (2012) reported MG antibodies were found 51.6% and 48.3% by SPA and iELISA test in Egypt. These results also supported by Osman *et al.* (2009) who revealed that MG specific antibodies were found 69.9% and 58.3% by SPA and iELISA test in Egypt. Feizi *et al.* (2013) reported that 42.22% in RSA, while 33.33% were positive in ELISA test.

But some contrary with other author Ayman *et al.* (2016) identified 46.11% and 53.88% by SPA and iELISA test respectively in Saudi Arabia. Zulfekar *et al*., 2015 stated 56.13% and 64.47% in SPA and iELISA test respectively in Bogra district.

SPA is a screening test and is prone to false positive reactions. This test is quick, relatively inexpensive and highly sensitive (Talha, 2003; OIE, 2012). But the iELISA test gave better results and exhibited higher sensitivity than the SPA test for the detection of specific MG antibodies. These findings also agreed with those recorded in another study Reda *et al.* (2012).

In my present findings 57.17%, 31.18% and 18.75% MG specific antibody were found by SPA, ELISA and HI test. Results supported by Luciano *et al.* (2011). These diagnostic methods (SPA, HI and ELISA) should be only used as screening tools in monitoring programs to detect avain mycoplasmosis in poultry flocks. Positive results should be confirmed by isolation using traditional microbiological methods or biomolecular assays (PCR).

On the other hand, the hemagglutination-inhibition (HI) is more specific compared to the SPA test, but some obstacles hinder its wide usage like the laboriousness, time consumption, and unavailability of reagents needed on the commercial scale (Kleven, 2008b; Kleven *et al*., 1988). The main disadvantages of the HI test are the inability to detect antibodies of variant MG strains and the low sensitivity as it might not detect antibodies before 3 weeks post infection. This is because the HI test detects the lately produced immunoglobulins, the IgG antibodies (Talkington and Kleven, 1983).

Haemagglutination Inhibition test and ELISA are most common techniques for screening of MG and status of immunity (Purswell *et al.,* 2012). However, chances of non-specific results are more. Serological techniques have disadvantage of cross reactivity between MG and MS isolates (Kleven *et al.*, 2000).

For isolation of MG in this research work was using PPLO medium and suitable incubation conditions. Several researchers have been employed PPLO medium for isolation and evaluated for their suitability growth of MG by Hanif and Najeeb (2007); Khalda *et al.* (2013); Jalaladdini *et al.* (2014); Ahmed *et al.* (2015); Jafar *et al.* (2015) and Malekhoseini *et al.* (2017).

Mg was isolated after cultuting in PPLO broth and agar. The PPLO broth colour was changed from red to orange and yellow with clear solution after 3 to 7 days of incubation indicated that the PPLO medium was suitable for isolation of mycoplasma and subcultured immediately into PPLO agar for pure isolates. Sometimes the changing of colour from red to yellow with turbidity within 2 days of incubation was found which was indicated the medium was contaminated. This finding is in accordance with Jalal addini *et al.* (2014), who observed turbidity in PPLO culture, indicating bacterial growth.

In my findings, overall result revealed only 9 tissues (5.77%) as culture positive out of 156 different tissues. It is slightly higher than 4.11%, 2.09% and 3.75% respectively by the findings of Khalda *et al.* (2013), Helilie *et al.* (2011) and Marois *et al.* (2002). The findings of our results is lower than 9%, 27.6%, 22.83% and 14% by Atif and Najeeb (2007), Rauf *et al.* (2013), Ahmed *et al*. (2015) and Jaffer *et al.* (2015). It can be speculated, that under field conditions, the reasons for low percentage of MG on isolation might be due to low prevalence of Chronic Respiratory Disease and environmental conditions in that particular area or when birds harbor other mycoplasmas or bacteria that hamper the isolation of MG, the cultural confirmation of such infections may be very difficult to achieve and may necessitate a high number of samples or alternative methods.

In my study MG recovered from different tissues which were highest 7 (13.46%) positive isolates were detected from 52 tracheal swabs followed 1 (1.92%) from 52 air sacs and 1 (1.92%) from 52 lung (Table 20) and results were significant. The results of mycoplasma isolation from different organs showed that tracheal swab is the main site of multiplication of this microorganism. It is supported by Gondal *et al.* (2015), Rauf *et al.* (2013) and Osman *et al.* (2009) who reported results regarding isolation of MG through traditional culture techniques depicts that upper respiratory tract is more prone to infection. The highest occurrence of this bacterium in tracheal swab might be attributed to a factor that it is the first organ of respiratory tract which is exposed to the infectious agent (Nascimento *et al.,* 2005). Localization of this bacterium is supported by ciliated epithelium of trachea.

Local environment supports its growth and propagation than any other organ of the respiratory system.

The findings of present study that isolation of the organism by cultivation is difficult because MG is slow-growing, relatively fastidious organisms. Culture techniques are laborious, slow and expensive and require sterile conditions and problems are overgrowth of faster-growing mycoplasma species and other organisms or no growth in subcultures and can take up to four weeks and even then, the result can be negative or be hampered by mixed infections. It is supported by Garcia *et al.* (2005), Evans *et al.* (2009) and Ferguson-Noel and Williams, (2015). Culturing of *Mycoplasma* could not isolate organism from chronic cases and medicated birds as MG concentration low in those conditions (Hyman *et al*., 1989).

In the present study, all 9 positive isolated organisms were characterized by Giemsa stain which was Gram negative, circular /coccoid shape. This observation revealed that the isolated organism was MG and consistent as reported by Bukte Swati Ramrao (2015).
Tubes containing positive colony agar fermented glucose was indicated by color changed (red to yellow) due to the pH indicator and similar with the findings of Bukte Swati Ramrao (2015) and Legesse Bekele (2015).

Results showed in my study 57.17%, 31.18% and 5.77% by SPA and ELISA and cultural test and results supported by Lamyaa and El-Samie (2012), Osman and coworkers (2009).

In the present study, MG specific 16S rRNA gene was amplified for detection of MG in clinical specimens. Several studies have been reported that detection of 16S rRNA gene of MG by PCR (Rasoulinezhad *et al*., 2017; Zakeri, 2016; Rauf *et al*., 2013; Hess *et al*., 2007; Garcie *et al*., 2005 and Lauerman, 1998).

The 16S rRNA gene of MG was successfully amplified by PCR reactions and the amplicon size was 185 bp, 732bp and supported by Rasoulinezhad *et al.* (2017), Hassan *et al.* (2014), Rauf *et al.* (2013).

The PCR test was functionalized into two approaches by direct PCR on tissues without culture and from culture positive isolates. The recovery rate by PCR was 14.58% (7 bands) which were 12.5% (6 bands) from direct tissues and 0.64% (1 band) from culture positive isolates in185bp and 12.5% (6 bands) showed successfully in 732bp from direct 48 different tissues and no band from positive culture isolates. It is supported by Khalda *et*

al. (2013) and Atique *et al.* (2017). Whereas Ramadass *et al.* (2006) reported lower (3.4%) prevalence in India and 4% (Michiels *et al*., 2016) in Belgium. This low prevalence can be due to difference in sampling strategy, surveillance program and season of sample collection. In Belgium there is reduction of vertical transmission due to the mandatory surveillance program in breeder poultry flocks that aims to prevent and control spread from breeder hens to their offspring and protect national and international trade markets (Michiels *et al*., 2016).

The tissue distribution for MG to some extent varied between different respiratory tissues. The highest percentage of MG for characterization by PCR from direct tissue were from tracheal swab followed by air sacs and lung which were 25% (4 band), 6.25% (1 band), 6.25% (1band) respectively and one band (1.92%) from tracheal swabs of culture positive isolate in 185bp and 25% (4 band), 6.25% (1 band), 6.25% (1band) respectively in 732bp and no band from culture positive isolates. The results of resent study for MG are in contrast to Ramadass *et al.* (2006), Rauf *et al.* (2013), Gondal *et al.* (2015) and Jafar *et al.* (2015), Hossam *et al.* (2016) and had documented that tracheal swab samples gave more isolations than from lung and air sac.

Results regarding characterization of MG through PCR depicts that upper respiratory tract is more prone to infection. The highest occurrence of this bacterium in trachea might be attributed to a factor that it is the first organ of respiratory tract which is exposed to the infectious agent (Nascimento *et al.,* 2005). Localization of this bacterium is supported by ciliated epithelium of trachea. Local environment supports its growth and propagation than any other organ of the respiratory system.

In my research findings 5.77% MG were isolated by culture and 12.5% were characterized from direct tissue without culture by PCR.

From my research work it is revealed that for detection of MG infection, PCR assay is a good, more sensitive and requires lesser time to offer result when compared to the tedious culture method. It is supported by (Marois *et al.,* 2002). Samples collected from suspected ailing birds yielded more positivity by PCR than compared to samples from dead birds. This might be due to contamination of tissues easily by secondary bacteria after death. This finding is agreement with Thilagavathi *et al.* (2017).

This study showed that MG could be detected from field samples directly without need of culturing of this fastidious organism and supported by McAuliffe *et al.* (2005). Samples collected from medicated birds were PCR positive (Stanely *et al.,* 2001; Finklin and Kleven, 2006). In agreement to present findings it is declare PCR assay is a good, specific, more sensitive tool and require lesser time for early identification of MG from infected birds and supported by Liu *et al.* (2001); Kleven *et al.* (2004); Barbour *et al.* (2005); Lysnyansky *et al.* (2005); Lierz *et al.* (2008); Raviv *et al.* (2007); Ghorashi *et al.* (2010); Nazarpak 2010). PCR based nucleic acid detection is considered as an alternative method to that of conventional isolation technique and supported by Feberwee *et al.* (2005); Ferguson *et al.* (2005); Callison *et al.* (2006); Hess *et al.* (2007); Raviv *et al.* (2007) and Evans and Leigh, (2008).

The 16S rRNA gene of uncultured MG sequences of the present study showed 99 to 100% per cent similarity with the sequence of the duck isolate from South Africa (AccessionNo. LN811535.1), chicken isolate of Spain (KC995374.1) and USA (Accession No.NR025912.1 and MF196178.1) as demonstrated by blast (NCBI).

In relation to this study further studies warranted as follows

- Further studies are needed to test the comparative efficacy, sensitivity and specificity of such PCR separately on different tissues, which will allow rapid and specific diagnosis of this infection which would subsequently help in its control.

- Further study should be conducted with larger sample size and geographic coverage to elucidate to role of *M.galliseptocum* in animals.
- A more dynamic detection method based on multiplex PCR, analysis can be developed to differentiate MG field strain.
- The researcher will be sent these isolates for serotyping using modern technique such as sequencing for strain identification near feature.
- A more efficacious vaccine may also be developed to control MG in Bangladesh, based on the identified characteristics of MG isolates in Bangladesh.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The present research work was performed with the main objective of serological and molecular characterization of *Mycoplasma gallisepticum* organism from field samples in northern part of Bangladesh covering with epidemiological investigation (Questionnaire survey, sign and symptom, postmortem lesions), SPA, ELISA, HI, cultural, morphological, biochemical and molecular test in the Molecular Biology and Bacteriology laboratories of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur and in the Laboratory of National Institute of Biotechnology (NIB) Savar, Dhaka, Bangladesh.

The commercial layer poultry flocks which were observed and obtained samples from northern five districts of Bangladesh namely Rangpur, Dinajpur, Thakurgaon, Panchagorh and Joypurhat. A total of 10 (ten) farms were selected where 2 (two) farms were from each district for this entire study. Each flock of birds was divided into 4 groups based on their ages- Group-1 (0-8 weeks), Group-2 (9-20 weeks), Group-3 (21-40 weeks), Group-4 (above 40 weeks). Eight flocks of commercial layer of Isa Brown and two flocks of Sonali cross breed were reared separately in cage system.

In association with the epidemiological investigation, the overall incidence rate was 11.54% based on sign and symptoms and post mortem examination. In this study, the birds of infected flock had observed some clinical signs such as nasal discharge, coughing, swelling of the facial skin and eyelids, increased lacrimation and reduced feed consumption, dull, depressed with ruffled feather, open mouth breathing.

In post mortem examination and serious involvement of trachea, lungs, air sacs, heart and liver and showed cloudy and air sacculitis in the air sac, pericarditis, congested and mucoid trachea and lung revealed dark red color and congested. The color of the liver was slightly changed to pale and fibrinopurulent covering perihepatitis was observed.

In the present study serological evidence of MG in the respiratory distress was recorded from 920 sera using Serum Plate Agglutination (SPA) test and overall seroprevalence of MG was found 57.17%.The incidence in Rangpur, Dinajpur, Thakurgaon, Panchagarh and Joyporhat were found 51.09%, 61.96%, 55.43%, 57.61% and 59.78% respectively. The

highest incidence was found in Dinajpur (61.96%) and the lowest in Rangpur (51.09%). In age variation maximum prevalence 64.78% was found above 40 weeks (group-4) followed by 59.30% in 21-40 wks (group-3), 56.52% in 9-20 wks (group-2) and 48.26% in 0-8 wks (group-1).To seasonal investigation of this study, 920 sera were collected from two seasons. The prevalence was found by SPA test 61.96% in winter and 52.39% in summer season. It is illustrated that the highest prevalence was found in winter compare to summer. In the present study the prevalence of MG antibody according to flock size was higher in larger flock size. Also found higher prevalence in Sonali breed than Isabrown breed.

SPA positive 526 sera again tested for detection of MG antibody through iELISA test and found 164 sera were positive. The overall prevalence was 31.18%.The highest prevalence was found 52.35% in above 40 weeks (group-4) and the lowest was 17.12% in 0-8 wks (group-1).

80 representative samples from 164 iELISA positive sera were screened by HI test. The overall prevalence was found 18.75%.The highest (40%) prevalence was found in above 40 weeks (group-4) and the lowest (10%) was in 0-8 wks (group-1).

In this research work, for isolation of MG, PPLO medium was using and suitable incubation conditions. Mg was isolated after cultuting in PPLO broth and agar. The PPLO broth colour was changed from red to orange and yellow with clear solution after 3 to 7 days of incubation indicated that the PPLO medium was suitable for isolation of mycoplasma and subcultured immediately into PPLO agar for pure isolates. Overall result revealed out of 156 organs only 9 organs (5.77%) showed 'fried egg' colony as culture positive.The highest 7 (13.46%) positive isolates were detected from tracheal swabs from live birds followed by 1 (1.92%) from air sacs and 1 (1.92%) from lung.

Culture positive isolates were performed by gimsa staining and organism found annular forms i.e. circular and elliptical pleuropneumonia like organism were characterized chiefly by the lack or thinness of stainable material in the central region of the organism.

All culture positive isolates were fermented glucose was indicated by color change (red to yellow) due to the pH indicator and phenol red.

PCR was carried out using primers to amplify 16S rRNA gene and amplified product was 185bp and 732bp. In the present findings the recovery rate by PCR protocol was 14.58%

(7 bands) which were 12.5% (6 bands) from 48 direct tissues and 0.64% (1 band) from culture positive isolates in185bp and 12.5% (6 bands) showed successfully in 732bp from direct 48 different tissues and no band from positive culture isolates.

Further, it was subjected to sequence analysis and confirmed 99-100% similarity as *M. gallisepticum* with South Africa, Spain and USA by NCBI blast analysis.

Concluding Remarks

- For the effective control of *M.gallisepticum* a concise informative epidemic behavior in the context of Bangladesh considering age, breed, season and location differences was established.
- Established efficacy of different serological test for detection of MG specific antibody based on age, breed, season, flock size and location.
- Detection of *M. gallisepticum* load in different tissues from field samples of poultry using conventional cultural method.
- Confirmatory characterized *M. gallisepticum* organism with molecular technique and sequence analysis was established in order to develop experimental vaccine candidate.

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APPENDICES

Appendix-1

Preparation of Horse serum collected from field for cultural isolation

Horse blood was collected from Abdus Subhan, Pachkur, Haji para, and Nujrul Islam, Naranpur, 8 no. Sonkorpur union, Dinajpur sadar, Dinajpur, in 50 ml sterial plastic disposable syringe, allow to clot for 1 hour at room temperature, separate the serum, centrifuged 10 minute for 2000 rpm and poured in vial and inactivation of serum in water bath for 30 minutes at 56°C and stored -20°C until use.

The procedure for proper heat inactivation of serum was

Step 1: Frozen serum was thawed under refrigerated conditions or at room temperature or 37°C water bath and serum was removed from the water bath as soon as after thawing.

Step 2: To start the heat inactivation process, the water bath was prepared by the adjusted of temperature of water to 56°C and a thermometer was allowed to equilibrate to the temperature of water (i.e. overnight). The thermometer was placed in a bottle of water, similar to the volume of the bottle of serum, to prevent of damage to the thermometer.

Step 3: Once the serum was thawed completely, serum was mixed by gently swirling the container to ensure that the solution is homogenous and transferred to water bath. The bottle should not submerged or allow the water level to extend to the caps of the bottles, as the compromise the integrity of the container.

Step 4: The temperature in the water bottle was monitored using the thermometer to ensure a steady temperature.

Step 5: During incubation, the contents of both bottles was swirl every five minutes to ensure that the contents are evenly heated.

Step 6: When the bottle of serum has been at 56°C for 30 minutes, the serum of bottle was transferred to ice to cool and proceed as needed. To prevent multiple freeze/thaw cycles, aliquot serum into smaller sterile containers, labeled with date and number, and transferred to a -20°C freezer.

To prevent protein denaturation, which leads to turbidity and the presence of globules in the serum, the following precaution was maintained

1. The speed of thawing using temperature should not be greater than 25°C.

2. Heat inactivate serum should not partially mixed/partially thawed.

3. Heat inactivate serum should not at a temperature greater than 56.3°C.

4. Heat inactivate serum should not for greater than 30°C.

5. Be sure to swirl the serum during the heat inactivation process.

Appendix-2

Reagents used for DNA extraction and Agarose Gel Electrophoresis

1. Phosphate Buffer Saline (PBS) 10x

Sodium chloride	8 gm
Potassium chloride	0.2 gm
Disodium hydrogen phosphate	2.8 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water	1000 ml

The pH of the solution was adjusted to 7.4 with 1N HCL.This 10x concentration solution was prepared with ultrapure water to 1x when required for molecular identification.

2. TAE buffer (50X)

Tris base	242 gm
Glacial acetic acid	57 ml
0.5 M EDTA	100 ml
Deionized water	upto 1000 ml

Dissolved by stirring and stored at room temperature. For preparation of 1X working solution 1 part stock buffer was added with 49 parts distilled water.

3. 1N HCL solution (v/v)

Concentrated HCL	0.365 ml
Distilled water	9.635 ml

The solution was used to adjust pH of buffer solution

4. 1M NaOH or 1N NaOH solution (v/v)

Sodium hydroxide	0.4 gm
Distilled water	10 ml

The solution was stored at 4 °C until used to adjust the pH of PBS solutions

5. 0.5 M EDTA (P^H 8.0)

Dissolve 18.612 g EDTA $2H_2O$ in 80 ml distill water and adjust pH to 8.0 using NaOH pellets. Adjust the final volume to 100 ml. Filter through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs for 15 min. Store at room temperature.

6. Agarose gel (1.5% and 2%)

Agarose power	1.5 gm / 2gm
1X TAE buffer`	100

7. 95% ethanol

95% (v/v) ethanol solution was prepared by diluting 95ml absolute ethanol in 5 ml distilled water. The solution was stored in airtight bottles at room temperature until used.

8. 10% SDS (Sodium dodecyl sulphate)

10% (w/v) SDS was prepared by mixing 10gm SDS with 100 ml distilled water properly. The solution was stored in airtight bottles at room temperature until used.

Appendix – 3

Media, stains and solutions

1) Biochemical tests

A) Carbohydrate fermentation media

Heart infusion broth (Difco)	-100 ml
Swine serum	-10 ml
Potassium penicillin G	-1000 units/ ml
Thallium acetate	-0.1 per cent w/v

The complete liquid growth medium was dispensed in two millilitre quantities in glass tubes and one per cent of glucose was added along with 0.002 per cent phenol red. The pH was adjusted to 7.6 to 7.8.

3) Giemsa stain (Himedia pvt. Ltd.)

Working Giemsa stain was prepared by adding 30 drops of stock solution in 30ml distilled H_2O.

Appendix- 4

Questionnaire for the surveillance/investigation of *Mycoplasma gallisepticum* infection in layer chicken

1. Particulars of the form owner :

iv)Upazila: ..... ….. v) District: .....

2. i)Type of farm: Commercial- Small ☐ Medium ☐ Large ☐

Native- Scavenging ☐ Semi- Scavenging ☐

i) Total No. of birds in flock: .....

3) **Source of egg/chicks:**

Name: ..... Breeder ☐ Shop ☐

4) **Management System:**

i) **Housing**

Type of housing: Litter ☐ Cage ☐

ii) Diet history

a) Food item: Ready food: Yes ☐ No ☐

Loose food: Yes ☐ No ☐

b) Time of feeding.....times/per day

iii) **Biosecurity and sanitary condition:**

a) Hygienic condition of the house: Poor ☐ Good ☐ Very good ☐

b) Cleaning time of house.....days/per week

c) Washing of house with: Antiseptic powder ☐ Disinfectant ☐ Water ☐

d) People close in contact with poultry: Visitors ☐ Workers ☐ Others ☐

e) Waste disposal time: Immediately ☐ Few minutes later ☐

5) **Disease history:**

i) History: 1.

2.

3.

ii) Treatment given: Yes ☐ No ☐

ii) Medication:

Name of medicine used	Remarks	Name of medicine used	Remarks

iv) Post-mortem lesion (if possible): 1..... 2.....

3..... 4.....

v) Mortality rate: .....

6) Sample collection:

i) Number of collection sample.....

ii) Name of sample.....

7) Vacccination:

Name of vaccine	**Age (days)**	**Name of vaccine**	**Age (days)**

Signature of investigator