**CHAPTER – I**

**INTRODUCTION**

Mycoplasmas are bacteria that lack of cell wall and belong to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae (Bradbury, 2001; Barua *et al*., 2006). They have no genes involved in amino acid biosynthesis and only a few genes involved in the biosynthesis of cofactors (vitamins) (Pollack, 2002).

Mycoplasmas are widespread in nature and infect a wide range of hosts. Species from the genus *Mycoplasma* have been isolated (over 110 species) from mammals, birds, reptiles and fish (Elgnay and Azwai, 2013). Avian mycoplasmosis is caused by several pathogenic mycoplasmas such as; *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. lowae* (Stipkovits and Kempf, 1996). Among them *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) are the most important and are listed as notifiable diseases by the OIE*.*

Infectious synovitis was first described and associated with *Mycoplasma* infection by Olson *et al*., during the early 1950's. The causative organism was designated as Avian *Mycoplasma* Serotype S by Dierks *et al*. (1967) and subsequently confirmed as a separate species, *Mycoplasma synoviae*, by Jordan *et al*. (1982). Now MS appeared to have worldwide distribution (Ziv and David, 2011).

*Mycoplasma synoviae* (MS) is primarily a disease of chicken and turkeys but also infects many other domestic and wild birds all over the world (Jordan and Amin, 1980; Bradbury *et al*., 1993). *Mycoplasma synoviae* is an important pathogen of poultry causing synovitis, chronic respiratory tract disease, and retarded growth in chickens and turkeys (Feberwee *et al*., 2009). *Mycoplasma synoviae* infection causes decreased egg production in chickens, turkeys and other avian species (Ley and Yoder, 1997). In addition to the overt disease, the infection causes decreased feed efficiency, poor carcass quality and sub-optimal egg production in layers (Ley and Yoder, 1997; Hossain *et al*., 2007).

*Mycoplasma synoviae* infections are transmitted both horizontally and vertically and it can remain in the flock constantly as a subclinical form (Bencina *et al*., 1988). MS can remain as carriers in chickens for many months after the acute phase of the infection and is considered to be a great problem for the breeder farms as there is possibility of transmitting the disease to the commercial layer farms through day old chicks from those breeder farms.

*Mycoplasma synoviae* can potentially be present in backyard poultry flocks (McBride *et al*., 1991). As in other poultry producing countries, mycoplasmosis is one of the important disease problems for poultry in Bangladesh, for both commercial and breeder farms (Hossain *et al*., 2007). In Bangladesh the seroprevalence of *Mycoplasma synoviae* was reported to be 61-67 % (Giasuddin *et al*., 2002).

Mycoplasmosis may be diagnosed by different methods, such as morphology of causal agents, cultural characteristics, physical, biochemical and serological properties (Ley and Yoder, 1997). Serology is the only reliable tool for detecting subclinical infection in a flock. Generally *Mycoplasma* infections are diagnosed by means of serological methods and, eventually, by PCR (Godoy, 1999)*.*Various serological tests have been developed for detection of antibodies against MS. The most commonly used procedures are serum plate agglutination test, tube agglutination test, haemagglutination inhibition test and enzyme-linked immunosorbent assay (OIE, 2008). The serum plate agglutination (SPA) test could be used as a tool for quick detection of avian *Mycoplasma* infection (Pradhan, 2002).  Tracheal and cloacal swabs are used in the identification of the agent by means of polymerase chain reaction (PCR) (Hong *et al*., 2004).*Mycoplasma synoviae* infections are often associated with other diseases, so the use of advanced techniques such as PCR is a tool of great importance in diagnosis of MS infections with achieving greater accuracy, which leads to a better understanding of the pathogen affecting avian populations (Raviv and Kleven, 2008).

Control of pathogenic *Mycoplasma synoviae* consists of three general approaches: maintaining flocks free of infection, medication, or vaccination. The most effective method of controlling MS infection is regular monitoring of the flocks and eliminating the positive flocks (Kleven, 2008). Reliable and rapid diagnosis is needed to prevent dissemination of infection (Kleven and Ferguson-Noeln, 2008; Lockaby, 1998). Chittagong is considered to be a poultry zone with a number of breeder farms in the area. Less emphasis has been given to the disease problems of the poultry farms and no work has been reported on *Mycoplasma synoviae* in the area.

**1.1 Aim of study**

The aim of the study was to determine the seroprevalence and identification of *Mycoplasma synoviae* among commercial chicken breeder farms of Chittagong in Bangladesh.

* + 1. **Objectives of the study**

To determine whether MS is present in the breeder farms at Chittagong and to determine the seroprevalence of the disease along with its risk factors that may shade a light on the measures to be taken to control the disease, the research work was taken with the following objectives:

1. To determine the prevalence of *Mycoplasma synoviae* in the breeder farms at Chittagong district using an appropriate sero-diagnostic method.

2. To detect *Mycoplasma synoviae* using a molecular diagnostic method polymerase chain reaction (PCR).

**CHAPTER – II**

**REVIEW OF LITERATURE**

**2.1 Avian Mycoplasma**

Mycoplasmas are the smallest known living organisms (Baseman and Tully, 1997; Vogl *et al*., 2008). They belong to the family Mollicutes (molis, soft; cutis, skin, in Latin) that includes all cell wall-less prokaryotes (Baseman and Tully, 1997). The cytoplasm of mollicutes are encased solely by a plasma membrane composed of approximately 60% membrane proteins and 40% membrane lipids, 4 to 20% of which are steroids (Razin *et al*.,1998). Mycoplasmas also have an unusually high percentage of lipoproteins when compared to other eubacteria (Razin *et al*., 1998). They have no genes involved in amino acid biosynthesis and only a few genes involved in the biosynthesis of cofactors (vitamins) (Pollack, 2002). Most mycoplasmas cannot synthesise any fatty acids and some even incorporate exogeneous phospholipids together with cholesterol in their cell membrane. Also the genes involved in the biosynthesis of nucleotides are very limited (Pollack, 2002). The use of carbohydrates is inefficient since both the tricarboxylic acid cycle and cytochromes are missing. Substrate-phosphorylation is the major route for ATP synthesis. Mycoplasmas mostly depend on the glycolysis for ATP, although sometimes other energy providing pathways, like the dihydrolysation of arginine, are assumed important. Probably mollicutes need less energy for their limited anabolic activity. Since phytoplasmas live intracellularly with an easy access to nutrients, they have seemingly even fewer genes related to metabolic functions and ATP-synthesis processes (Oshima *et al*., 2004). The degenerative evolution of mycoplasmas can also be observed from their number of genes related to DNA replication, transcription and translation. Mycoplasmas use a simplified DNA replication complex resembling polymerase III of Gram-positive bacteria. Another polymerase without proofreading activity, resembling PolC of *E*. *coli*, has been described as well (Barnes *et al*., 1994). The number of genes with respect to DNA repair systems is much lower compared to other bacteria and the missing or ineffective uracil-DNA glycosylase may explain the low G-C content (typically lower than 35%) (Glass *et al*., 2000). The DNA-dependent RNA polymerase in mycoplasmas is similar to those found in other bacteria. Also translation is carried out using a minimal set of genes. Mycoplasmas contain only around 30 tRNA genes (Dandekar *et al*., 2000). Interestingly, mycoplasmas have a tRNA gene that translates the UGA codon into tryptophan, instead of recognizing it as a stop codon. Possibly owing to their low GC-content, this UGA codon is far more frequently used than their cognate UGG codon (Westberg *et al*., 2004). Vasconcelos (2005) stated that, *Mycoplasma synoviae* has one circular chromosome that is made up of 799, 476 bp with a total of 694 ORFs and a G+C content of 28.5mol%. There are 672 protein genes and 41 RNA genes. This information is based on *Mycoplasma* *synoviae* strand 53, isolated from a broiler breeder chicken from Brazil.

The minute size and lack of a cell wall of *Mycoplasma* baffled scientists about their identification for decades. Nocard and Roux (1898) first cultivated *Mycoplasma* successfully *in* *vitro*. The condition designated as “chronic respiratory disease” was described by Delaplane and Stuart (1943). In the early 1950’s, Markham and Wong (1952) and Van Roekel and Olesiuk (1953) reported the successful cultivation of the organisms from chickens and turkeys and noted their similarity, and suggested they were the members of the pleurpopneumonia group. Edward and Freundt (1956) proposed a system for classification and nomenclature of organisms of the pleuropneumonia group, according to which all species were arranged into the genus *Mycoplasma*, Family Mycoplasmataceae, and order Mycoplasmatales. Edward and Freundt (1956) also proposed Mollecutes as a name of the class for the order mycoplasmatales. Different strains of pleuropneumonia like organisms of avian origin have been described, which are now designated as different species of *Mycoplasma* (Adler *et al*., 1957).

**2.2 Economic impact of Avian Mycoplasmosis**

Losses attributed to mycoplasmosis are due to decrease in egg production and egg quality, poor hatchability (high rate of embryonic mortality and culling of day-old birds), poor feed efficiency, increase in mortality and carcass condemnations, besides medication costs (Mohammed, *et al*., 1987; Yoder, 1991; Ley and Yoder, 1997). Mohammed *et al*. (1987) reported that, a MG-infected chicken lays 15.7 eggs less than a healthy one, contributing to a loss of 127 million eggs in the USA in 1984, which corresponded to an annual loss of 125 million dollars. According to Projeto (1994), there was a loss of 34 thousand tons of broilers in the end of the production cycle due to respiratory diseases, which corresponded to 30 million dollars in 1994, using slaughter data from the Federal Inspection Service in Brazil. Particularly for MS, losses have been attributed to transient immune depression, increase of 1 to 4% in the mortality rate of broilers in the final phase of production (Shapiro, 1994), decrease of 5 to 10% in egg production and 5 to 7% in hatchability (Mohammed *et al*., 1987; Stipkovits and Kempf, 1996). Moreover, MG infection alone is considered one of the diseases that cause more losses to the poultry industry (Yoder, 1991; Charlton *el al.*, 1996; Ley and Yoder, 1997).

Balen and Fiorentin (1990) reported that, the prevalence of MS in chicken flocks is increasing since the 80's, overcoming that of MG in breeding flocks in Brazil. Although MS is mostly involved in asymptomatic infections and sometimes considered harmless to chickens, it is pathogenic for birds (Stipkovits and Kempft, 1996). Besides, MS has been proven to affect the humoral response of chicks vaccinated with a La Sota strain of Newcastle disease virus (ND). Hemagglutination inhibition protection values for Newcastle (ND HI) (GMT > 4.0, titer > 1:16) were detected in non-MS-infected birds up to 45 days after a single ND vaccination, but not in birds that were MS-infected and ND-vaccinated. Protection of MS-infected broilers was induced only after a second dose of vaccine (Nascimento *et al*., 2003; Silva, 2003).

**2.3 Classification of *Mycoplasma***

The mycoplasmas belong to a class of microorganisms known as mollicutes (Table 2.1) (Jordan, 1972). They have the following distinctive features (a) The absence of cell wall; (b) presence of trilaminar membrane; (c) small size (as the smallest free -living microbes just within the limits of resolution of the microscope); (d) possession of a few cellular organelles, particularly those for metabolism and reproduction; and (e) a small genome of about 5 x 108 daltons (Freundt and Edward, 1979; Razin, 1981). The classification includes eight genera, *Acholeplasma, Anaeroplasma, Mesoplasma, Entomoplasma, Spiroplasma, Mycoplasma, Asteroplasma and Ureaplasma* (Dybvig and Voelker, 1996) (**Table 2.1**). Classification of any isolate within the family depends on biochemical and serological tests (Tully *et al*., 1993). The characteristics of mycoplasma species isolated from avian sources are presented in **Table 2.2**.

**2.4 Etiology of Avian Mycoplasmosis**

Avian mycoplasmosis is caused by several pathogenic mycoplasmas of which *M.* *gallisepticum* and *M*. *synoviae* are the most important; they are the only ones listed by the Office International Des Epizootics (OIE, 2008). *Mycoplasma* *gallisepticum* and *M*. *synoviae* are present a pathogenic species within the genus *Mycoplasma* and stain well with Giemsa, but is weakly Gram negative. It is generally coccoid and approximately 0.25-0.5 µm (Razin and Freundt, 1984). The organism shows a filamentous or flask-shaped polarity of the cell body, which is due to the presence of terminal organelles or bleb (Maniloff and Quinlan, 1973). It has now been established that these terminal organelles govern motility, attachment to the host cell and pathogenicity (Levisohn and Dykstra, 1987).

**Table 2.1:** Classification and major features of members of the class of *Mollicutes*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Order** | **Family** | **Genus** | **Genome size (kbp)** | **%GC** | **Cholesterol requirement** | **Habitat** |
| *Mycoplasmatales* | *Mycoplasmataceae* | *Mycoplasma* | 580-1350 | 23 - 40 | Yes | Animals and humans |
| *Ureaplasma* | 760- 1170 | 27- 30 | Yes | Animals and humans |
| *Entomoplasmatales* | *Entomoplasmataceae* | *Entomoplas ma* | 790-  1140 | 27-29 | Yes | plants and insects |
| *Mesoplasma* | 870-  1100 | 27-30 | No | plants and insects |
| *Spiroplasmataceae* | *Spiroplasma* | 940-  2220 | 25-31 | Yes | plants and insects |
| *Acholeplasmatales* | *Acholeplasmataceae* | *Acholeplas ma* | 1500-  1650 | 25-36 | No | animals, plants and insects |
| *Phytoplasma* | 640-  1185 | 23-29 | ND | plants and insects |
| *Anaeroplasmatales* | *Anaeroplasmataceae* | *Anaeroplas ma* | 1500-1600 | 29-34 | Yes | Bovine and ovine rumen |
| *Asteroleplasma* | 1500 | 40 | No | Bovine and ovine rumen |

Source: Stakenborg Tim (2005).

**Table 2.2:** Characteristics of avian mycoplasmas species

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Species* | Usual host | Glucose  fermentation | Arginine  hydrolysis | Phosphate  activity |
| *A. laidlawiia* | Various Duck | + | - | + or - |
| *M. anatis* | Various Duck | + | - | + |
| *M. anseris* | Hawk | - | + | - |
| *M. buteonis* | Goose | + | - | - |
| *M. cloacale* | Buteo hawk | - | + | - |
| *M. Columbinasale* | Turkey | - | + | - |
| *M. columbinum* | Pigeon | + | - | - |
| *M. columborale* | Pigeon | + | - | - |
| *M. corogypsi* | Black vulture | + | - | - |
| *M. falconis* | Saker falcon | - | + | - |
| *M. gallinaceum* | Chicken | + | - | - |
| *M. gallinarum* | Chicken | - | + | - |
| *M. gallisepticum* | Chicken, Turkey | + | - | - |
| *M. gallopanovia* | Turkey | + | - | - |
| *M. glycophilum* | chicken | + | - | +or - |
| *M. gypis* | Griffon vulture | - | + | + |
| *M. imitans* | duck, goose, Partridge | + | - | - |
| *M. iners* | Chicken | - | + | - |
| *M.iowae* | Turkey | + | + | - |
| *M. lipofaciens* | chicken | + | + | - |
| *M. meleagridis* | Turkey | - | + | + |
| *M. Pullorum* | Chicken | + | - | - |
| *M. synoviae* | Chicken, Turkey | + | - | - |
| *U. galloraleb* | Chicken | - | - | - |

Source: (www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg) (2007)

Key:

a*Acholeplasma* species do not require sterols for growth

b*Ureaplasma* species are characterized by splitting of urea.

Mycoplasmas require a protein-rich medium for their growth, containing 10-15% added animal serum. A medium described by Frey *et* *al*. (1968) or a medium described by Bradbury (1977) is commonly used for the cultivation of avian mycoplasmas. Growth generally is optimal in medium at approximately pH 7.8 incubated at 37- 38˚ C. They produce tiny, smooth, circular translucent colonies on agar plate with a dense, raised central area (Frey *et al*., 1968; Kleven, 1994; Ley and Yoder, 1997).

**2.5 Virulence factors of *Mycoplasma synoviae***

The immunodominant *M. synoviae* surface membrane proteins, are associated with hemadherence, (Noormohammadi *et al*., 1997) and are associated with the evasion of the immune response (Noormohammadi *et al*., 1998). *M. synoviae* has two major immunogenic membrane proteins, MSPA and MSPB, both of which are phase variable and coordinately involved in adhesion of the organism to erythrocytes (Noormohammadi *et al*., 1997). MSPA and MSPB, each containing two members (MSPA1, MSPA2 & MSPB1, MSPB2 respectively). A 25- to 30-kDa membrane protein (MSPC) which is antigenically related to the MSPB proteins (Noormohammadi *et al*., 1997). The *vlhA* gene of *M. synoviae* (WVU 1853 strain) is a hemagglutinin and responsible for encoding a variably expressed lipoprotein (VlhA protein). In addition, it is stated that MSPA and MSPB proteins are transcribed from a single gene, *vlhA* (Noormohammadi *et al*., 1998).

**2.6 Transmission of *Mycoplasma synoviae***

*Mycoplasma synoviae* may be transmitted horizontally through infectious aerosols coughed and sneezed by infected birds, and through contaminated feed, water, contact, personnel and communicant animals, mainly birds (Jordan, 1985). The most frequent source of mycoplasma infections of poultry appears to be ovarian (vertical) transmission (MacOwan *et al*., 1984; Bencina *et al*., 1988). Infection of the ovary or oviduct with *M. synoviae* may be by haematogenous spread or by direct spread from infected air sacs (Charlton *et al*., 1996). Shedding in the egg can vary; egg transmission is more frequent in birds infected during laying than in birds infected before they mature (Charlton *et al*., 1996). Infected birds carry *M. synoviae* for life, and can remain asymptomatic carriers until they are stressed (OIE, 2008). Horizontal transmission can occur from bird to bird by aerosol, droplets in respiratory infections with *M. synoviae* from birds in the incubation period of disease, clinically affected ones, or carriers (Jordan, 1985; Nascimento *et al*., 2005). Olson *et* *al*. (1964) and Olson and Kerr (1967) have demonstrated *M. synoviae* in the respiratory tract of contact birds about 1 – 4 weeks after infection of inoculated birds. Lateral spread has also been demonstrated by Hemsley (1965), and Kerr (1965) between groups of chicken kept up to 45 feet apart. Wyeth (1975) reported lateral spread to in-contact birds from birds infected by the intravenous and respiratory routes.

Airborne and contact transmission had been described previously (Fahey and Crawley 1954; Jordan, 1985; Bradbury, 2001). Both eggs and airborne transmission may be influenced by concurrent infections and probably by other non-infectious factors which stimulate multiplication of the *Mycoplasma* (Jordan, 1985). Arthropod transmission has been reported by Turner *et al*. (1963) who demonstrated *Mycoplasma synoviae* in the blood of mosquitoes and mites. Rodents frequently visit chicken farms in search of food which may also be responsible for the mechanical transmission between flocks and farms (Johnson *et al*., 1981; Soliman *et al*., 1988), particularly in intensive or semi intensive systems.

In ducks and guinea fowls, high SPA antibodies titre had been demonstrated (Onunkwo and Onoviran, 1978; Adesiyun and Abdu, 1985). These birds, particularly those kept in various households could mix freely and readily with other free-range domestic poultry and establish or maintain a definite cycle of infection (Adesiyun and Abdu, 1985). They therefore, constitute yet another major reservoir for these organisms.

Wyeth (1975) found localized lesions and minimal spread of *M. synoviae* to susceptible birds after intravenous inoculation. There was increased spread of the infection after intrasinus or aerosol exposure accompanied with no lesions. Olson *et al*. (1964) showed that *M*. *synoviae* may persist in the absence of lesions for seven weeks in the turbinates of chickens infected via the sinuses whereas Springer (1971) found that lesions resulting from air sac inoculation remained localized. Pruthi *et al*. (1985) and Kempf *et al*. (1989) noted that the yok sac route was more effective than the tracheal route of *Mycoplasma* infection in establishing the disease in day-old chicks. However, natural infection of ducks and geese with *M. synoviae* had been reported (Bencina *et al*., 1988).

**2.7 Incubation period**

Experimentally, infected poultry develop symptoms such as conjunctivitis, coughing, synovitis and sneezing after 6 to 21 days (Bozeman *et al*., 1984; Luttrell *et al*., 1996). In natural infections, the incubation period is variable; infected birds may be asymptomatic for days or months until stressed (Bozman *et al*., 1984; Jordan 1985). In finches, incubation periods of 4 to 14 days have been reported (Luttrell *et al*., 1996).

**2.8 Pathogenesis**

It is presumed that *Mycoplasma synoviae* enters the respiratory tract by inhalation of aerosols or via the conjunctiva, but it is not clear how it surmount bird’s natural defense mechanism (Bradbury, 2001). Mycoplasmas attaches to mucosal cells by its well-organized terminal organelles or blebs, which govern motility or host-pathogen interactions and ultimately pathogenicity (Levisohn and Dykstra, 1987). Cell division of *Mycoplasma synoviae* occurs through binary fission, which is synchronous with DNA replication (Quinlan and Maniloff, 1973). *Mycoplasma* antigens, especially those with adhesion or haemagglutinin properties, may play key roles in the pathogenesis of, and immune response to, infection (Markham *et al*., 1992; Avakian and Ley, 1993; Kheyar *et al*., 1995). *Mycoplasma synoviae* has sophisticated ways of varying its surface antigens and that these changes may permit subpopulations to avoid the immune response and persist in the host for long periods (Bradbury, 2001). Markham *et* *al*. (1992) described a haemagglutinin of *Mycoplasma synoviae*, which was found to be encoded by a family of genes, whose hypothetical function is immune evasion. Although it is not confirmed how the lesions and clinical signs of *Mycoplasma* infection originated, many of the lesions seem to result from the host immune and inflammatory response rather than the direct effects of the organism (Razin *et al*., 1998). Sometimes, chickens may not show the signs of the disease, but they remain as carriers for many months after the acute phase of the infection (Bencina and Dorrer, 1984). *M. synoviae* have been considered extracellular organisms, but Vogl *et al*. (2008) proved that mycoplasmas invade cells. In order to survive within the host to induce disease and evade the host immune system, mycoplasmas use some pathogenicity mechanisms. The mechanisms include adherence to host target cells, mediation of apoptosis, innocent by-stander damage to host cell due to intimate membrane contact, molecular mimicry that may lead to tolerance, and mitotic effect for B and/or T lymphocytes (Vogl *et al*., 2008). The mitotic effect for B and/or T cells could lead to suppression of function and/or production of cytotoxic T cell. Mycoplasmas also produce by products, such as hydrogen peroxide and superoxide radicals (Vogl *et al*., 2008). Moreover, mycoplasmas has the ability to stimulate macrophages, monocytes, T-helper cells and natural killer (NK) cells, resulting in the production of substances, such as tumour necrotizing factor (TNF-α), interleukin (IL-1, 2, 6) and interferon (α, β, γ) (Razin and Tully, 1995; Razin *et al*., 1998). These mechanisms may explain the transient suppression of humoral and cellular immune responses during *Mycoplasma* infection in birds, the immune tolerance and auto immune diseases, as well as the massive lymphoid cell infiltration in the respiratory tract and joint tissues of infected fowls (Razin and Tully, 1995; Razin *et al*., 1998). Besides these mechanisms that may be used *M. synoviae*, latency is common to them (Razin *et al*., 1998). Thus, these pathogens induce disease after the host is affected by other disease-causing organisms, such as bacteria and viruses and/or after an episode of host weakness (Yoder, 1991; Whitford *et al*., 1994). The latent status, *i*.*e*., when the *Mycoplasma* is not recognized by the host immune system, may be explained by its intracellular location due to environmental pressure, as can be exemplified by the presence of antimicrobials in host tissues for the treatment of *M. synoviae* infection of birds (Razin *et al*., 1998). Silva *et* *al*. (2003) and Nascimento *et al*. (2005) in their works provided experimental evidences that *M. synoviae* causes immune depression by affecting the chicken cellular and humoral immune system. Moreover, mycoplasmas are more susceptible to mutations than other bacteria (Woese *et al*., 1985), and this can be explained by their defective DNA repair system (Ghosh *et al*., 1977). The frequent changes on surface antigens (antigenic variations) allow *Mycoplasma synoviae* to evade the host immune system (Markhan *et al*., 1994).

**2.9 Age of the birds**

Chicks and turkey poults are generally more susceptible than older birds and resistance is said to increase with age (Jordan, 1979). However, Seifi and Shirzad (2012) found, that prevalence of *Mycoplasma synoviae is* higher in the age group of above 60 weeks than young birds.

**2.10 Concurrent infections of *Mycoplasma synoviae* with other disease agents**

Organisms that have been found in concurrent infection with *M. synoviae* include Newcastle disease virus (Villegas *et al*., 1976), infectious bronchitis virus and *E*. *coli* (Springer *et al*., 1974), influenza A virus (Bahl *et al*., 1974), *M.* *meleagridis* (Rhoades, 1977), reovirus (Al-Afaleg *et al*., 1989), *Staphylococcus* *spp* (Moorhead *et al*., 1967) and *Pseudomonas* *spp* (Peterson, 1975). Giambrone *et al*. (1977) also showed that early exposure of chicks to infectious bursal disease virus lead to an increase in susceptibility to air sac inflammation.

**2.11 The role of stress factors on *Mycoplasma* infection**

Debilitating factors, such as scarification of the trachea (Corsvet and Saddler, 1964), excess ammonia (Sato *et al*., 1973) and social stress (Gross and Colmano, 1971) play active role in the exacerbation of poultry *Mycoplasma synoviae* infections. Jordan (1972) reported that changes in intensive poultry husbandry practices, multiple breeding and increased population density are factors that enhance respiratory disease due to *Mycoplasma synoviae* infection. It is not known whether debilitating factors are of significance in precipitating disease of synovial tissues of *M. synoviae*. Yoder *et al*. (1977) showed that low environmental temperatures increased the incidence and severity of air sacculitis caused by *M. synoviae* in 3 -4 week old infected chickens.

**2.12 Diagnosis of Avian Mycoplasmosis**

Diagnosis of *Mycoplasma synoviae* is based on clinical signs, gross and microscopic lesions and serological tests (Jordan, 1985).

**2.12.1 Clinical signs associated with *M. synoviae***

The clinical signs seen in chickens infected by *M. synoviae* include paleness of the face and comb, lameness and retarded growth (Kemps and Gibson, 1981). There are swellings around joints, especially in the areas of the hock and footpad, breast blisters are also common. Birds become listless, dehydrated and emaciated (Jordan, 1975). Lameness is usually associated with hot, painful, swollen hocks or foot joints, and in very severe cases all the body joints may be affected (Morrow *et al*., 1990). In the respiratory form, chickens may show slight rales in 4-6 weeks or may be asymptomatic (Olson, 1972). Mortality is low, varying from 2-6% but reports of morbidity vary greatly between 2-75% with an average in natural infections of 20% (Olson, 1972). In turkey, there is sinusitis (Jordan, 1975). Feberwee *et al*. (2009) have also shown that MS causes egg apex abnormalities in layers in the Netherlands and that this can reduce egg quality and increase the number of broken eggs.



Figure 2.3 Egg apex abnormality

Figure 2.2 Lameness

Figure 2.1 Arthritis

**2.12.2 Gross lesions associated with *Mycoplasma synoviae* infection in chickens**

Lesions of the respiratory tract may be very mild, or consist only of excess mucous or catarrhal exudates in the nares, sinusitis, trachea and lungs with oedema of the airsac walls (Yagihashi *et al*., 1988). Caseous exudates may appear later particularly in the airsacs. Fibrinous or fibrinopurulent pericarditis and perihepatitis are usually present when infections are complicated with pathogenic strains of *E*. *coli* (Gross, 1961; Fabricant). Salpingitis has also been reported (Domermuth and Gross, 1962). Arthritis and synovitis, particularly of the hocks have been seen in chickens (Uchida, 1984), and in turkeys (Fabricant and Levine, 1963). Lesions in the brain which appear as pale areas of 1-2mm in the cerebrum have been reported (Varley and Jordan, 1978a; b). Acute swelling of the capillary endothelium has been described (Thomas *et al*., 1966). The association of *M. synoviae* with arthritis in chicken has been reported (Sentíes-Cué et *al*., 2005). In the early stages of the infection, vicious creamy exudate appears in the synovial space of the keel (Morrow *et al*., 1990). As infection progresses, the exudates becomes caseous (Sells, 1971). In chronic cases, inhibition of the sesamoid bone development in the tendons and degenerative osteoarthritis may also occur (Olson and Kerr, 1970). Olson and Kerr (1970) described lesions encountered in 2- week old chicks inoculated via foot pad and found that 80% of the chickens inoculated had gross lesions in the pelvic limbs, while only 27% of contact- infected birds developed lameness and tendo-vaginitis. Occasionally, considerable mucus is found in the trachea following intranasal inoculation of *M*. *synoviae* (Olson, 1972). It has been reported that *M. synoviae* on its own is capable of causing airsacculitis (Rhodes, 1978). It could cause the condition when concurrently infected with Newcastle disease and /or infectious bronchitis virus (Kleven *et al*., 1975). Ghazikhanian *et al*. (1973) observed thickened air sacs containing non adherent yellow exudates in turkeys seven days after inoculation via the air sacs. Bradbury and Howell (1975) reported air sac inflammation in 44% of birds infected *in* *vivo* with *M. synoviae*. Endocarditis and valvular lesions had been reported in chickens affected between 2 and 20 weeks of age (Kerr and Olson, 1967) and pericarditis, epicarditis and myocarditis in chick embryo (Kerr and Bridges, 1970). Cardiac lesions have also been observed in chickens following footpad inoculation (Kerr, 1971). Kerr and Olson (1970) found that cardiac lesions in contact – infected chickens paralleled those in inoculated chickens. Splenomegaly followed in some infected chickens by hepatomegaly and nephromegaly (Olson, 1972). Kawakubo *et al*. (1990) recorded atrophy of the bursa of Fabricius and thymus in experimentally infected birds. Jordan (1979) noted that none of the clinical pathological manifestations was pathognomonic of mycoplasmosis in the turkeys. Sentíes-Cué *et al*. (2005) in their necropsy work on broilers reported that the livers were mildly to severely enlarge with a prominent reticular pattern, and occasionally had a greenish tinge; the spleens were moderately to severely enlarge with white mottling. Sentíes-Cué *et al*. (2005) also observed that there were mildly to moderately enlarged hocks with an accumulation of fibrinous and/or caseous exudate in five birds. Other lesions included a mild to severe accumulation of fibrinous to caseous exudate in the air sacs, pericardium and capsule of the liver; congestion of the lungs; moderate to severe atrophy of the bursa of Fabricius and thymus; moderately enlarged and pale kidneys; moderately thickened proventricular wall and a moderate increase in mucus in the trachea.

**2.13 The avian immune response to Mycoplasma infection**

The response to microbial infection involves interaction of both innate and acquired immunity. The development of the immunity involves both T and B cells in eliminating infection. However, this varies with disease (Nighot *et al*., 2002). It has been reported that the first antibody population to appear after *M. synoviae* infection in chickens is IgM (Roberts, 1967). In *Mycoplasma* infected chickens, Kuniyasu *et al*. (1967) reported that antimycoplasma IgM persist for 77 days. These antibodies were followed by IgG antibodies (Kuniyasu, 1969; Roberts, 1969; Leslie, 1975). Antibodies against *M. synoviae* in chicks which react with plate agglutination test are associated with both IgM and IgG (Rhoades, 1978).

**2.14 Laboratory diagnosis of *Mycoplasma synoviae***

Several serological tests have been utilized for the detection of antibodies against *Mycoplasma synoviae*. The most commonly used procedures are the serum plate agglutination test (SPA), tube agglutination (TA), haemagglutination-inhibition test (HI) and enzyme-linked immunosorbent assay (ELISA) (OIE, 2008).

**2.15 Serological identification of *Mycoplasma synoviae***

Serological tests provide an essential basis for identification and classification of avian species of *Mycoplasma* isolated from different field cases by comparing them with standard species. Reliable serological tests are prerequisites for any control measures, for determining the magnitude of the problem, monitoring the effectiveness of eradication techniques and for preventing the re- establishment of infection (Snell and Cullen, 1978). Various procedures developed over the years are rapid serum plate and tube agglutination, haemagglutination-inhibition, agar gel precipitation and direct identification by specific fluorescent antibodies. All these serological methods are for the identification of serotypes (Whitford *et al*., 1994). Three serotypes of major concern have already been given separate species designation: *M.gallisepticum, M.synoviae* and *M. meleagridis* (Bradbury, 1982). Serological tests for identification of antibodies are used routinely in the control programmes for chicken and turkeys in the United States of America and as a part of the National Poultry Improvement Plan (NPIP) (Yoder, 1975). *Mycoplasma* infections of poultry may be readily confused with other respiratory diseases and in fact are frequently observed only in conjunction with Newcastle disease or infectious bronchitis virus infection from field exposure or vaccination (Springer *et al*., 1974). Secondary bacterial invasion associated with airsacculitis is often significant and *E.* *coli* is the species isolated most frequently (Springer *et al*., 1974). This makes it important to employ appropriate cultural and serological procedures for the diagnosis of *Mycoplasma* infections. Serological tests form an essential part of identification and classification of a new *Mycoplasma* isolate (Rozina, 2000).

**2.15.1 Serum plate agglutination test.**

Serum plate agglutination test was described by Adler (1954), and subsequently, used by other investigators for the detection of antibodies (Engureanu and Jonita-Jonescu 1986; Salami, 1986; Thayer and Beard, 1998). The basic principle of the test is to detect the presence of antibodies in the sera of birds. It is simple, sensitive and fast; however it has the disadvantage of non-specific reactions (Wise and Fuller, 1975). The test has been described to work best with fresh clear serum and easier to read if conducted soon after serum collection (Rocke *et al*., 1985). Several factors associated with the serum and/or the antigen can influence the specificity and sensitivity of the test (Woods and Wood, 1984; Rhoades, 1984) and the test is considered to be of greatest value for indicating flock rather than of individual bird infection (Jordan, 1979). Serum plate agglutination test has also been used for characterizing the serotypes of different mycoplasmas. The use of saline diluted yolk from fresh eggs as a source of antibodies employed with rapid plate test antigen is described by Devos *et al*. (1968).

**2.15.2 Tube agglutination test**

Test tube agglutination test was found more reliable than the plate test by Jungher *et al*. (1955). The antigen prepared for the plate test can also be used in the tube agglutination test with some modifications (Rhoades, 1978; Salami, 1986).

**2.15.3 Haemagglutination inhibition (HI)**

Haemagglutination inhibition test has been used by several workers for detecting antibodies in the sera of birds (Newnham and Audrey, 1964; Yoder and Hofstad, 1964; Dierks *et al*., 1967). It can also be used for differentiating between two species (Vardaman and Yoder, 1970). It is laborious and time consuming test and mainly performed as a confirmatory test on sera reacting positively to the serum agglutination test. Chabra and Goel (1980) studied the immune response of chicken against *M.synoviae* and determined the antibody response by HI test.

**2.15.4 Agar gel precipitation test (AGP)**

Species of *Mycoplasma* are readily typed by the agar gel precipitin test (Nonomura and Yoder, 1977). The precipitin reaction is highly specific. Aycardi *et al*. (1971) devised a relatively simple agar gel precipitin test (AGP) and classified various serotypes of avian mycoplasma. The AGP test could be used for the identification of mycoplasma isolates from field flocks (Nonomura and Yoder, 1977).

**2.15.5 Enzyme-linked immunosorbent assay technique**

Enzyme-linked immunosorbent assay has been developed for the diagnosis of avian *Mycoplasma* infections (Talkington *et al*., 1985), but the first commercial kits did not show the specificity and sensitivity desired for routine serological diagnosis of *Mycoplasma* infection (Avakian *et al*., 1988). Yagihashi and Tajima (1986) used enzyme- linked immunosorbent assay to measure antibodies in sera and respiratory secretions from chickens infected with *Mycoplasma*. Enzyme-linked immunosorbent assay kits vary between manufacturers, therefore non-specific reactions may occur (Avakian and Kleven, 1990).

**2.15.6 Immnuoflourescent technique**

Immunoflourescent technique (IF) has proved useful in the identification of *Mycoplasma* and detection of antibodies against them (Bencina and Dorrer, 1983). Many investigators have used immunoflourescent technique as a serological method for examining *Mycoplasma*.

Immunoflourescent technique is an excellent procedure because it allows rapid identification and simultaneous detection of mixed cultures (Bencina and Dorrer, 1983). It should be realized that serological techniques are not equally valuable unless carried out with pure cultures, which are not easily possible to obtain without adequate biological characterization and determinations of culture purity. However, this is not the case with the immunoflourescent technique as it can be used in mixed cultures (Al-Aubaidi and Fabricant, 1971). Immunoflourescent technique has been used rather extensively in the identification of mycoplasmas by many researchers (Ertel *et al*., 1970). Both direct and indirect methods of immunoflourescent technique have been used. The indirect method requires preparation of only one antispecies conjugate; however, Corstvet and Sadler (1964) reported that indirect immunoflourescent technique was not as useful for identifying avian mycoplasmas. Baas and Jasper (1972) noted that direct immunoflourescent technique was more suitable for the agar block technique. Other researchers have tested mammalian mycoplasmas by direct and indirect immunoflourescent technique (Rosendal and Black, 1972) but the avian strains have not been examined as thoroughly as the mammalian species.

Buys (1976) described the use of the direct test with conjugates for 10 avian *Mycoplasma* species for identifying such isolates *in* *situ* on agar plates. There were reports of the use of indirect immunofluorescence to examine serological relationship between the avian serotype, but there were no reports on the application of this technique for identifying field isolates (Bradbury, 1982).

Direct immunoflourescent staining of *Mycoplasma* colonies grown on agar medium has also been used (Stewart, 1967; Al-Aubaidi and Fabricant, 1971). In another technique, *Mycoplasma* colonies were transferred from agar blocks onto glass slides by submerging the agar blocks and slides in boiling water and the colonies were heat fixed to the slides while, the agar melted (Lemeka and Leach, 1968; Karbe and Helmboldt, 1968). Other researchers grew colonies on coverslips to eliminate the necessity of transferring agar grown organisms, centrifugation preparation and use of chick embryo (Ertel *et al*., 1970). Clark *et al*. (1963) studied the direct fluorescent antibody technique for the identification of *Mycoplasma* in broth cultures and in tissue culture fluid and they concluded that this method has the distinct advantage of rapid identification and determination of *Mycoplasma* growth. Perreau *et al*. (1969) also used this technique to classify 13 murine *Mycoplasma* strains and concluded that it was clearly the most useful and distinct criterion for classifying the murine serotypes. *M*. *bovimastidis*, a bovine type, was successfully identified by the Immunoflourescent technique. Studies on the identification of *M*. *mycoides* var. mycoides, *M*. *agalactiae*, *M*. *capri*, and other strains by the immunoflourescent method have reported to be satisfactory (Kleven *et al*., 1972).

**2.16 Nucleic acid detection techniques**

**2.16.1 Nucleic acid recombinant technique**

A rapid approach to diagnosis of *Mycoplasma* infections involve the use of nucleic acid probes (Razin, 1985). Specific recombinant DNA probes have been developed for the rapid detection of *Mycoplasma* (Hyman *et al*., 1989; Levisohn *et al*., 1989).

**2.16.2 Polymerase chain reaction (PCR)**

The use of PCR is very beneficial in the rapid diagnosis of clinical disease (Nascimento *et al*., 1993). The PCR seems to be not only specific but a sensitive method for amplifying low amounts of nucleic acid (Nascimento *et al*., 1993). In early infections or during carrier stage when conventional serological and cultural procedures may fail, this sensitive method for detecting the organism would facilitate diagnosis of the infection (Nascimento *et al*., 1993). The ability of the PCR to detect extremely low levels of DNA however requires some caution on the use of this method for diagnosis of *M. synoviae*. PCR test for the detection of *Mycoplasma* is 1000-10,000 times more sensitive than DNA probe technology (Nascimento *et al*., 1993). It is a simple and rapid procedure that does not require sophisticated equipment. PCR using 16S rRNA gene sequences as a target for the detection and identification of *Mycoplasma* spp is more common. It is due to several reasons like, (a) 16S rRNA genes are well conserved within a species; (b) the 16S rRNA gene sequences of almost every known bacterium are available and the specificity of selected primers can be precisely assessed; (c) 16S rRNA genes consist of highly conserved regions and regions with higher interspecific variability (Woese *et al*., 1980**)**. *Mycoplasma synoviae* PCR product can be visualized by agarose gel electrophoresis within 7 hours. Pourbakhsh *et al*., (2010) found that,a 8 μl aliquot of each PCR products mixing with 2 μl loading buffer (6X) and separated by electrophoresis in an 1% agarose gel with 0.5 μl /ml ethidium bromide (100 volts for 1 hour) following visualization using UV transillumination is effective.

**2.17 Prevalence of *Mycoplasma synoviae***

*Mycoplasma synoviae* (MS) infection occurs worldwide in commercial poultry flocks and may result in severe economic losses. Seifi and Shirzad (2013) reported that the highest prevalence of *Mycoplasma synoviae* was found 41.2% in the year 2003 among 315 broiler breeder farms in Iran. They also found highest prevalence in above 60 weeks of age (43.1%), but at 10-20 weeks it was lowest (12.7%). Seifi and Shirzad (2013) found prevalence of MS was lowest (30.1%) in coastal areas than other parts (36.9%) of Iran. In Australia a MS prevalence study was conducted by Gole *et al*. (2012) and they found (69%) prevalence. Elgnay and Azwai (2013) reported 6.4% seroprevalence in day old broiler chickens in Libya. Pérez *et al*. (2011) reported the prevalence of MS to be 64.12% in layers and 55.87% in broiler breeders in Venezuela. Onunkwo and Onoviran (1978) found a seroprevalence of 22% for *M. synoviae* among 1,604 chickens in Plateau State of Nigeria. They further recorded a seropositive rate of 15% for *M. synoviae* among 234 guinea fowls. Nawathe *et al*. (1982) screened 175, 117, 165, 61, 61, 30 and 92 chicken sera from Lagos, Anambra, Imo, Benue, Kaduna, Plateau and Kano States of Nigeria, respectively. For *M. synoviae* they recorded the following corresponding prevalence of 66.1%, 35.3%, 54%, 45%, 40%, 40% and 58.5%. Abdu *et al.* (1983) in Zaria of Nigeria found that 99% of the 93 chickens sampled from 37 flocks were seropositive for *M. synoviae*. A 66.33% prevalence of MS was reported by Heleili *et al.* (2012) in Algerian Commercial farms. Suzuki *et al.* (2009) reported 53% seroprevalence in backyard checkens in Paraguay. In the Brazilian states of São Paulo, Paraná and Pernambuco 72.7% seroprevalence was reported by Buim *et al.* (2009). Uchida *et al.* (1987) reported 40% and 20% prevalence in commercial layers and broiler chicken respectively in the year 1986 in Japan. In Denmark 73% and 10% seroprevalence of MS in commercial layer and broiler breeder farms was reported by Feberwee *et al.* (2008). A study on Thai native chickens in the area of Nakornpathom province of Thailand was conducted by Pakpinyo *et al.* (2009). They found 60% seroprevalence in birds of the age of 3-4 months. In Pakistan 76.57% seroprevalence in commercial broiler farms was reported by Ehtisham-ul-Haque et al. (2011). In Bangladesh 61-67 % birds have been found to be sero-positive to *Mycoplasma* *synoviae* (Giasuddin *et al*., 2002).

**2.18 Sample collection and isolation of *Mycoplasma synoviae***

*Mycoplasma* can be detected in tissue fragments of affected organs like trachea, air sacs and lungs. Besides synovial, ocular and infraorbital sinus exudates good sources are swabs from trachea and air sacs, and pipped embryos (Sentíes-Cué *et al*., 2005; Vogl *et al*., 2008). Swabs from trachea and choanal cleft constitute excellent specimens, mainly for isolation or PCR, which are used as confirmatory tools for monitoring *M*. *gallisepticum* and *M*. *synoviae* infections in live birds (Salami, 1986).

Isolation and propagation have received a noticeable advancement in avian mycoplasmology. Any of the cultural procedures, namely artificial media, embryonated eggs and tissue culture or a combination of these may be employed for isolation and propagation of *Mycoplasma* from suspected tissues, eggs and exudates (Bencina *et al.,* 1988 a, b). Extraneous bacterial contamination is usually controlled by the inclusion of thallium acetate and penicillin in the original inoculums of medium used (Adegboye, 1977).

Frey’s medium (Frey *et al*., 1968) or a modification of Frey’s medium (Kleven, 1994) is commonly used for isolation of *M. gallisepticum*. Frey’s medium supplemented with NAD is used for isolation of *Mycoplasma synoviae* (Frey *et al*., 1968). Direct plating of exudates or tissue swabs onto agar medium (Kleven, 1994) may give rise to colonies after 4-5 days of incubation, but initial enrichment in broth is generally a more sensitive method (Heleili *et al*., 2011). Cultures are incubated at 37˚ C for 5-7 days when growth may not be evident, but 2 or 3 serial passages at 3 to 7 day intervals may increase the number of isolations (Ley and Yoder, 1997).

Confirmation of *Mycoplasma* isolates as *Mycoplasma synoviae* is commonly done by antibody-based procedures. Direct immunofluorescence employing colonies on the surface of agar plates, or colony imprints, has been very effective for culture and identification (Talkington and Kleven, 1983). These techniques and their modifications are particularly useful for identification of *Mycoplasma synoviae* in cultures containing other *Mycoplasma* species (Bencina and Bradbury, 1992). The agar gel precipitin test is also used to identify cultures (Nonomura and Yoder, 1977).

**2.19 Treatment and control of *Mycoplasma synoviae***

Mycoplasmosis can be successfully treated using a range of antibiotics, although resistance to some antibiotics has been recorded (Nascimento *et al*., 1999). Mycoplasmas are resistant to antibiotics that act on cell wall, such as penicillin, but are sensitive to tetracyclines (oxytetracycline, chlortetracycline and doxycycline), macrolides (erythromycin, tylosin, spiramycin, lincomycin, and kitasamycin), quinolones (imequil, norfloxacin, enrofloxacin and danofloxacin) or tiamulin (Nascimento *et al*., 1999). Drugs that accumulate in high concentrations in the mucosal membranes of the respiratory and genitourinary tracts, such as tiamulin and enrofloxacin (Nascimento *et al*., 1999) are often preferred (Stipkovits and Kempf, 1996). Areas of control of avian mycoplasmosis should include careful choice of source of chickens, good management and hygiene practices along with periodic serological testing of chickens and maintaining of flocks free of the organisms (Kleven *et al*., 1984).

Flock testing and eradication programmes have been applied and found to be valuable as a control measure in some countries that have been able to pursue the measure diligently (Yamamoto *et al*., 1983). Vaccination against *Mycoplasma synoviae* (MS) can be a useful long-term solution in situations where maintaining flocks free of infection is not feasible, especially on multi-age commercial egg production sites **(Kleven, 2008).**

**CHAPTER – ӀӀӀ**

**MATERIALS AND METHODS**

**3.1 Study area and season**

The study was conducted in the breeder farms of Chittagong District from January 2012 to December 2012 encompassing **Summer (March to May)**, **Winter** **(November to February)** and **Rainy (June to October)** seasons. The research work was carried out in the Poultry Research and Training Centre (PRTC) Laboratory, Chittagong Veterinary & Animal Sciences University, Khulshi, Chittagong.

**3.2 Sample size**

Sample size was determined using the standard formula adopted from Araoye (2004):

N=z2pq/d2

Where

N= sample size

Z=1.96 confidence interval

P= prevalence, which is 61% for MS (Giasuddin *et al*., 2002)

D=5% allowable error

Q=1-p

Using this formula the minimum sample size was calculated to be around 365 for the breeder chickens for the Chittagong district in Bangladesh.

**Experimental Design**

Collection of Blood sample (1 – 1.5 ml /bird) from unvaccinated (against MS) poultry breeder farms of Chittagong.

Blood samples were kept in stable condition for at least 40 minutes and then the straw colored fluid was centrifuged at 1500 rpm for 10 minutes.

The clean straw colored serum was poured into new eppendorf tube and stored at -200C until used.

For SPA test a 25 μl of serum sample and 25 μl of standard antigen were placed side by side on a clean glass plate and mixed well by stirring with a small tooth pick followed by gentle rocking.

Results were read within 2 minutes over a light source.

Serum plate agglutination (SPA) test negative

Serum plate agglutination (SPA) test positive

Tracheal swab was collected from SPA positive flocks and Swab samples were stored in PBS buffer at 4˚C over night

Five samples of each flock were pooled and then DNA extraction was performed

PCR

PCR products were analyzed by electrophoresis on 1% agarose gel and examined under UV light using an image documentation system.

**Figure 3.1** Flowchart showing different steps to be followed during this study**.**

**3.3 Blood collection and serum preparation**

A total of 365 blood samples were randomly collected from the breeder farms where the birds were not vaccinated against MS. About 1-1.5 ml of blood was collected from wing vein using a fresh disposable plastic syringe (3 ml volume) for each bird. The blood samples were kept at room temperature for about 1-2 hours and then the straw colored fluid was centrifuged at 1500 rpm for 10 minutes using a bench centrifuge (VELOCITY 18RTM refrigerated centrifuge). A clean straw colored serum was seen up and was poured into a labeled eppendorf tube and stored at -200C until used.

**3.4 Serum plate agglutination (SPA) test**

The SPA test was conducted using the method as described in the work of Sarkar *et al*. (2005). In this study crystal violet stained *M. synoviae* commercial antigen (obtained from Bio Vac.) was used. A 25 µl volume of antigen was placed side by side on a glass plate with 25 µl of the serum using a micropipette. The serum and the antigen were then mixed well by stirring with a small tooth pick followed by gentle rocking. Results were read within 2 minutes over a light source. In positive cases granules were formed slowly which could be seen during rocking. In the negative cases, no such granules were formed. The strength of the agglutination reaction was measured according to the following scheme:

− = No clumps, no background clearing

+ = Small clumps, no background clearing

++ = Medium sized clumps, almost complete background clearing

+++ = Large clumps, complete background clearing





Figure No agglutination

Figure 3.3. Visible agglutination

Figure 3.2. No agglutination

**3.5 Polymerase chain reaction (PCR)**

**3.5.1 Specimen for PCR**

For PCR test, tracheal swab samples were taken randomly from each of the sero –positive breeder flocks among the studied breeder farms. Five tracheal swab samples of each flock were pooled for DNA extraction. According to the protocol adopted by Silveria *et al*. (1996) swab samples were stored in PBS at 4˚C over night then DNA extraction was performed.

**3.5.2 DNA Isolation**

DNA was extracted using the protocol described in OIE Terrestrial Manual 2008. In brief:-

1. Swab samples (five pooled) suspended in 1 ml of PCR-grade PBS in a1.5 ml snap-

cap Eppendorf tube.

1. The suspension was centrifuged for 30 minutes at 14,000 g at 4˚C.
2. The supernatant was carefully removed with a Pasteur pipette.
3. The remaining pellet was suspended in 25 µl PCR- grade water.
4. The tubes with the contents were boiled for 10 minutes and then placed on ice for

10 minutes.

1. Then centrifugation at 14,000 g for 5 minutes.
2. The supernatant containing the DNA was used as template DNA for PCR.

**3.5.3 Selection of Primers**

Targeted fragment (214 bp) of 16S rRNA gene of *Mycoplasma synoviae* was selected for PCR amplification. Details of the primers used for PCR are shown in **Table 3.1**.

**Figure 3.4.** Nucleotide sequence (5′-3′) of 16S rRNA gene fragment (214 bp) amplified by the selected primers (Forward marked- in yellow and Reverse in green).

**Mycoplasma synoviae 53 strain 53 16S ribosomal RNA, complete sequence**

GenBank accession no: NR\_074745.1

1 GATGAACGCTGGCTGTGTGCCTAATACATGCATGTCGAGCGGAATTTAGCAATAAATTTA

61 GCGGCGAATGGGTGAGTAACACGTACTTAACGTACCTTTTAGACTGGAATAACGGTGAGA

121 AATTATCGCTAATGCCGGATATATAAAAAAATCGCATGATTTTTTTAAGAAAGAAGCGTT

181 TGCTTCACTAAGAGATCGGGGTGCGGAACATTAGCTAGTTGGTAGGGTAATGGCCTACCA

241 AGGCAATTATGTTTAGCGGGGTTGAGAGACTGAACCGCCACACTGGGACTGAGATACGGC

301 CCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTTCCACAATGGGCGAAAGCCTGATGGA

361 GCAACACAGCGTGTAGGATGAAGGCCTTCGGGTTGTAAACTACTGTTATATGGGAAGAAA

421 AACTAGTATAGGAAATGATATTAGCTTGACGGTACCATGTCAGAAAGCAACGGCTAACTA

481 TGTGCCAGCAGCCGCGGTAATACATAGGTTGCAAGCGTTATCCGGAATTATTGGGCGTAA

541 AGCGTCTGTAGGTTGTTTGTTAAGTCTGGTGTTAAAACTTGGAGCTCAACTCCAAATTGC

601 ATTGGATACTGGCAGACTAGAATTGTTTAGAGGTTAGCGGAATTCCTTGTGAAGCGGTGG

661 AATGCGTAGATATAAGGAAGAACACCAACATGGCGAAGGCAGCTAACTGGGAACATATTG

721 ACACTGAGAGACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTG

781 TAAACGATGATGACTAGTTGATGGAAACCATCGACGCAGCTAACGCATTAAGTCATCCGC

841 CTGAGTAGTATGCTCGCAAGAGTGAAACTTAAAGGAATTGACGGGGATCCGCACAAGCGG

901 TGGAGCATGTGGTTTAATTTGAAGATACGCGTAGAACCTTACCCACTCTTGACATCTTCT

961 GCAAAGCTATAGAGATATAGTGGAGGTTAACAGAATGACAGATGGTGCATGGTTGTCGTC

1021 AGCTCGTGTCGTGAGATGTTCGGTTAAGTCCTGCAACGAGCGCAACCCTTGTCCTTAGTT

1081 ACTTTATCTAAGGAGACTGCCCGAGTAATTGGGAGGAAGGTGGGGACGACGTCAAATCAT

1141 CATGCCTCTTACGAGTGGGGCAACACACGTGCTACAATGGACGATACAAAGAGAAGCAAA

1201 ATAGTGATATCAAGCAAATCTCAAAAAATCGTTCTCAGTTCGGATTGTAGTCTGCAACTC

1261 GACTACATGAAGTCGGAATCGCTAGTAATCGTAGATCAGCTACGCTACGGTGAATACGTT

1321 CTCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCTGGTAATGCCCGAAGTCGGTT

1381 TGTTAACTTCGGAGACGACTGCCTAAGGCAGGACCGGTGACTGGGGTGAAGTCGTAACAA

1441 GGTATCCCTACGAGAACGTGGGGATGGATTACCTCCTTTCTTACGGAGTACATTAATTTT

1501 ACAAAAGGCATTTTTTATTAATTGAAAGCT

**Table 3.1.** Details of the primers used for PCR

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Primer name** | **Gene** | **Nucleotide Sequence (5′-3′)** | **Product size** | **Reference** |
| MS - F  MS - R | 16S r RNA | 5´ GAG AAG CAA AAT AGT GAT ATC A-3´  5´ CAG TCG TCT CCG AAG TTA ACA A-3´ | 214 bp | Pérez *et al*., 2011 |

**3.5.4 Polymerase chain reaction mixture and thermal cycles**

**Table 3.2.** Composition of 2X PCR Master mix solution (i-Taq TM, iNtRON)

|  |  |
| --- | --- |
| **2x PCR Master mix solution (i-TaqTM)** | **0.5 ml x 2ea (1 ml x 5ea)** |
| TaqTM DNA polymerase (5U/µl) | 2.5 U |
| dNTPs | 2.5 Mm each |
| PCR reaction buffer | 1X |
| Gel loading buffer | 1X |

The reaction mixture was prepared in a separate clean area using a set of dedicated pipettes. For one 20 µl PCR reaction the mixture was as follows:

**Table 3.3.** Composition of reaction mixture

|  |  |
| --- | --- |
| **Components** | **Volume** |
| 2X PCR Master mix | 10 µl |
| DNA Template | 2 µl |
| F primer (10 pmole/μl) | 1 µl |
| R primer (10 pmole/μl) | 1 µl |
| UP Water | 6 µl |
| Total Volume | 20 µl |

1. 10 µl of 2x PCR Master mix Solution was dispensed into each PCR tubes.
2. Then 2 µl of Template DNA was added into the PCR tube.
3. Then 1 µl (10 pmol / µl) of each (forward and reverse) primer was added.
4. Finally 6 µl distilled water was added into each PCR tube.
5. The mixture was then mix thoroughly.
6. The reaction mixture was overlaid with a few drops of light weight mineral oil.
7. Positive and negative controls were used in each run.
8. The tubes were then placed in a thermal cycler for the following cycles

The thermal cycler was programmed according to the program outlined in **Table 3.4** to run the PCR.

**Table 3.4.** Steps and conditions of thermal cycling for PCR

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sl No.** | **Steps** | | **Temperature** | **Time** |
| Step 1 | Initial denaturation | | 94°C | 4 min |
| Step 2 | 40 Cycles | Denaturation | 94°C | 30 sec |
| Step 3 | Anneling | 55°C | 30 sec |
| Step 4 | Extension | 72°C | 60 sec |
| Step 5 | Final Extension (1cycle) | | 72 °C | 5 min |
| Then soaked at 4˚C. | | | | |

**3.5.5 Electrophoresis**

PCR products were analyzed by electrophoresis on 1% agarose gel and examined under UV light using an image documentation system.

The procedure was performed by following ways:

1. Gel casting tray was assembled with gel comb of appropriate teeth size and number.
2. 1% agarose solution was prepared in 50 ml (1x)TAE buffer by melting in a microwave oven.10µl of ethidium bromide was added to molten agarose to have a final concentration of 0.5µg/µl.
3. Molten agarose was poured on to the casting tray and allowed to solidify on the bench.
4. The hardened gel in its tray was transferred to the electrophoresis tank containing sufficient TAE buffer. Then the comb was gently removed.
5. 5µl of each PCR product was mixed with 1µl of (6x) loading dye and the sample was loaded to the appropriate well of the gel.
6. 2µl DNA (100 bp) size marker was loaded in the first well.
7. The leads of the electrophoresis apparatus were connected to the power supply and the electrophoresis was run at 100 V for 1 hour.
8. Then the gel was gently placed on the UV transilluminator (Biometra, Germany) in the dark chamber of the image documentation system. The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in computer.

**3.6 Statistical analysis**

All data were entered into a spreadsheet programme (Excel 2007, Microsoft Corporation) and transferred to Stata 11 (Intercooled Stata 11, Stata Corporation, College Station, Texas, USA) for analysis. The difference in the seroprevalence rate of *Mycoplasma synoviae* in between variables was shown using a χ2 test.

**CHAPTER – ӀV**

**RESULT**

To study the seroprevalence of *Mycoplasma synoviae* (MS) serum plate agglutination test was done based on the previous research works where the sensitivity and specificity of serum plate agglutination test was compared with the culture, polymerase chain reaction (PCR) and various commercial enzyme linked immune sorbent assay tests (Feberwee *et al.*, 2005; Pourbakhsh *et al*., 2010; Seifi and Shirzad, 2013). In the present study sera samples were collected from the different breeder farms of Chittagong district where the birds were not vaccinated against MS. After collection of the samples serum plate agglutination (SPA) test was done followed by polymerase chain reaction (PCR) as a confirmatory test for identification of the organism as the culturing of MS is costly, time-consuming and inconclusive (Ewing *et al* 1998). A total of 365 sera samples were tested using serum plate agglutination (SPA) test, among them 220 were positive and 145 were negative. Details of the Seroprevalence of *Mycoplasma synoviae* with different risk factors (variables) are shown in **Table 4.1**.

**Table 4.1**. Seroprevalence of *Mycoplasma synoviae* with different risk factors

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Risk  Factors | Level of Risk  Factors | Seronegative (%) | Seropositive (%) | Total  tested  sample | χ2 | P value |
| Farm | 01 | 31 (37.80) | 51 (62.20) | 82 | **24.451** | **0.000** |
| 02 | 16 (34.04) | 31 (65.96) | 47 |
| 03 | 35 (71.43) | 14 (28.57) | 49 |
| 04 | 25 (34.25) | 48 (65.75) | 73 |
| 05 | 16 (30.77) | 36 (69.23) | 52 |
| 06 | 22 (35.48) | 40 (64.52) | 62 |
| Flock  size | 4000-7000 | 61 (46.21) | 71 (53.79) | 132 | **6.531** | **0.038** |
| 7001-10000 | 56 (40.58) | 82 (59.42) | 138 |
| >10000 | 28 (29.47) | 67 (70.53) | 95 |
| Age  (weeks) | 10 – 19 | 41 (57.75) | 30 (42.25) | 71 | **12.978** | **0.024** |
| 20 – 29 | 27 (38.57) | 43 (61.43) | 70 |
| 30 – 39 | 16 (38.10) | 26 (61.90) | 42 |
| 40 – 49 | 19 (35.85) | 34 (64.15) | 53 |
| 50 – 59 | 20 (34.48) | 38 (65.52) | 58 |
| >60 | 22 (30.99) | 49 (69.01) | 71 |
| Seasons | Summer | 65 (42.48) | 88 (57.52) | 153 | **1.109** | **0.574** |
| Winter | 31 (35.63) | 56 (64.37) | 87 |
| Rainy | 49 (39.20) | 76 (60.80) | 125 |

**Table (4.1)** shows that seroprevalence of MS was highest 69.23% in farm 05 among the different breeder farms under the study. The seroprevalence was found to be lowest 28.57% in the farm 03. There was significant (p<0.01) differences in the seroprevalence of MS with farm 03 to other breeder farms under study in Chittagong district.

The seroprevalence of MS infection in the breeder farms was highest 70.53% with the flock size of >10000 whereas it was lowest 53.79% with the flock size ranging between 4000- 7000 birds. From this result it became evident that higher the flock size greater the seroprevalence of MS in the breeder farms of Chittagong district. There was significant (p<0.05) variation in terms of seroprevalence of MS among the different flocks of the breeder farms in Chittagong district **(Table 4.1)**

As shown in **(Table 4.1)**, the seroprevalence of MS in breeder farms of Chittagong district was lowest 42.25% in the lowest age group (10-19 weeks) and highest 69% in the highest age group (>60 weeks) of birds. This gives an indication that as the age of birds advanced the seroprevalence of MS infection in the breeder farms was also advanced. There was significant (p<0.05) difference in the seroprevalence of MS in the different age groups of birds from various breeder farms of Chittagong district.

The seroprevalence of MS in the winter season was found as high as 64.37% whereas it was lowest 57.52% in the summer season. However, the difference in the seroprevalence of MS was statistically not significant (p>0.05) for the winter, summer and rainy seasons in the breeder farms of Chittagong district.

**Table 4.2**. Overall Seroprevalence of *Mycoplasma synoviae* in poultry breeder farms of Chittagong.

|  |  |  |
| --- | --- | --- |
| Farm | % positive | Average |
| 01 | 62.20 % | 60 % |
| 02 | 65.96 % |
| 03 | 28.57 % |
| 04 | 65.75 % |
| 05 | 69.23% |
| 06 | 64.52 % |

The highest seroprevalence was found 69.23% and lowest seroprevalence was found 28.57% among the studied breeder farms in Chittagong district. Overall Seroprevalence of *Mycoplasma synoviae* was found (60%).

**4.1 Confirmation of *Mycoplasma synoviae* infection by Polymerase chain reaction (PCR).**

Polymerase chain reaction (PCR) test was done to confirm the presence MS organism among the studied breeder farms in Chittagong district. For PCR tracheal swab samples were taken randomly from each of the sero –positive breeder flocks among the studied breeder farms. Five tracheal swab samples of each flock were pooled for DNA extraction. Fragment of 16S rRNA gene was amplified from genomic DNA extracted from tracheal swab samples using the forward primer MS - F and reverse primer MS - R (Pérez *et al*., 2011). In the PCR reaction, 32 pooled samples amplified 214 bp fragments. The PCR for 16S rRNA gene was optimized accordingly and the specific band was found at a template volume of 2 µl, 40 cycles of PCR amplification, 550C annealing temperature, while primer concentration was 0.5pmol/µl and total reaction volume was 20µl. Fragment length of the PCR products were verified by comparing with Sizer ®100 bp marker on 1% Agarose gel. The results of PCR identification of *Mycoplasma synoviae* is shown in **Figure 4.4**. It is important to note that every farm under study showed positive result in PCR. Details of PCR result are shown in **Table 4.3.**

Table 4.3. Result of Polymerase chain reaction (PCR).

|  |  |  |  |
| --- | --- | --- | --- |
| Farm ID | Sample No. | No. of Pooled Sample | No. Positive |
| 01 | 30 | 6 | 3 |
| 02 | 20 | 4 | 2 |
| 03 | 35 | 7 | 2 |
| 04 | 25 | 5 | 3 |
| 05 | 30 | 6 | 5 |
| 06 | 20 | 4 | 3 |
| Total | 160 | 32 | 18 |

**Figure 4.1.** Results of PCR for 16S rRNA gene of MS; Lane M: 100 bp ladder; Lane N: Negative control; Lane 1-5: 16S rRNA gene-sized (214bp) amplicon; Lane P: Positive control.



**CHAPTER – V**

**DISCUSSION**

The present study was conducted to determine the sero- prevalence of *Mycoplasma synoviae* infection among the breeder farms of Chittagong district. A total of 365 sera samples were collected during January 2012 to December 2012 encompassing **summer (March to May)**, **winter** **(November to February)** and **rainy (June to October)** seasons. Sera samples were tested using Serum Plate Agglutination (SPA) test. To determine the true existence of *Mycoplasma synoviae* infection among the sero-positive breeder farms tracheal swab samples were collected and polymerase chain reaction (PCR) was performed.

Among the studied farms seroprevalence of *Mycoplasma synoviae* infection was highest 69.23% in farm No. 05 and lowest 28.57% seroprevalence was found in farm No. 03. In case of flock size Seroprevalance was highest 70.53% in flocks having >10000 birds and lowest 53.79% in flocks having 4000- 7000 birds. Seroprevalance varied among different age groups, highest 69% seroprevalence was found in >60 weeks age groups and lowest 42.25% in age group 10-19 weeks. The **Table 4.1** shows that, there was significant (p<0.05) difference in the seroprevalence of MS in different farms, age groups and flocks in various breeder farms of Chittagong district. Though, there was statistically no significant (p>0.05) difference in seroprevalence of MS among the winter, summer and rainy seasons in the breeder farms of Chittagong district, the highest 64.37% and lowest 57.52% were found in winter and summer season respectively.

*Mycoplasma synoviae* can be transmitted vertically and horizontally (Stipkovits and Kempf, 1996). Results showed that the prevalence of MS infection highest 69.23% and lowest 28.57% in farm 05 and 03 respectively. In farm 03 strict culling of flocks showing *Mycoplasma* like signs were practiced. It reduces the risk of vertical transmission of *Mycoplasma* to the offspring. This is in agreement with a similar report demonstrated by Feberwee *et al.,* (2008) who reported lowest MS infection 6% in farms where intensive culling was practiced. The high prevalence 69.23% of *M. synoviae* infections in breeder stock can be explained by the frequent occurrence of multiple age housing and lower biosecurity standards in this sector (Stipkovits and Kempf, 1996; Kleven and Ferguson-Noel, 2008). The higher prevalence of *Mycoplasma* infection might be due to the replacement of breeding stock with the progeny of the same flock (Hossain *et al*., 2007). However, intensive nature of poultry farming provided opportunity for recycling of the pathogens due to population density (Pradhan, 2002). The other factors that contribute MG infection are poor ventilation, contamination of litters and no restriction on the movement of the technical personnel, visitors and such other persons as well as other bio-security measures (Dulali, 2003). There is no relation between a special breed with MS infection status (Seifi and Shirzad, 2013; Dufour-Gesbert *et al.*, 2006).

Size of flock does not have an impressive effect in appearance of *Mycoplasma* *synoviae* infection but this problem is worse in the greater sizes of flocks (Seifi and shirzad, 2013). Seroprevalence investigation shows **Table 4.1** that the *Mycoplasma synoviae* infection was found highest 70.53% in the large flock having >10000 birds. Whereas it was found lowest 53.79% in small size flock having birds ranges from 4000- 7000. The same finding was in agreement with Heleili *et al*., 2012; Talha, 2003, they showed that the highest infection rate 76.97% in large flocks (18000 birds) in comparison to small (500-1000 birds) flocks. Seifi and shirzad (2013) showed the highest infection rate 57% in large scale flocks (>40,000 birds) in comparison 41.9% to small (up to 30,000 birds) flocks. Similar report was demonstrated by Dufour-Gesbert *et al.* (2006). This variation may be due to horizontal transmission of infection, deficiency in management and low biosecurity (Feberwee *et al*.,2008; Hossain *et al*., 2007; Pradhan, 2002; Dulali, 2003).

Age is a very important parameter influencing the incidence of mycoplasmosis (Whitford *et al*., 1994). The seroprevalence of *Mycoplasma synoviae* was found higher in advancing age. The highest 69% and lowest 42.25% prevalence found in age group (> 60 weeks) and (10- 19 weeks) respectively. This finding was in agreement with other research groups Feberwee *et al*. (2008) reported that, the seroprevalence of *Mycoplasma synoviae* was highest (60%) in age group above 51 weeks. Same type of findings (12%) seroprevalence in age group (10-20 weeks) and (43%) seroprevalence in age group above 60 weeks was suggested by Seifi and Shirzad (2013). The prevalence study of Hagan *et al.* (2004), which was based on the detection of *M. synoviae* antibodies in eggs, reported a prevalence of 78.6% in commercial layer flocks in East England. In another study (Mohammed *et al.*, 1986), a *M. synoviae* prevalence of 87% was found in commercial layer flocks in Southern California. The infection was associated with older flocks that had been moulted or frequently medicated. Stipkovits and Kempf (1996) suggested that it may be due to more exposure of *Mycoplasma synoviae* organism to birds as their age advances because *Mycoplasma synoviae* can be transmitted both vertically and horizontally.

In the present study seasonal variation for the prevalence of *Mycoplasma synoviae* was observed. But the statistical analysis showed no significant (p > 0.05) variation among the prevalence of seasons, the seroprevalence was highest 64.37% in winter and lowest 57.52% in summer **Table 4.1**. This finding was in agreement with Seifi and Shirzad (2013), Heleili *et al*. (2011), Hossain *et al*. (2010) and Sikder *et al*. (2005) where they suggested that it might be due to the influence of cold weather. But the present findings was disagree by Heleili *et al*. (2012) and Arbelot *et al*. (1997) where they found that the seroprevalence was higher in summer season.

In the present study the overall seroprevalence of MS was 60% which is in agreement with Giasuddin *et al*. (2002). He reported that the seroprevalence of *Mycoplasma synoviae* in Bangladesh was 61- 67%.

For eradication of MS infection, rapid and accurate identification of MS is of great importance and molecular methods such as the PCR have been developed to improve this. Earlier MS specific PCRs were based on the 16S rRNA gene Lauerman *et al.* (1993) and Garcia *et al.* (1995) and more recently, some have been based on haemagglutinin genes (Hong *et al*.,2004 and Ben Abdelmoumen Mardassi *et al* ., 2005). In this study, species specific primers of Pérez *et al*. (2011) were used. These MS primers were selected from the 16S rRNA gene. The PCR method of Garcia *et al.* (1995) used by Galeh Golab et al. (2005, 2008) is also based on the 16S rRNA gene. The PCR used in this study is species specific and has been used in recent years by other workers successfully (Buim *et al*.,2010; Pérez *et al*., 2011). In this study, all sero- positive farms showed positive result in polymerase chain reaction (PCR). With these findings it may be concluded that those farms were harboring MS infection. It is possible to determine the presence of serum antibody using serum plate agglutination (SPA) test but not possible to determine whether the antibody is due to active infection or vaccination. To determine active infection or presence of *Mycoplasma synoviae,* culture of organism and PCR can be used. But *Mycoplasma* is a facultative organism. Its culture is time consuming and laborious (Ewing *et al.,* 1998). On the other hand PCR is comparatively easy, less time consuming and most reliable diagnostic tool.

These results strongly support the use of this PCR assay as an efficient alternative or supplement to culture and serological identification, which are labor-intensive, extremely time-consuming, and often provide confusing results. Overall, it is suggested that the PCR could be an alternative method for accurate identification of the MS infection especially in breeder chicken flocks.

**CHAPTER – VI**

**CONCLUSION**

*Mycoplasma synoviae* (MS) infection was found to be prevalent among the poultry breeder farms in Chittagong district. The overall seroprevalence of the disease was 60% with the highest and lowest rate of 69.23% and 28.57% respectively. The prevalence varied significantly among the different farms due to varied farm conditions, age groups and flock sizes however; there was not any seasonal variation. All sero-positive flocks were also found to be positive in PCR when tested for the presence of active infection with MS that was performed with the tracheal swab samples. Through this study PCR for the detection of MS have been established in PRTC laboratory of Chittagong Veterinary & Animal Sciences University that could be used as an easy, fast, specific and less laborious method for detection of MS against the conventional method of time consuming and difficult bacterial culture. The prevalence rate of MS that has been observed in this study may not be true for the whole country. To determine the actual prevalence rate on MS in Bangladesh a detail study covering the whole country may be conducted. Economic impact due to MS infection in poultry and the strain of MS involved was not assessed through this study. For proper control of the disease strain identification of MS has significant value. Further study is needed to be conducted in order to have all those questions answered.

**CHAPTER – VII**

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**APPENDIX**

**Questionnaire used during sample collection**

1. Name of the farm:…………………………........… 2. Date: …………....……..

………………………………………………

3. Name of the owner: ………………………………………………............................

1. Address of owner: …………………………………………

………………………………………..

1. Mobile No.: ……………………………………………....

4. Location of the farm:

1. Name of the Village: ……………………………………….
2. Name of the Upazilla: ………………………………………
3. Name of the District: ………………………………………

5. Person interviewed: …………………………………………………………………

1. Occupation: ………………………………………………
2. Relation with owner: ……......…iii. Mobile No. …………

6. Type of Farm: Broiler breeder. Layer breeder. 7. No. of birds…………

8. Name of the strain maintained: ………………………………………………….

9. Management details:

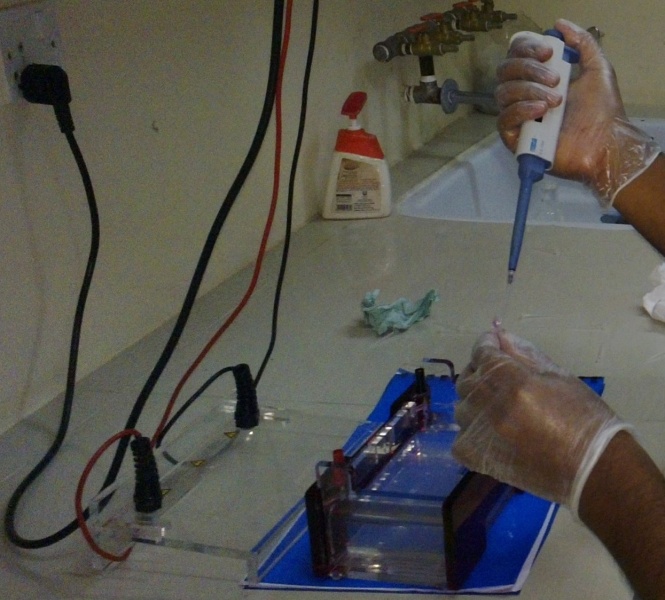
1. Bio- security status: Very well / Well / Good.
2. Type of house: Conventional housing / Environmentally controlled.
3. Type of rearing system: Case system / Litter system / Dual system.
4. Source of parent stock chicks: From own Grandparent / Purchased.

10. Vaccination history: Use of *Mycoplasma synoviae* vaccine. Yes / Not

11. Farm No.: ………. 12. Flock No.: ……….. 13. Age of the birds (weeks) …...…

14. Number of Blood sample: ………. 15. Number of tracheal swab sample: ……….

16. Miscellanies:

**Figure A1.** Some activities of my thesis work.A)Collection of blood from wing vein of chicken; B) Collection of tracheal swab sample; C) Performing serum plate agglutination (SPA) test; D) Observation of SPA test result; E) Loading of PCR product into appropriate well of gel; C) Visualization of PCR product band.

**B**

**D**

**F**

**C**

**A**

**E**