Seroprevelence of *Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella pullorum* and *Salmonella gallinarum* in different poultry Flocks.

A thesis submitted in partial fulfillment of the requirement for the Degree of

Master of Philosophy

In

Microbiology

BY

MUHAMMAD SHOAIB

DEPARTMENT OF MICROBIOLOGY

FACULTY OF BIOLOGICAL SCIENCES

QUAID-I-AZAM UNIVERSITY

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Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this elsewhere for any other degree.

Muhammad Shoaib

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Certificate

This thesis submitted by ***Muhammad Shoaib*** is accepted in its present form by the Department of Microbiology, Quaid-I-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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Muhammad Shoaib

Abstract

The Sero prevalence of *Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella pullorum* and *Salmonella gallinarium* infections in breeder, broiler and layer was determined during the period August 2013 to March 2014. A total of 5239 sera samples from different types of chickens were collected to conduct this study. Serum Plate Agglutination (SPA) test was used to check the presence of antibodies against all the four pathogens. Commercial *Mycoplasma gallisepticum* (MG) antigen (code SL 212, inactivated coloured antigen for SPA test, Soleil diagnostics France), *Mycoplasma synoviae* (MS) antigen (code SL 222, inactivated coloured antigen for SPA test, Soleil diagnostics France) and *Salmonella pullorum /gallinarium* (SPG) antigen (code SL 242, inactivated coloured antigen for SPA test, Soleil diagnostics France) were used respectively for *Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella pullorum /gallinarium.* The overall sero prevalence of *Mycoplasma gallisepticum, Mycoplasma synoviae* and *Salmonella pullorum /gallinarium* for broiler was 7.14%, 10% and 5.35% respectively. In the layer birds, sero prevalence was 44.9%, 42.6% and 51.32% for *Mycoplasma gallisepticum, Mycoplasma synoviae* and *Salmonella pullorum /gallinarium* respectively. The overall sero prevalence of *Mycoplasma gallisepticum, Mycoplasma synoviae* and *Salmonella pullorum /gallinarium* in case of broiler breeder was 59.6%, 50.13% and 44.1% respectively. The highest prevalence was found in the period from October to December. The study further showed that incidence was higher in female birds for *Mycoplasma gallisepticum* and in male birds for *Mycoplasma synoviae* and *Salmonella pullorum /gallinarium.* Results further revealed that incidence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* was higher in breeder than layer or broiler and for *Salmonella pullorum /gallinarium* incidence was highest in layers. The study showed that there is a need of appropriate strategies for successful prevention and control of these pathogens in the area. It was concluded from study that serum plate agglutination test could be used as quick tool to detect infection caused by *Salmonella* and *Mycoplasma* species.

Introduction

Poultry industry in Pakistan alleviates the poverty by offering enormous opportunities to millions of people in the country. Poultry is the second largest industry of the Pakistan after textile. The major byproducts of the poultry industry are meat and eggs. Pakistan poultry industry comprises of 400 hatcheries, 150 feed mills and 25000 poultry farms (Bhatti, 2007). Role of livestock in the gross domestic product (GDP) is 11.9 %, out of which share of poultry is 1.2% at constant cost factor. This sector produces 108.2 thousand tones of poultry meat and 3878 million eggs (Economics Survey, 2012-13). In spite of all these facts and a huge infrastructure, availability of poultry meat in Pakistan is 3.90 Kg per capita while 55 Kg per capita in Kuwait, 50 Kg per capita in USA and 12 Kg per capita in the world per annum (Bootwala, 2007). Similar situation prevails regarding the consumption of eggs. The above cited data indicates that poultry industry in Pakistan still have enormous potential to develop. However, recently poultry industry suffered great losses economic losses. The main threats are diseases caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) and *Salmonella gallinarium* and *Salmonella pullorum* (Marois et al., 2001). Main prevailing bacterial diseases in Pakistan are salmonellosis and mycoplasmosis (Rehman et al., 2013).

Although birds of all ages are susceptible but relatively young birds are more prone to Mycoplasmosis (Seifi and Shirzad, 2012). Mycoplasmosis is transmitted via eggs and hatchery disseminated and is economically devastating disease of chickens. *Mycoplasma* is a worldwide avian pathogen that causes immense losses in the poultry industry by decreasing egg production, reducing growth and increased condemnation at slaughterhouses (Kleven et al., 2008). About 10-20% losses in egg production occur in the flocks affected from mycoplasmosis (Bradbury, 2001). *Mycoplasma gallisepticum* (MG) is bacterium that does not possess cell wall and causes chronic respiratory disease (CRD) in the chickens (Siddique et al. 2012). Gasping, respiratory rales, coughing, nasal discharge and rhinitis are the major signs of the CRD. Sometimes MG may cause arthritis, salpingitis, conjunctivitis and fatal encephalopathy (Much et al., 2002). In the layer birds MG causes marked decrease in egg production and embryo mortality (Mukhtar et al., 2012).

Mycoplasma synoviae is also very important poultry pathogen worldwide. It may cause losses in terms of decreased egg production, growth retardation, and condemnation at slaughterhouse. It causes infectious synovitis respiratory infection and in the chickens it may result in sub clinical infection. Recent reports show that MS causes infectious synovitis and air sacculitis relatively more frequently in chickens and turkeys (Bencina et al., 2001). It causes air sacculitis that may also result in a co-infection with MG and *E. coli*. When infection becomes systemic, it causes inflammation of synovial membranes of joints and tendon sheaths causing synovitis, tenovaginitis and bursitis (Kleven et al., 2008).

Immune system of host fails to deal effectively with *Mycoplasma* specie because of chronic nature of the infection. *Mycoplasma* evades the host immune response by antigenic variation of surface proteins (Glew et al., 2000). Both MG and MS are transmitted either by vertical method or by direct contact between ill and susceptible birds (Marois et al., 2001). Ability of the *Mycoplasma* to survive within the host cell allows the pathogen to resist immune response of host and antimicrobial therapy (Winner et al., 2000). Age and size of the flock and locality are the factors which affect the severity of the disease. Great economic losses occur due to mycoplasmosis in broiler, breeder and layer birds in the terms of condemnation of carcass, reduced egg production and feed efficiency, more hatchability losses and increased cost for the treatment of the infection (Hassan et al., 2012). For timely treatment and control of mycoplasmosis early and timely diagnosis is necessary. Isolation of MG and MS is not reliable due to least tolerance in adverse environment and the fastidious nature of the organism (Levisohn and Kleven, 1984). *In vitro* cultivation of *Mycoplasma* is very difficult, expensive and time consuming. It requires three to four weeks to grow and even then there can be mixed growth or no growth. In the cultures, *Mycoplasmas* are over grown by the fast growing or apathogenic species of *Mycoplasmas*. Serological tests and molecular techniques are reliable methods for diagnosis of the disease. Serological tests like serum plate agglutination (SPA) test, ELISA and haemagglutination inhibition tests are mainly used. Serum plate agglutination test is a quick tool for flock screening although it may give false positive results because of cross reactivity of MG and MS (Kleven et al., 2000). While conducting serology of MG and MS cross reactivity of antigens is common problem (Ehtisham et al., 2010). Polymerase chain reaction (PCR) essays are commonly used for rapid detection of the MG and MS (Ahmed et al., 2009). Test and

slaughter policy is the most effective control measure for control of mycoplasmosis but this is expensive practice and impossible (Ley, 2003).

Fowl typhoid (FT) and pullorum disease (PD) are two most important bacterial diseases of chickens. *Salmonella gallinarium* and *Salmonella pullorum* (SPG) are the causative agents of fowl typhoid and pullorum disease (Tadele et al., 2014). *Salmonella gallinarium* and *Salmonella pullorum* are gram- negative and facultative anaerobes. Pullorum disease and fowl typhoid have become a wide spread problem due to expansion in poultry farming. Both these diseases show similar clinical signs and are of serious concern in all types of young and adult chickens (Berchieri et al., 2001). If eggs infected with *Salmonella gallinarium* and *Salmonella pullorum* are hatched, dead and dying chicks are often observed. These diseases are both vertically transmitted through egg to embryos and horizontally transmitted (Ivanics et al., 2008). In commercial poultry of many countries including Pakistan, *Salmonella pullorum* is quite prevalent (Ayesha et al., 2014). Increase in the number of multi-drug salmonella has developed a worldwide apprehension (Barrow et al., 2011). Incubation period of both the organisms is 4-6 days. Pullorum disease is the septicaemic disease of young chickens while Fowl typhoid is disease of growing period and observed in mature birds. Depression, weakness, somnolence, loss of appetite, drooping wings, huddling, dehydration and ruffled feathers are the common non generalized clinical signs observed in case of FT and PD (Kwon et al., 2000). Typical septicaemic birds, increased mortality and poor quality hatched chicks are clinical signs of the FT. Anemia, depression, labored breathing and diarrhea causing adherence of feaces to the vent are observed in chronic cases in the adult birds. *Salmonella pullorum* may cause mild disease or sub clinical disease. Birds of 2-3 week of age and at the point of lay are more susceptible to PD (Wigley et al., 2005).

Peritonitis, congestion of tissues and an inflamed unabsorbed yolk sac are general post mortem signs of PD in newly hatched chicks. Misshapen or shrunken ovaries and follicles with fibrous stalks are the post mortem signs observed in PD in adult birds (Eswarappa et al., 2009). Generalized septicemia and enlarged, dark and friable liver with bronze appearance are post mortem signs of the FT. Spleenomegaly and dark brown bone marrow are also observed (Shivaprasad, 2000). For PD and FT the most evident pathological findings are congested and necrotic foci on enlarged liver, Spleenomegaly, deformed ova, salpangitis and unabsorbed yolk (Hossain and Islam, 2004). *Salmonella pullorum* and *Salmonella Gallinarium* are frequently

diagnosed by culturing and serological test. Infected flocks are detected by serum agglutination test and used to find prevalence of infection in the flock (Proux et al., 2002). *Salmonella gallinarium* and *Salmonella pullorum* is host adapted to chicken and pose a minimal Zoonotic threat (Liu et al., 2002). Clinical signs flock history, mortality and post-mortem lesions help in the diagnosis of FT and PD but for definite diagnosis isolation and culture is required. Isolation and culture, serology and molecular techniques especially PCR are the major methods used for diagnosis of FT and PD (Barrow et al., 2012). Other than extensive use of antibiotics and vaccination, use of lytic bacteriophages in the poultry feed is emerging strategy for control of *Salmonella pullorum* and *Salmonella Gallinarium*. Currently, in poultry sector of Pakistan gigantic economic losses occur due to the avian Salmonellosis in terms of mortality, carcass condemnation, reduced weight and egg production, less feed conversion ratio (FCR) and hatchability losses.

Keeping in mind the economic importance and high incidence of the mycoplasmosis and salmonellosis, there is dire need to design the prevalence study to define and quantify the load of avian mycoplasmosis and salmonellosis in the region. Best measures for control of Mycoplasmosis and salmonellosis include biosecurity, hygiene, good management, monitoring and removal of infected flocks, routine vaccination and farmer awareness. To reduce the risk of transmission of disease to other poultry populations, there should be continued monitoring of flocks for MG, MS and SPG. This study might help the practitioners and the farmers for effective and strategic control of these diseases. In addition, it may help the farmers to follow the chemotherapeutic or immune- prophylactic methods to control the diseases and in turn mitigate the economic losses. The current study accentuates the importance of these diseases in the area and reports the sero-prevalence of MG, MS and SPG in the different types of poultry birds in the Rawalpindi region.

Aims and Objectives

Aim:

- The purpose of the study was to screen the poultry birds for *Mycoplasma* and *Salmonella* species. To achieve this, the objectives of my study are as follows.

Objectives:

- Isolation of *Mycoplasma* species from the tissues samples taken from birds at post mortem examination.
- Serological screening of *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella gallinarium* and *Salmonella pullorum* by serum plate agglutination test.
- To find the effect of age, gender, type of bird and season on sero prevalence of these pathogens in the poultry birds.

CHAPTER-2:
LITERATURE REVIEW

Review of Literature

In the last few decades poultry industry of Pakistan has made great progress involving switch from backyard poultry to commercial poultry industry. Despite of this growth poultry industry is facing numerous problems in terms of infectious diseases due to inadequate control measures and poor conditions (Mukhtar et al., 2012). Along with the surge of different viral outbreaks from time to time, there is consistent prevalence of variety of diseases in the standing poultry population (Alam et al., 2012). It is important to remain updated about the prevalence of different poultry disease and health issues of commercial birds because poultry diseases causes severe economic and production losses. Mean prevalence (%) of poultry diseases in commercial chicken including Broilers, layers and breeders from June 2011 to July 2012 in the Punjab province of Pakistan is given in table 2.1.

Table 2.1: Mean prevalence (%) of poultry diseases in commercial chicken (Rehman et al., 2013).

Sr. no	Diseases	Prevalence %
01	Infectious bursal disease	5.9
02	Infectious bronchitis	1.6
03	Avian influenza	0.8
04	Merek's disease	0.4
05	Newcastle disease	10.3
06	Hydro pericardium syndrome	2.5
07	***Mycoplasma gallisepticum***	**8.7**
08	***Mycoplasma synoviae***	**1.5**
09	**Pullorum disease**	**2.7**
10	**Fowl typhoid**	**7.1**
11	Fowl cholera	1.4
12	Collibacilossis	10.1
13	Infectious coryza	4.4
14	Endoparasite	3.0

15	Ectoparasite	0.7
16	Coccidiosis	13.1
17	Mycotoxicosis	6.1
18	Others	17.6

According to this study by Rehman et al., (2013) it is clear that among all the poultry diseases in Pakistan there is significant share of diseases caused by *Mycoplasma* and *Salmonella* species. Among the bacterial diseaseś mycoplasmosis and salmonellosis have got highest prevalence which accentuates the importance of these diseases in the Pakistan. Of these many health hazards faced by poultry industry in Pakistan, mycoplasmosis and salmonellosis are the major bacterial threats to the industry at the time.

2.1 Mycoplasma gallisepticum* and *Mycoplasma synoviae:

The term *Mycoplasma* was first used by A. B. Frank in 1889 referring these organisms as a fungus (Krass & Gardner, 1973). *Mycoplasmas* are free living, self replicating which are known to have smallest genome (Nicolas and Ayling, 2003). Cell wall is absent in *Mycoplasmas* and have low G+C content of 23-40%. The cell membrane of these organisms is incorporated with sterols which differentiates these from other organisms (Kleven, 2003). Based on 16S rRNA analysis *Mycoplasmas* belong to phylum Firmicutes, class Mollicutes and family Mycoplasmataceae (Ley, 2003).

Out of 22 known species of *Mycoplasmas* in the birds, the four common pathogenic species include *M. gallisepticum*, *M.synoviae*, *M. meleagridis* and *M. iowae* (Bradbury, 2001). Of all avian *Mycoplasma* pathogens, MG and MS are important species and MG is considered most pathogenic (Kleven, 2003). Turkeys, quails, partridges, pheasants and pigeons are also natural host of MG other than chicken (Ley, 2003). *Mycoplasma gallisepticum* causes chronic infections in both chickens and turkeys and is most virulent of all the *Mycoplasmas* species (Liu et al., 2001). *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cause respiratory disease both in chicken and turkeys while *M. iowae* and *M. meleagridis* cause disease only in poultry (Fan et al., 1995a).

2.1.1 Status in Pakistan:

In Pakistan for mycoplasmosis serological evidence is reported as early as 1964 (Mukhtar et al., 2012). The mean prevalence of diseases caused by MG and MS was found to be 8.7% and 1.5%

respectively in Punjab from June 2011 to July 2012 (Rehman et al., 2013). Incidence of *Mycoplasma* was found to be 12.69% in Faisalabad in 1997 in the flocks with respiratory problems (Tariq et al., 1987). The increase in sero prevalence was noted in 2000 and prevalence was 90% and 15.5% respectively for MG and MS (Mukhtar et al., 2000). The overall sero prevalence of MG in Baluchistan in broilers was 10% and 19.76% by serum plate agglutination (SPA) test and ELISA respectively. In case of layers sero prevalence of MG was 19.4% and 31.6% by SPA and ELISA respectively. In Baluchistan overall prevalence was 8% and 15% respectively by SPA and ELISA (Atique et al., 2012). A study conducted by (Mukhtar et al., 2012) showed that sero prevalence of MG in the layer birds in the Faisalabad region was 49.38%. A study conducted by Rehman et al., (2013) in Punjab province of Pakistan showed that prevalence of MG was found 31%, 39% and 45% in broiler, layer and breeder respectively. For MS the sero prevalence was found to be 12.4%, 36.3% and 34.1% for broiler, layers and breeders respectively. Both MG and MS are responsible for huge economic losses to poultry industry of Pakistan (Rehman et al., 2011).

2.1.2 Chronic respiratory disease (CRD):

Mycoplasma gallisepticum and *Mycoplasma synoviae* both cause Chronic Respiratory Disease (CRD) in the all types of chickens (Kleven and Bradbury, 2008). The primary causative agent of Chronic Respiratory Disease (CRD) is MG and it causes disease under stress and poor management conditions or when bird is suffering from some other respiratory problem (Papazisi et al., 2002). In the expanding poultry industry *Mycoplasma* is the most virulent avian pathogen and causes worldwide outbreaks leading to immense economic losses (Evans et al., 2005). *Mycoplasma gallisepticum* primarily damages respiratory tract by colonizing it and then secondary bacteria like *E. coli* and viruses causes severe infections (Liu et al., 2001). Extensive antibiotic treatment is used to keep *Mycoplasma* under check and attenuated vaccines are used to prevent the disease but complete eradication of pathogen is very difficult. *Mycoplasma gallisepticum* is the only avian *Mycoplasma* specie which is invasive *in vitro* as well. This is the reason why MG not only resists host defense and antibiotics but also enters the blood and causes systemic infection (Winner et al., 2000).

2.1.3 Transmission chain and economic losses:

Mycoplasma gallisepticum causes disease in birds of all age but young birds are more susceptible to this pathogen (Nunoya et al., 1995). In case of MG infection the organism first colonizes the

respiratory tract. In the different strains of MG the tissue tropism, cell injury, attachment and pathogenicity may vary (Domanska et al., 2008). *Mycoplasma gallisepticum* causes great economic losses in all types of chickens. It is vertically transmitted through egg and is hatchery disseminated. Decreased hatchability and low egg production count for the major economic losses caused by MG. Birds infected from MG produce low quality day old chicks and slower growth rate. This also leads to increased medication and control procedure costs in the farming (Ley, 2003).

2.1.4 Pathogenesis of Mycoplasmosis:

Saloglycoprotein receptors in the respiratory epithelium are required for the attachment of the *Mycoplasmas* and initiation of the disease. The process is mediated through cytadherence. To escape the innate host defense attachment is very important process. Since many metabolic pathways are absent in the *Mycoplasmas*, so for their survival *Mycoplasmas* need very close interaction with the host cell (Simecka et al., 1992). Adherence mechanisms of the *Mycoplasmas* are very similar to adhesion of *M. pneumonia* which is better studied and adhesion is mediated through cytadhesin genes (Goh et al., 1998).

Mycoplasma species have ability to cause direct cell injury although exact mechanism of cell injury is not well understood. Mycoplasmosis may cause cell injury by depriving nutrients, producing toxic substances and alteration in the host cell metabolites. *Mycoplasma* species may produce enzymes like phospholipases, proteases, and nucleases. These enzymes may cause membrane damage to host cell and can increase the chances of genetic alteration in host cell which may lead to auto immune disease (Bhandari & Asnani, 1989). *Mycoplasma* species produce hydrogen peroxide which play very important role in cell injury. Hydrogen peroxide released by mycoplasma causes oxidative stress to host cell and may also cause hemolysis. During adherence process *Mycoplasma* produces hydrogen per oxide which causes damage to cell membrane and facilitates the entry of *Mycoplasma*. Nascent oxygen (O^{-}_{2}) is produced from hydrogen per oxide by catalase enzyme. This Nascent oxygen (O^{-}_{2}) causes oxidative damage inside the host cell and responsible for major cell injury. To counter this oxidative damage antioxidant enzymes like glutathione (GSH) and superoxide dismutase (SOD) are produced by the host cell. In this way host cell directs its energy for production of these enzymes to counter oxidative damage caused by *Mycoplasma* (Razin, 2006).

Mycoplasmas may be transmitted vertically through egg or horizontally through close contact, air borne droplets and contaminated dust (Papazisi et al., 2002). It is difficult to maintain *Mycoplasma* free flocks because rapid expansion of poultry increases the risk of transmission of pathogen (Lysnyansky et al., 2005).

2.1.5 Signs and symptoms:

The clinical signs in the birds infected with MG include open mouth breathing, rales, and respiratory sounds. Nasal discharge, coughing and sometimes conjunctivitis are also seen in the infected birds (Saif et al., 2003). Lacrimation and depression is also observed in infected birds (Forrester et al. 2012). Fatal encephalopathy, arthritis and salpangitis are sometime seen in MG infected birds (Much et al., 2002).In the case of infected broiler breeder and commercial layer sharp decrease in egg production takes place. There is marked increase in embryo mortality in eggs of infected birds (Ley, 2003). The clinical signs in the case of MS are somewhat similar to MG. *Mycoplasma synoviae* causes subclinical upper respiratory tract infection and synovitis in chickens and turkeys is one of very important clinical finding (Khan, 2002). *Mycoplasma synoviae* causes air sacculitis more frequently than infectious synovitis (Bencina et al., 2001). *Mycoplasma synoviae* disseminates very quickly after it is introduced at farm because lateral spread of MS is quick both by direct contact and between cages (Kleven, 2003).

The major pathological finding in MG infection is the air sacculitis while in some birds upper respiratory tract infection may also be present (Hong et al., 2005). Pathogenic mechanisms of *Mycoplasmas* are controlled by number of factors which include ability of pathogen to attach host cell, type of cell injury and ability to resist host immune response.

2.1.6 Chemotherapeutics of MG and MS:

Since *Mycoplasmas* lacks cell wall, so cell wall inhibitors like penicillin etc are ineffective against the pathogen. Antibiotics that inhibit metabolic processes of microorganism like macrolides, tetracyclines, fluoroquinoles and others are effective against Mycoplasma (Ley, 2003). Tylosine and gentamycin are effective against *Mycoplasmas* but at higher doses Tylosine may be toxic to embryos and reduce the hatchability (Nascimento et al., 2005). Tilmicosin has lowest minimum inhibitory concentration (MIC) followed by tylosine for the *Mycoplasma* species (Hassan et al., 2012). For the control of *Mycoplasma* there is need to completely eradicate the organism from breeder stock and maintain *Mycoplasma* free stock by periodic

serological screening such as plate agglutination, haemagglutination inhibition (HI) test and ELISA kits.

2.1.7 Diagnosis of *Mycoplasma*:

For the diagnosis of mycoplasmosis a number of methods including serological methods, molecular techniques and isolation and identification have been used. Cultivation is most reliable method for the confirmatory diagnosis of Mycoplasma (Ley, 2008). Due to the limitations of diagnostic tests and the similarities in the disease caused by *Mycoplasmas*, specific diagnosis is very difficult. It is very important to characterize and identify the *Mycoplasma* species and strain variability. Brief review of various methods used for diagnosis of *Mycoplasma* is given below.

2.1.8 Isolation and identification:

Direct isolation and identification of *Mycoplasma* is not part of routine procedure used for diagnosis of *Mycoplasma* (Zain & Bradbury, 1996). The main reason for this is the fastidious and slow growing nature of the *Mycoplasma species*. *Mycoplasmas* require one to three weeks or even more for their growth and identification. Another major problem in isolation of *Mycoplasma* is the growth of fast growing non pathogenic *Mycoplasma* species and growth of other bacteria and fungi (Garcia et al., 1995). Selective pressures on populations of *Mycoplasmas* that differ substantially *in vivo* and *in vitro* are also an important factor. Pathogenic properties of the strain may be lost during passage in the culture media. *Mycoplasma* has very small genome and little capacity of biosynthesis and is dependent on host cell for its requirements. *Mycoplasma* is dependent on host for cholesterol, amino acids, fatty acids, vitamins, nucleotides and other nutrients that is why *in vitro* growth is very difficult. *Mycoplasmas* do not have regulatory gene involved in gene expression and cannot respond to the changing environmental conditions *in vitro*, it makes extremely fastidious to work with this organism (Razin et al., 1998). *Mycoplasmas* once isolated from their host tend to die rapidly if not placed in suitable medium and environment (Zain & Bradbury, 1996). Handling of samples between collection and inoculation is very critical for isolation of *Mycoplasmas*. Swabs dipped in *Mycoplasma* broth and placed at 4°C were more viable than dry swabs. Due to these reasons isolation of *Mycoplasma* is laborious, time consuming expensive and difficult task. Small size and lack of cell wall make morphological characterization of *Mycoplasma* very difficult. Due to these factors isolation and identification of *Mycoplasma* may not give true picture of *in vivo* presentation.

serum plate agglutination (SPA), ELISA, and haemagglutination inhibition (HI) (Kleven, 1998). Although serological tests are quick and fast they have their own disadvantages and limitations. Serological tests are based on detecting antibodies in the serum produced in response to antigens and subsequent detection of these antibodies. However, to prevent the spread of infection rapid diagnosis of *Mycoplasma* is necessary which is achieved through serological screening. Serological methods do not detect the sub clinical infections. We cannot use serological tests for detection of early infections as antibodies are produced minimum one week after the infection and it requires three weeks post infection to conduct haemagglutination inhibition test (Kempf et al., 1993). *Mycoplasma* can alter their surface antigenic proteins due to variation in antigenic make up. Another major problem of serological tests is their sensitivity and specificity. Sensitivity and specificity of SPA test are almost same as HI test and ELISA. Although more reliable, ELISA is not feasible for sero monitoring because it is more time consuming and costly (Higgins and Whithear, 1986). A very high prevalence by SPA test may be due to the false positive results which are because of cross reactivity, use of inactivated vaccine, contaminated sera and age of flock (Luciano et al., 2011). Major constraints in the use of SPA test for diagnosis are its low specificity and higher incidence of false positive results (Pourbakhsh et al., 2010). Serum plate agglutination test can be used for screening flocks but not for screening individual birds. For proper diagnosis and control, programs based on sero conversion may be inadequate, so sero monitoring should be combined with culture and molecular techniques (Luciano et al., 2011). *Mycoplasma gallisepticum* is shown to be cross reactive with closely related *Mycoplasma imitans* (MI) that would also lead to aberrance in prevalence of specific *Mycoplasma* species (Bradbury et al., 1993). This is because both MG and MI have many similarities including same antigenic and phenotypic properties and same terminal attachment structure (Abdul-Wahab et al., 1996). Flocks showing no clinical signs may be serologically positive if the flock recovered from the infection at younger age (Ley, 2003).

Non-pathogenic species such as *M. gallinarum* and *M. gallinaceum* are also related to MG and MS, for this reason serological test used should differentiate between these species (Hong et al., 2005). Due to extensive use of live vaccine serological tests should be able to distinguish between field strain and vaccine strain. Therefore, it is necessary that test should not only differentiate at species level but also at strain level (Ferraz & Danelli, 2003).

Hassan et al., (2012) carried out a study to see the sero prevalence of MG and MS by SPA test. The results showed that 74.28% samples from turkeys were positive for MG and 25.71% samples from turkeys were positive for MS. A study was planned to check the sero prevalence of MS in the respiratory distress cases of broiler. About 76.57% samples were positive when checked by SPA test. When all the positive samples were confirmed by culture, isolation rate was 42.3% while PCR detected positive results from 98% of sero positive samples. In SPA test the false positive results were observed because of cross reactivity of MG and MS (Ehtisham et al., 2010). The overall sero prevalence of MG in Baluchistan in broilers was 10% and 19.76% by serum plate agglutination (SPA) test and ELISA respectively. In case of layers sero prevalence of MG was 19.4% and 31.6% by SPA and ELISA respectively. In Baluchistan overall prevalence was 8% and 15% respectively by SPA and ELISA (Atique et al., 2012). IgM antibodies in serum are detected by SPA test within a week of an infection. The overall sero prevalence of MG in chickens by ELISA in the Jordan was 73.5%. The prevalence in case of broilers was 70% and in case of layers prevalence was 71% (Gharibeh and Roussan, 2008).

2.1.10 Use of molecular techniques:

For differentiation of *Mycoplasma* strains several molecular techniques have been developed including protein profile analysis, restriction fragment length polymorphism (RFLP), ribotyping, strain-specific DNA probes. Under the field conditions for discriminating vaccine strains no other method is as successful as RAPD as there are limited problems of this technique (Ferguson et al., 2005).

2.1.11 Polymerase chain reaction:

Due to high sensitivity and increasing specificity of the Polymerase chain reaction (PCR), it has become valuable tool in the diagnosis of *Mycoplasma* species. The principle of the PCR is the direct detection of the nucleic acid of the *Mycoplasma* (Kempf et al., 1993). PCR is better than other diagnostic methods because it is rapid, easy, highly sensitive and inexpensive. It eliminates the need to isolate and culture the organism.

Since PCR is dependent on the target, its specificity is highly flexible. It can be specie specific or strain specific by targeting unique gene in particular specie or conserved region in the specific strain. For four main avian pathogenic mycoplasma species, PCR essays are developed in 1990s (Raviv & Kleven, 2009). Earlier PCR methods targeted 16S rDNA region but the recent PCR essays target the specie specific regions and the surface proteins (Liu et al., 2001; Garcia et al.,

2005; Raviv et al., 2007). PCR essays that target 16S rDNA region are less specific and may cross react with the other avian *Mycoplasmas* because 16S rDNA region is highly conserved among phylogenetically related groups (Garcia et al., 2005). Those PCR essays are less sensitive which target surface protein because of high levels of intraspecific genetic polymorphism (Raviv et al., 2007). For detection of *M. gallisepticum* many PCR methods are applied including commercial kits produced by IDEXX Laboratories, Genekam Biotechnology AG, and others. Polymerase chain reaction essays are developed to target various genes including 16S rRNA gene, pvpA, gap A, lipoprotein, mgc2 and 16S-23S intergenic spacer region (Domanska-Blicharz et al., 2008). For the detection of MG mgc2-PCR is highly specific and sensitive (Garcia et al., 2005).

Polymerase chain reaction developed by targeting 16S rRNA gene has its own limitations and shortcomings. Although this region is highly conserved but 16S rRNA gene of *M. gallisepticum* and *M. imitans* is very much similar and both the organisms are amplified by the organism (Garcia et al., 2005). Keeping in mind the above mentioned limitations of PCR essays based on 16S rDNA region and 16S rRNA gene, we can say that PCR cannot be solely used to identify *M. gallisepticum* without possibility of false positive results.

Surface proteins on which PCR essays are based help the *Mycoplasma* cell to bind to the host cell membrane-receptors. These proteins which mediate the attachment are called cytadhesins. After the firm attachment of *Mycoplasma* to host cell, pathogenesis and host cell alterations occur (Goh et al., 1998; Winner et al., 2000). One of the important cytadhesins is encoded by mgc2 gene (Boguslavsky et al., 2000). In *M. gallisepticum* mgc2 gene is fairly conserved and is used for molecular detection of isolates. Essay based on mgc2 gene is able to differentiate between field strain and the vaccine strain (Lysnyansky et al., 2005). Other cytadhesins are encoded by gapA gene (Goh et al., 1998), PvpA gene (Boguslavsky et al., 2000) and MGA 0319 gene (Garcia et al., 2005). About 42.4% tracheal samples were positive when *Mycoplasma* specific primers were used. The reason for high prevalence by PCR is that it detects DNA from both viable and non viable *Mycoplasma* (Marois et al., 2002). When we compare PCR with the cultural isolation, PCR is fast, less expensive, effective and more reliable method.

2.1.12Control of MG and MS:

Vertical transmission is the one of the major reason for ineffective control of *Mycoplasma* (Papazisi et al., 2002). Rapid expansion of poultry industry and high concentration of multi aged

birds in the close proximity are major reasons for the high incidence of mycoplasma. Due to these factors and poor biosecurity measures it is difficult to maintain *Mycoplasma* free flock (Lysnyansky et al., 2005). In the areas where complete eradication is difficult, live vaccines are used as alternative control strategy. There are three commercially available live vaccines for control of MG which include the F strain Ts-11 and 6/85 strain (Liu et al., 2001). The MG-F strain was describes as typical pathogenic and naturally occurring strain and the advantage is that single dose is needed with this strain (Ley, 2003). The Ts-11 strain came from Australia and 6/85 originated from U.S.A (Ferraz & Danelli, 2003). Ts-11 and 6/85 are live vaccine and contain poorly transmitted strains. That is why these strains are regarded safer than MG-F strain.

2.2 *Salmonella pullorum* and *Salmonella gallinarium* (SPG):

Fowl typhoid (FT) and pullorum diseases (PD) are most important diseases of poultry. Fowl typhoid and PD are septicaemic diseases of poultry and other game birds. These conditions are caused by two very closely related organisms which were thought to be two different species but now are considered biovars of *Salmonella enterica subsp. enterica* (Grimont & Weill, 2007). *Salmonella pullorum* causes pullorum disease and *Salmonella gallinarium* causes fowl typhoid in poultry. Both are non motile, gram-negative and are pathogenic avian serotypes which are highly host adapted.

2.2.1 Signs and symptoms:

Fowl Typhoid is acute or chronic septicaemic disease of adult birds although young birds are less susceptible. Pullorum disease is an acute septicaemic disease which affects young birds (Kwon et al., 2000). Clinical signs of FT include typical septicaemic birds, increased mortality and poor quality hatched chicks. In the adult birds in the case of chronic diseases anemia, depression, labored breathing and diarrhea causing adherence of feaces to the vent are frequently observed. In the case of *S. pullorum* disease is mild or may be sub clinical. Birds at point of lay and 2-3 week of age are most susceptible to PD (Wigley et al., 2005). Post mortem signs of FT include generalized septicemia and enlarged, dark and friable liver with bronze appearance. Some times Spleenomegaly and dark colour bone marrow are also seen (Shivaprasad, 2000). Post mortem signs of PD in newly hatched chicks are peritonitis, congestion of tissues and an inflamed unabsorbed yolk sac. The post mortem signs observed in PD in adult birds are misshapen or shrunken ovaries and follicles with fibrous stalks (Eswarappa et al., 2009).

2.2.2 Fowl typhoid and Pullorum disease as worldwide problem:

Exact data regarding occurrence of FT and PD in many countries is not available because of lack of proper disease reporting system. Most of the cases occur in backyard poultry. Although many countries are reported to be free of FT and PD but wild avian species are thought to be main reservoirs of these serovars. According to the updated World Organization for Animal Health database last case of FT was reported in 1981 in USA (OIE, 2010a, b). In case of PD last case has been noted in backyard flocks in 2009 (USDA, 2009). Same situation exists in the UK and Europe where they have eliminated FT but are not able to free themselves from PD (Davies & Breslin, 2003). The major reasons for unsuccessful elimination are poor biosecurity measures, extensive farming and presence of various wild life vectors (Auri et al., 2010). Although UK was registered free of FT in 1986, but *Salmonella gallinarium* was isolated from cage layer and from backyard chickens in 2005 and 2007 respectively (Defra, 2007). In Africa numbers of reports are available on outbreaks of FT and PD. In Nigeria an outbreak affected 11000 birds with 25% mortality (Ezema et al., 2009).Both FD and PD occur frequently in Asian countries. In India FT and PD outbreaks were reported in 2008 and 2002 respectively. Between 20005 and 2008, more than 11000 outbreaks of PD were reported in China (OIE, 2010a,b). Based on the above discussion we can say that it is difficult to precisely predict the occurrence of FT and PD and most incidences are underestimated.

2.2.3 Status in Pakistan:

The mean prevalence of the FT and PD in the Punjab province of Pakistan from June 2011 to July 2012 was found to be 7.1 and 2.7% (Rehman et al., 2013). This is very high prevalence rate as these diseases have been eradicated from USA and Western Europe. There is need of strict culling policy at grandparent level to control the infections in the commercial poultry population. A study conducted by Rehman et al., (2013) in Punjab showed that sero prevalence of SPG in the broiler, layer and breeder was found to be 21.8%, 32.3% and 35% respectively. The prevalence of *Salmonella pullorum* infection was 56.3% when detected through cloacal swab samples and sero prevalence of SPG was shown to be 52% in the Faisalabad district of Pakistan (Ayesha et al., 2014). In 2003 the sero prevalence of SPG was reported to be 39.02% in the Hyderabad. Pakistan (Rehman et al., 2003).

2.2.4 Drug resistance and SPG:

A number of drugs are used to treat FT and PD and reduce mortality but are not able to eliminate infection. This is due to the fact that birds become carrier and can be reinfected from environment. Furazolidone has been extensively used to treat FT; this has led to development of resistance against this drug (Smith et al., 1981). In *Salmonella gallinarium* resistance has increased from 0% to 6.5% for Enrofloxacin and to 82% for Ofloxacin (Lee et al., 2003). This is due to the mutation in the gyrA gene (Lee et al., 2004). *Salmonella gallinarium* is resistant to number of other antibiotics including ampicillin (13%), gentamycin (43%) and kanamycin (69.6%). Multi-drug resistance is also increasing in this pathogen. Class 1 integrons are isolated from *Salmonella gallinarium*, due to which resistance is becoming structural part of integrons increasing the transfer between the strains (Kwon et al., 2002).

Salmonella pullorum strains are found to be highly resistant to ampicillin, carbenicillin, streptomycin, tetracycline, trimethoprim and sulphafurazole (Pan et al., 2009). This multi-drug resistance can be transmitted to humans via food chain. This makes it a problem of international concern (Tollefson & Miller, 2000).

2.2.5 Taxonomy of SPG:

Salmonella enterica subsp. enterica serovars gallinarum and *pullorum* are now not considered as separate species. *Salmonella gallinarum* and *S. pullorum* cannot be differentiated by normal sero typing as both of these belong to sero group D. Both of these species are non motile as they are not flagellated and contain O antigens 1, 9, 12. Both of these are considered as bio type of same serovar (Christensen et al., 1993). The strains which are intermediate between *S. gallinarum* and *Salmonella pullorum* are confirmed by comparative genomics by micro arrays (Porwollik et al., 2005). *Salmonella gallinarum* and *Salmonella pullorum* possess serological relationship with other members of sero group D including *S. enteritidis* in chicken and *S. dublin* in calves which shows the phylogenetic relationship (Mortimer et al., 2004). *Salmonella Typhimurium, S. Enteritidis,* and *S. Heidelberg* affect the wide range of host while *S. gallinarium* and *S. pullorum* are host specific for chickens (Foley et al., 2007). Close taxonomic relationship between *S. enteritidis* and *S. gallinarum* by Comparative genome sequence analysis shows that both of these may have split from a common ancestor by acquisition of a number of fimbrial genes, for example lpf, pge and ste (Clayton et al., 2008). *Salmonella enteritidis* and *S. gallinarum* have sane O antigen (O9) of the lipopolysaccaride on their cell surfaces, which led to competition

between these two serovars in the poultry (Velge et al., 2005). *Salmonella enteritidis* was competitively excluded from poultry by *S. gallinarum*. Therefore, in the USA the ecological niche which was created by eradication of *S. gallinarum* was filled by *S. enteritidis* (Foley et al., 2007).

2.2.6 Pathogenesis of Fowl typhoid and Pullorum disease:

Salmonella gallinarum and *S. pullorum* causes the infection in the birds through oral route where they invade intestinal epithelial cells or lymphoid tissue and mainly are localized in the payer's patch and caecal tonsils. In the gut of the bird bacterial uptake is through lymphoid tissue, that's why bacterial recovery is easy from payer's patch and caecal tonsils than secretory tissue of the intestine (Chadfield et al., 2003). Multiplication of bacteria takes place in liver, spleen and bone marrow when infected phagocytes and free bacteria move to these sites. Then these bacteria re enter the lymphoid tissue in the intestine by unknown mechanism and are shed in the feaces. These bacteria very seldom enter the human food chain because poorly colonize the gut of the bird in the absence of clinical disease. *Salmonella gallinarum* has lost several metabolic pathways including the inability to use long chain maltodextrins, D-glucarate and hydrogenase 1. Importantly, there is an inability to utilize 1, 2-propanediol due to mutations in the certain genes (Thomson et al., 2008). *Salmonella gallinarum* and *S. pullorum* are not able to produce glycogen due to mutations in the glgA, B and C (McMeechan et al., 2005). *Salmonella gallinarum* and *S. pullorum* both are host adapted and are non motile. They lack the flagella because of mutations in the genes required for synthesis of flagellar machinery. Due to absence of flagella both of these pathogens are able to invade intestinal tract of bird without provoking strong inflammation and cause systemic disease and this might be the cause of specific adaptation to the avian host (Iqbal et al., 2005). Intra cellular multiplication of these bacteria is thought to take place the activities of homologous/orthologous genes which contribute to virulence (Jones et al., 2001). Both *S. gallinarum* and *S. pullorum* contain virulence plasmid spvRABCD genes and are essential for clinical disease. Although these plasmids are essential for systemic disease but these have little role in the specie adaptation as these plasmids are interchangeable in *S. gallinarum*, *S. pullorum* and *S. typhimurium* without affecting the virulence phenotype of variant strain (Rychlik et al., 2006).

Haemolysis and the presence of necrotic lesions in the heart and alimentary tract are the factors which characterize the acute and systemic disease caused by *S. gallinarum* and *S. pullorum*. This

is correlated with multiplication of bacteria. Myofibrillar necrosis with heterophils, lymphocytes and plasma cells being replaced by histiocytes are the characteristic features of the heart lesions (Shivaprasad & Barrow, 2008). These lesions are mostly seen in the chronic disease caused by *S. gallinarum* and *S. pullorum.*

Birds of all age are susceptible to FT variable to high mortality depending on the bacterial strain and immune status of the host. Brown egg layers are more susceptible to FT than white egg layers (Barrow et al., 1999). Cells of the macrophage monocyte lineage play important role in the chickens which are resistant to systemic salmonellosis (Wigley et al., 2002).

Susceptibility of poultry to *Salmonella* colonization is affected by many factors. These factors include age of the bird, *Salmonella* serotype and initial dose, environmental stress presence of feed additives. Presence of compatible colonization site, competition with gut flora and survival of the Salmonella in the wide range of pH also affect the colonization of the pathogen in the gut of the poultry. From hatching to 96 hour of age, chickens are very much susceptible to *Salmonella* infection and colonization (Bailey, 1988).

2.2.7 Transmission of SPG:

Salmonella gallinarum and *S. pullorum* are transmitted both vertically and horizontally but horizontal transmission is more important for *S. gallinarium.* Eggs produced by infected or reactor birds can be contaminated with *S. gallinarium* but there is very little proof for transmission of the *S. gallinarium* via eggs (Berchieri et al., 2000). Environmental factors which are responsible for *Salmonella* contamination in poultry farms include air, litter, unclean premises and vectors such as rodents and humans (Amick-Moris, 1998). After the *Salmonella* has established in the primary breeding stock, poultry is infected by hatcheries via both vertical and lateral spread (Lister 1988).

Salmonella pullorum can persist within the mcrophages of spleen and persistence was seen despite the high titre of circulating antibody which shows the establishment of intracellular infection (Wigley et al., 2001). The survival of *S. pullorum* in the splenic macrophages is responsible for slower clearance of this pathogen from bird (Chappell et al., 2009). This might be due to fact that infected macrophages are not visible to T cells due to down regulation of MHC expression or immune response itself may be regulated.

2.2.8 Predilection site and susceptibility:

When the birds gain sexual maturity, number of bacteria increase in the spleen and bacteria migrate to the reproductive tract of the birds. More concentration of the bacteria was found in the lower oviduct as compared to the upper oviduct (Wigley et al., 2005). After the infection both male and female birds become carrier, but multiplication of bacteria and spread to the reproductive tract is only restricted to female birds that may be related with the onset of laying. At the onset of laying T cell response to *Salmonella* decrease in the infected and carrier birds. This fall in T cell response is responsible for the increased risk of PD at the start of lay point. After the three weeks of onset of laying response of T cell increases and number of bacteria declines. This explains why laying hens are more susceptible to PD at onset of laying (Wigley et al., 2005).

2.2.9 Diagnosis of Fowl typhoid and Pullorum disease:

A tentative diagnosis of FT and PD can be made from clinical signs, flock history, mortality and lesions, but for definite diagnosis isolation and identification of organisms is required. The main methods used for the diagnosis of SPG include culture and isolation, serology and molecular methods specially PCR (Barrow et al., 2012).

2.2.10 Culture and isolation:

Both *S. gallinarium* and *S. pullorum* can be detected from all the tissues, organs and feaces during acute phase of disease. *Salmonella gallinarium* is mostly recovered from liver, spleen and reproductive tract, and occasionally in the caecal tonsils while *S. pullorum* is mostly recovered from the ova and oviduct and it is occasionally recovered from other organs and tissues like gastro intestinal tract. These organisms are 1.0–2.5 μm in length and 0.3–1.5 μm in width and are gram negative rods (Grimont & Weill, 2007). Both *S. gallinarium* and *S. pullorum* are non motile but some variable strains of *S. pullorum* may show motility. Samples should be taken from live birds, fresh carcasses and egg materials. Liver, ileo-caecal junction, ovaries and oviduct are the preferred site for investigation. Samples should be first cultured by direct inoculation in broth such as selenite cysteine or selenite F and then plating on selective media such as brilliant green agar (Parmar & Davies, 2007). *Salmonella pullorum* may grow slowly and incubation should be done for 48 hours. MacConkey agar, brilliant green agar and xylose lysine deoxycholate agar are the selective media for these organisms. On non selective media colonies of *S. gallinarium* are round, translucent and smooth while colonies of *S. pullorum* are little

smaller and translucent. On the selective media, the colonies of both the organisms varies according to the media used (Kang et al., 2011). Pink colour colonies of *Salmonella pullorum* are observed on Brilliant Green Agar. Colonies of *Salmonella pullorum* with black centers on Salmonella Shigella Agar are seen (Ayesha et al., 2014).

Serological methods like serum plate agglutination test can be used for flock monitoring but cross reactivity with other members of D sero group may occur. To overcome this problem ELISA can be used for specific pathogen. Molecular methods are used to detect and differentiate these two serovars (Kwon et al., 2000). *Salmonella gallinarum* and *S. pullorum* possess polymorphisms at codons 316 and 339 of fliC gene. These polymorphisms can be confirmed by combining PCR with restriction fragment length polymorphism (RFLP). *Salmonella gallinarum* and *S. pullorum* can be differentiated by detecting polymorphic nucleotides by using PCR-RFLP for this gene (Park et al., 2001). Recently polymorphic regions of the genes glgC and spec in *S. Gallinarum* are targeted by duplex PCR primers and can be used to differentiate it from *S. pullorum* (Kang et al., 2010).

2.2.11 Biochemical confirmation:

For biochemical confirmation pure culture from non selective media should be used. Biochemical test used for *S. gallinarum* and *S. pullorum* are mentioned in the table 2.2.

Table 2.2: Biochemical confirmation of *S. gallinarum* and *S. pullorum* (OIE, 2012)

Test	*S. pullorum*	*S. gallinarium*
TSI glucose (Acid fermentation)	+	+
TSI glucose (gas fermentation)	variable	_
TSI lactose	_	_
TSI sacharrose	_	_
TSI hydrogen sulphide	variable	variable
Gas from glucose (medium with Durham tube)	+	_
Urea hydrolysis	_	_
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	_
Maltose fermentation	_ or late +	+

Dulcitol	–	+
Motility	–	–

2.2.12 Serological techniques:

Serological tests are indicative of flock status rather than the individual bird because individual bird may vary according to stage of infection. Random sampling should be done and the expected prevalence and level of confidence desired will determine the number of the samples. For *S. gallinarum* and *S. pullorum* most common serological test include whole blood agglutination, serum plate agglutination test, tube agglutination, micro-agglutination, haemagglutination and ELISA (USDA, 1996). While performing serology of *S. pullorum*, other *Salmonella* such as *S. enteritidis* and vaccination may give false positive results. *Salmonella gallinarum* and *S. pullorum* have O antigen 9, 12 and 1 in common (Brooks et al., 2008), so same antigen is used for serology of both pathogens (Proux et al., 2002). To detect the antibodies against *S. gallinarum* and *S. pullorum*, ELISA is also used (Oliviera et al., 2004). Most sensitive and specific serological test for flock screening of *S. gallinarum* and *S. pullorum* is indirect ELISA using lipo polysaccharide antigen. It quantifies the titre of antibodies and is easier to perform (Wray & Wray, 2000). For *S. gallinarum* and *S. pullorum* yet no commercial ELISA kits are available. The overall sero prevalence of SPG in the different poultry farms in Bangladesh was found to be 26.67%. The birds at 24th week of age at Mirpur farm showed highest sero prevalence of 45%. The lowest sero prevalence (16.6%) of SPG was found in the birds of 10 week of age (Hossain and Islam, 2004).

2.2.13 Molecular techniques:

Molecular techniques used to detect *S. gallinarum* and *S. pullorum* include plasmid profile analysis, pulsed field gel electrophoresis PCR-restriction fragment length polymorphism (RFLP) and ribotyping. Molecular techniques especially PCR is the accurate method for the diagnosis of FT and PD (Barrow et al., 2012). Random amplified polymorphic DNA (RAPD) is one of most powerful technique for detection of *S. gallinarum* and *S. pullorum* (Habtamu-Taddele et al., 2011). Due to high level of clonality, it is better to use combination of different methods and mixture of different restriction enzymes. Most effective technique will differ from region to region because of different circulating clones. Complete sequencing can be used for *S.*

gallinarum and *S. pullorum* but it is not economically feasible for outbreak investigation (Richardson et al., 2011).

2.2.14 Control of Fowl typhoid and Pullorum disease:

Control of FT and PD depends on the level of infection and varies from region to region. In the regions where infection is low serological testing is important and test and slaughter policy can be used to achieve elimination. Where infection rate is high and elimination is not the goal chemotherapy with side effects and vaccination are the measures used to control the FT and PD. Recently some killed vaccines are used, but live vaccines are more effective than the inactivated vaccines (Okamura et al., 2007). *Salmonella pullorum* is completely resistant to penicillin, ampicillin, chloramphenicol, tetracycline and nitrofurantoine and partially resistant to gentamycin, contrimoxazile and nalidixic acid. *Salmonella pullorum* has shown less resistance against fluoroquinolone (Ayesha et al., 2014). Live vaccines include 9R and 9S, 9S is more effective than 9R as 9R does not produce the lipo polysaccharide specific circulating antibodies. 9R vaccine is also protective against *S. enteritidis* (Feberwee et al., 2001). The most extensively used vaccine is 9R but the problem is its retained virulence for hatched and young chicks (Lee et al., 2005). Live vaccines work by principle of competitive exclusion. The live vaccine induces resistance to the colonization by pathogenic species. The protective strain occupies the metabolic niche required for colonization by pathogenic or challenge strain (Chacana & Terzolo, 2006). Autogenic vaccines can be used to prevent clinical FT and PD, but strain instability leading to reversion to virulence must be kept in mind (Okamoto et al., 2010). Biological approach to control the salmonellosis includes use of probiotics. Probiotics contains *Lactobacilli* which produce large quantities of volatile fatty acids like formic acid. These acids inhibit growth and colonization of *Salmonella* in gut of the poultry. This is also basis of incorporation of formic acid into the poultry feed (Sterzo et al., 2007). Main approaches for controlling *Salmonella* include use of antibiotics, vaccination of birds, competitive exclusion mechanism and use of probiotics and use of lytic bacteriophages (Barrow et al., 2012).

CHAPTER-3:
MATERIALS AND METHODS

Materials and Methods

3.1 Culture and isolation of *Mycoplasma gallisepticum*:

3.1.1 Sampling of field isolates:

Different poultry farms suspected of MG infection located in Rawalpindi region were selected for sampling. Swab samples were taken from sinuses, pharynx, and trachea. On post mortem examination parts of tracheal tissues, air sacs containing creamy exudates and portion of lungs were also collected. To increase the recovery rate of isolates, many precautionary measures were adopted. The swabs used were pre dipped in *Mycoplasma* broth. Tissue samples collected were put in tubes containing *Mycoplasma* broth. Samples were transported carefully and cultured immediately on arrival in the *Mycoplasma* Laboratory of Poultry Research Institute, Disease Diagnosis Section Rawalpindi. Names of the farms were kept confidential from which samples were collected.

Six tissue samples were taken from broiler farm (4 week of age) suspected of MG infection (Farm 1). Gasping, rales and Dysponea were observed on clinical examination. There was reduced weight gain and some birds were freshly dead. The flock was tested serologically positive for MG (described later in the serological monitoring of MG). Extensive antibiotic treatment was being used. Tissue samples were taken from trachea, lungs and air sacs of moribund and freshly dead birds.

Nine tissue samples were taken from day old chicks (Farm 2). The parents of these chicks were serologically positive for MG by SPA test. Samples were taken from trachea and future cultured in the *Mycoplasma* laboratory. A layer farm (W-36 and 22 week of age) was selected because the birds were showing severe respiratory distress (Farm 3). Decreased egg production and high mortality was noted despite the usage of Tylosine-Doxycycline combination in one shed. In the second shed no treatment was done. Seven samples were taken from this farm and transported to lab carefully.

Eleven Samples were taken from ceva-22 layer birds (55 week of age) suspected of MG infection (Farm 4). The flock was treated with Tiamulin hydro chloride. Respiratory problems and reduced egg production were observed. Five tissue samples were taken from breeder farm (Farm 5). The birds were showing respiratory distress and were sero positive for MG by SPA test. No information regarding treatment protocol was provided.

Some samples were taken from birds presented for post mortem in the disease diagnostic laboratory; poultry research institute (PRI) Rawalpindi. These 20 samples comprised of trachea, lungs and air sacs. A total of 58 tissue samples collected from different types of birds which were processed for isolation of MG.

3.1.2 Sterilization of glass ware:

All the glass ware used in the culture and isolation of MG was sterilized in hot air oven. Test tubes, petri plates, glass flasks, pipettes and beakers were wrapped in paper and sterilized. The plastic capped tubes were used and flasks were plugged with sterilized cotton wool stoppers before sterilization.

3.1.3Preparation of culture media:

3.1.3.1 PPLO broth medium:

Initially tissue samples were inoculated in PPLO broth medium for isolation of MG. Following ingredients mentioned in table S were mixed to prepare PPLO broth medium according to Kleven, (1998). Table A-1in the appendix shows the composition of PPLO broth medium.

3.1.3.2 Modified Frey's solid medium:

Tissue samples were streaked on Frey's solid medium for isolation of MG. Following ingredients mentioned in table R were mixed to prepare Frey's solid medium according to Kleven, (1998). Table A-2 in the appendix shows the composition of Frey's medium.

Mycoplasma broth (for broth) or agar (for agar) base, glucose, yeast extract, phenol red and thallium acetate (part A) were added in distilled water and autoclaved at 121°C for 15 minutes at 15 lb. sq. in. pressure. Horse serum, cysteine hydrochloride and ampicillin (part B) were filter sterilized by using 0.2um and 0.45 um membrane filters. Autoclaved part A was cooled down to 50°C and was aseptically added into filter sterilized part B. Plates were poured into depth of 5mm in the safety cabinet.

3.1.4 Isolation of microorganisms:

PPLO broth was used for the direct inoculation of the tissues and the swabs and then bacterial growths were transferred on agar medium. Some of the swabs were directly streaked on the agar plates. Tubes containing broth inoculated with sampled swab or tissue were incubated at 37°C. Damped cotton wool was placed in the incubator to increase the humidity. Colour change from red to yellow because of glucose fermentation by *Mycoplasma* was indicative of growth in the broth. Sometimes the colour changes produced by MG are masked by arginie hydrolyzing species of *Mycoplasma*. Tubes were placed in the incubator for 21 days before discarding as

3.2.5 Antigens used for serology:

Standard MG antigen (code SL 212, inactivated coloured antigen for SPA test), manufactured by Soleil diagnostics France were used for used for detecting antibodies in sera by serum plate agglutination test.

Standard MS antigen (code SL 222, inactivated coloured antigen for SPA test), manufactured by Soleil diagnostics France were used for detecting antibodies in sera by serum plate agglutination test.

Standard SPG antigen (code SL 242, inactivated coloured antigen for SPA test), manufactured by Soleil diagnostics France were used for used for detecting antibodies in sera by serum plate agglutination test.

3.2.6 Serum plate agglutination (SPA) test:

The SPA test was performed by placing side by side 20 ul of serum and 20 ul of antigen on glass slide with help of micropipette. Antigen and serum were mixed properly with help of stirrer and results were recorded within two minutes. Samples were considered positive where agglutination or granule formation occurred, otherwise samples were marked negative. Positive samples were graded (+) to (++++) according to extent of agglutination. The test was performed in similar way for detection of MG, MS and SPG antibodies in the serum samples. Known positive and negative control sera were used for validating evaluation of SPA test. Figure 3.1 shows the SPA test for MG, MS and SPG.

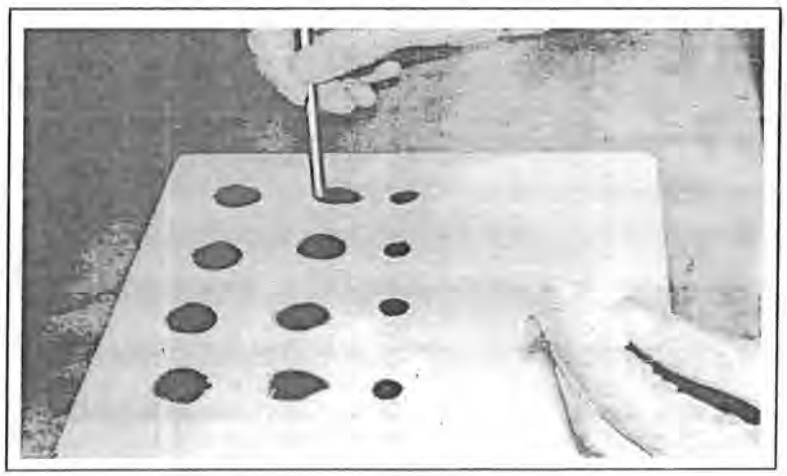

Fig 3.1: Mixing of sera and antigen

The sero prevalence of MG, MS and SPG infections were compared in different groups based on type of chickens, age, sex and month of infection.

3.2.7 Age wise Sero prevalence of MG, MS and SPG:

Age is very important factor which affect the incidence of mycoplasmosis and salmonellosis. Sero prevalence of the three pathogens was compared in the different groups based on age in the case of layer and breeder birds and effect of age was checked.

Table 3.2: Sample size from different groups of birds based on age

	Layer		**Breeder**	
	0-20 week	> 20 week	0-20 week	> 20 week
MG	187	546	261	608
MS	236	478	228	485
SPG	211	574	322	741

3.2.8Gender wise Sero prevalence of MG, MS and SPG:

Sero prevalence of the three pathogens was compared in the different groups based on sex of birds in the case of broiler breeder.

Table 3.3: Sample size in different groups based on sex

	Male birds	**Female birds**
MG	180	430
MS	161	475
SPG	212	540

Sero prevalence of the three pathogens was compared in the different groups based on sex of birds in the case of broiler breeder.

3.2.10 Season wise Sero prevalence of MG, MS and SPG:

Effect of season on sero prevalence of MG, MS and SPG was checked by determining the prevalence of these pathogens in the different months.

Table 3.4: Sample size in different groups of layer based on season

	July-Sept.	**Oct-Dec**	**Jan-Mar**
MG	272	192	243
MS	191	185	203
SPG	290	204	248

Table 3.5: Sample size in different groups of breeder based on season

	July-Sept	**Oct-Dec**	**Jan-Mar**
MG	265	336	271
MS	230	257	243
SPG	227	453	393

CHAPTER-4:

RESULTS

Results

4.1 Isolation of *Mycoplasma gallisepticum*:

At post mortem examination of the birds infected by *Mycoplasma*, mucoid material was observed in the trachea. Lungs of the infected birds were consolidated and bloody in appearance as shown in figure 4.1.

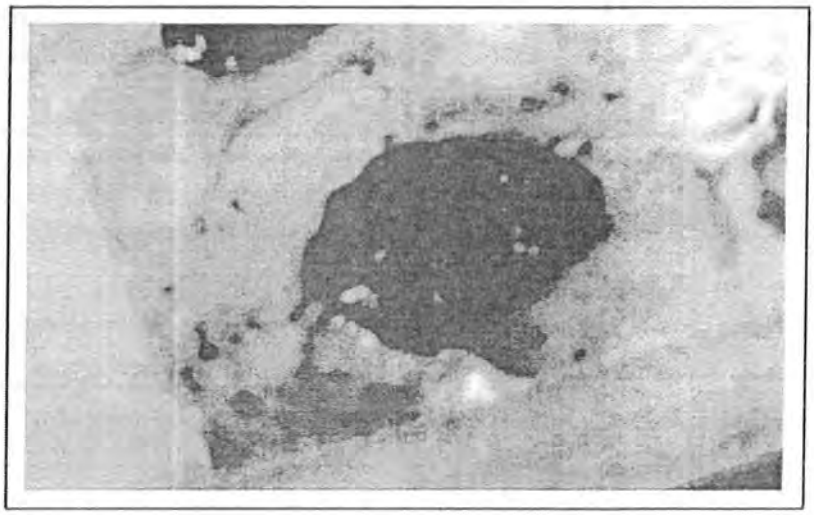

Fig. 4.1: Lungs of the birds affected by *Mycoplasma*

The air sacs of the birds affected by *Mycoplasma* were creamy yellowish in appearance and copious caseous exudates were accumulated as shown in figure 4.2.

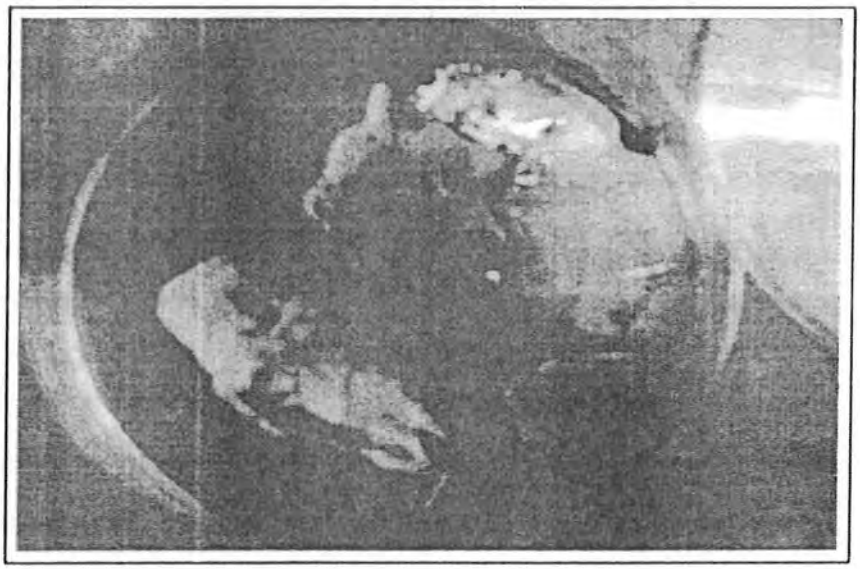

Fig. 4.2: Air sacs of birds affected by *Mycoplasma*

Out of 6 tissue samples taken from farm 1, only one of which changed the *Mycoplasma* broth colour. The colour changed from the red to orange due to acid production because of glucose fermentation by *Mycoplasma gallisepticum* as shown in figure 4.3.

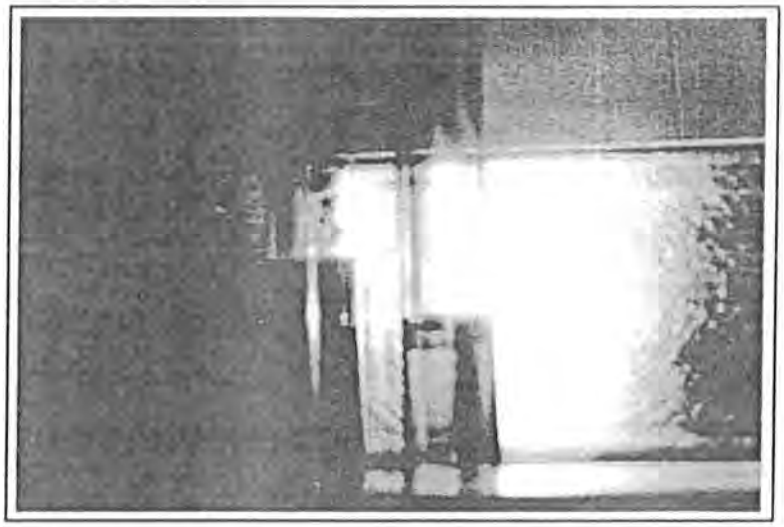

Fig. 4.3: Photograph showing change in colour of *Mycoplasma* broth in the samples taken from farm 1 due to acid production, tube on the left side showing normal colour of broth and tube on right side shows acid fermentation.

Although colour changed in only one broth tube, all the samples were inoculated on agar because arginine hydrolyzing species can inhibit the acid formation and colour production. However in only two agar plates growth resembling the *Mycoplasma* was seen. After two weeks when viewed under the stereo microscope, it was confirmed that it was not *Mycoplasma* but the fungal growth. Out of 9 tissue samples taken from farm 2, none of which changed the *Mycoplasma* broth colour. These samples were from day old chicks of serologically positive parents and samples were taken at hatchery. All the 9 samples were streaked on the Frey's agar. None of the samples grew on the solid medium.

Out of 7 tissue samples taken from farm 3, only four of which changed the *Mycoplasma* broth colour. Colour of *Mycoplasma broth* was changed very early even after 24 hours. 1 mL from the tubes where colour changed was passaged in fresh 5 mL of *Mycoplasma* broth and colour gain changed. Figure 4.4 shows the colour in broth after incubation for four days.

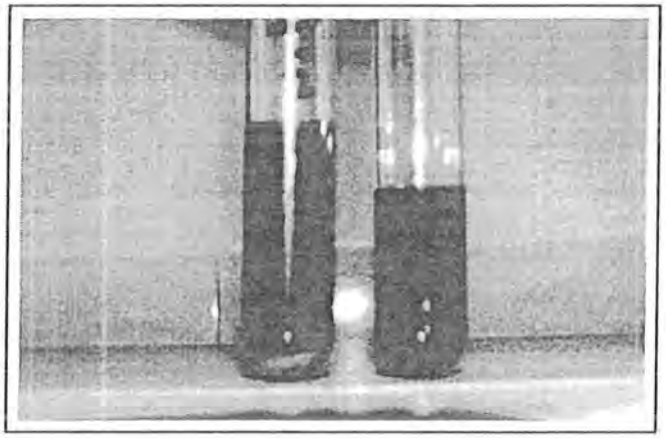

Fig: 4.4: Change in colour of broth from dark red to orange from the samples taken from farm 3

These four samples were transferred to the solid medium and plates were observed daily for two weeks. Out of these four three samples grew on plates faintly with hazy appearance as shown in figure 4.5.

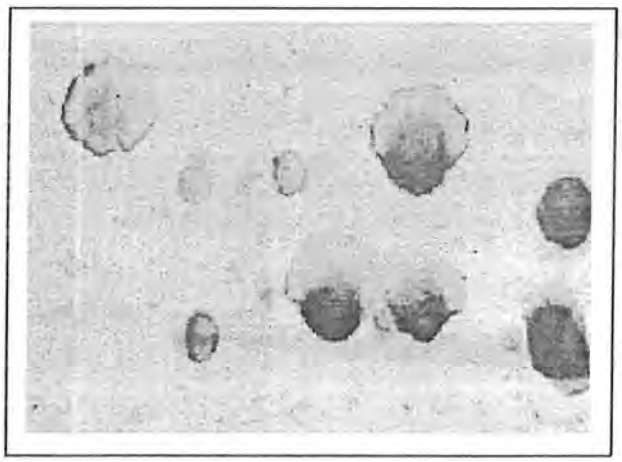

Fig. 4.5: Suspected colonies of *Mycoplasma* showing translucent and egg fried appearance.

The colonies were transferred into fresh agar plates and observed after seven days. Colonies appeared were larger and pigmented with slimy appearance when colonies grew rapidly. Further investigations showed that it was contamination and the agent was gram negative with short rods and identified as *Pseudomonas*.

Out of 16 samples 7 samples changed the colour of *Mycoplasma* broth. Only three samples grew on solid medium. There was extensive growth of fungi and it was confirmed under stereo microscope by mycelia and their relative size. Out of 20 miscellaneous samples taken at post

mortem from PRI laboratory, 4 samples changed the colour of PPLO broth and none of the sample grew on the solid medium.

4.2 Serological screening of MG, MS and SPG:

The overall prevalence of MG, MS and SPG in all types of chickens was found to be 45.96%, 40.43% and 41.80% respectively. Sero prevalence of these pathogens in different types of birds is mentioned in the table 4.1.

Table 4.1: Overall Sero prevalence of MG, MS and SPG in different types of birds

Type of chicken	MG (%)	MS (%)	SPG (%)
Broiler	7.14	10.0	5.36
Layer	44.9	42.6	51.3
Breeder	59.6	50.1	44.1

4.2.1 Sero prevalence of MG:

According to the present study the overall sero prevalence of MG in chicken on average is 45.96%. The sero prevalence of MG in the broiler, layers and breeders is found to be 7.14%, 44.9% and 59.6% respectively. Figure 4.6-A and 4.6-B shows the SPA test negative results and positive 1 (+) results respectively for SPA test.

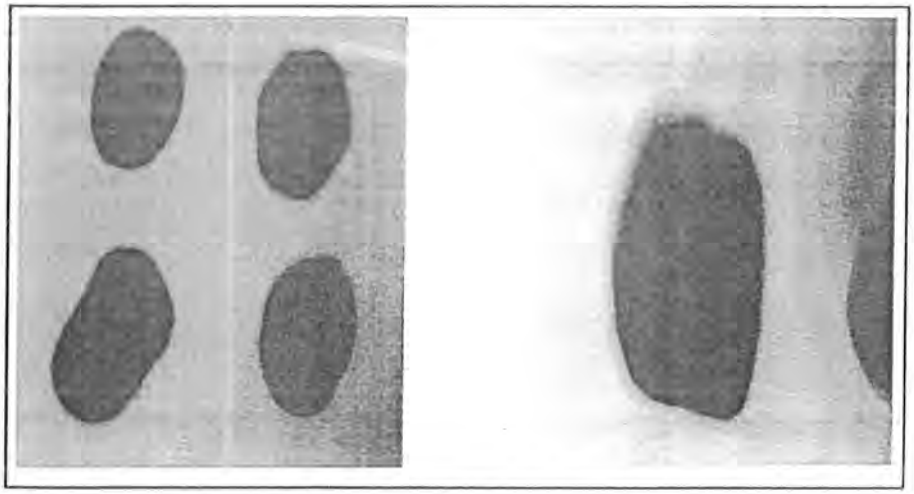

Fig. 4.6-A Fig.4.6-B

4.2.2 Sero prevalence of MS:

The overall sero prevalence of MS in all types of chickens was found to be 40.43%. The sero prevalence of MS in the broiler, layers and breeders is found to be 10.0%, 42.6% and 50.14% respectively. Figure 4.7-A and 4.7-B shows the (++++) and (++) results respectively for SPA test.

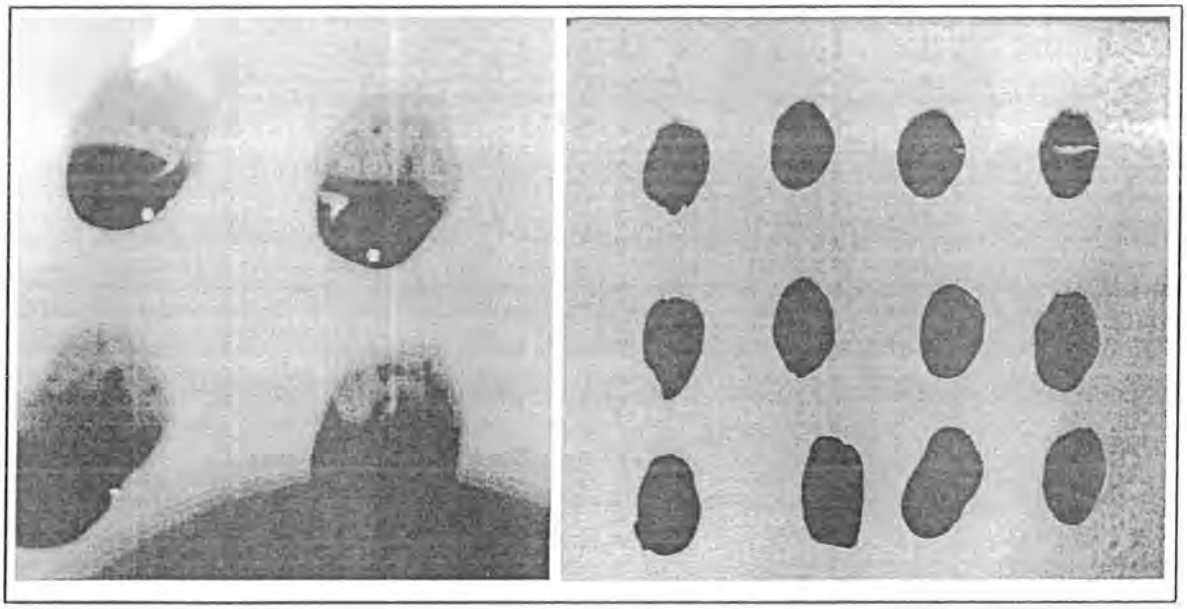

Figure 4.7-A Fig. 4.7-B

4.2.3 Sero prevalence of SPG:

The overall sero prevalence of SPG was found to be 41.8% in all types of chickens. The sero prevalence of SPG in the broiler, layers and breeders is found to be 5.36%, 51.32% and 44.11% respectively.

4.2.4 Age wise Sero prevalence of MG, MS and SPG:

Age is very important factor which affect the incidence of Mycoplasmosis and Salmonellosis. Sero prevalence of the three pathogens was compared in the different groups based on age in the case of layer birds. Table 4.2 shows the effect of age on sero prevalence of these pathogens.

Table 4.2: Age wise sero prevalence of MG, MS and SPG

	Layer		Breeder	
	0-20 week	> 20 week	0-20 week	> 20 week
MG	33.15%	50.92%	73.94%	51.15%
MS	23.53%	52.09%	42.10%	48.04%
SPG	23.22%	40.07%	41.61%	54.52%

Results show that in the case of layer birds, the sero prevalence of all the three pathogens was more in case of birds which were more than 20 week old. In the case of breeders, the sero prevalence of MG was more in birds which were less than 20 week old. The sero prevalence of MS and SPG was more in the birds which were more than 20 week old.

4.2.5 Gender wise Sero prevalence of MG, MS and SPG:

Sero prevalence of the three pathogens was compared in the different groups based on sex of birds in the case of broiler breeder.

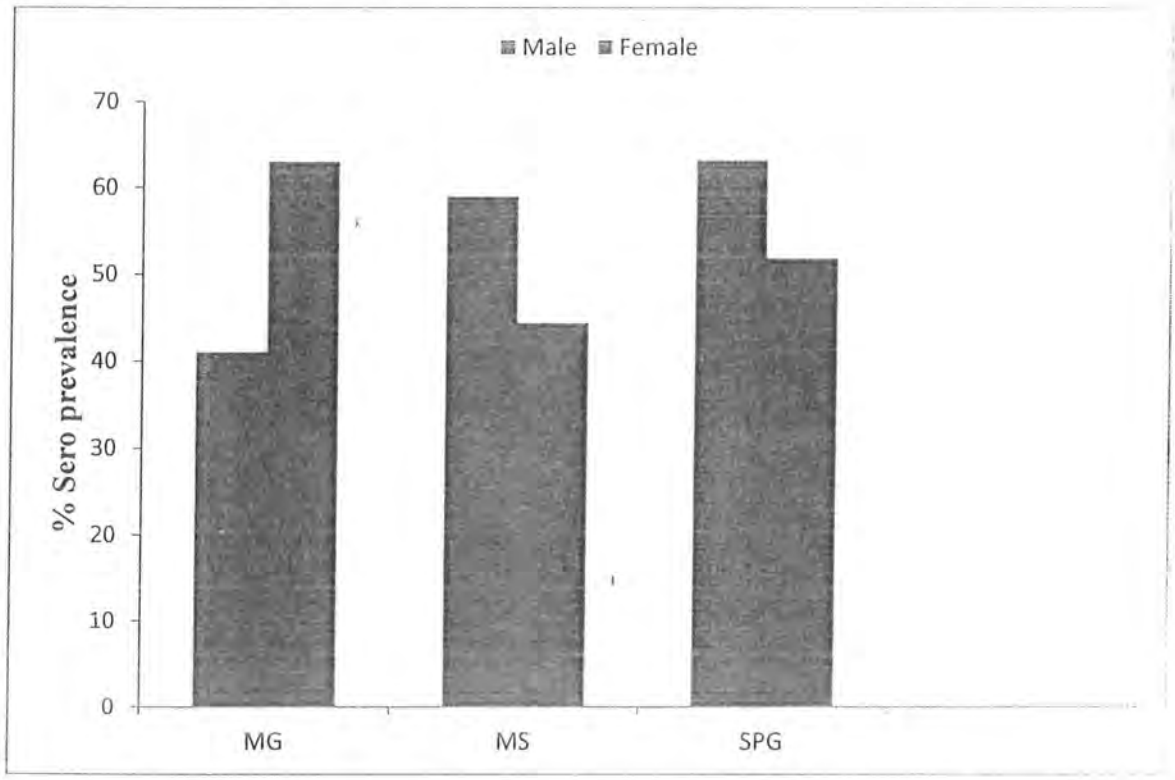

Fig. 4.8: % Sero prevalence of MG, MS and SPG in male and female birds in breeders

Results show that in case of broiler breeders, sero prevalence of MG was more in female birds while sero prevalence of MS and SPG was more in male birds as compared to female birds.

4.2.6 Season wise Sero prevalence of MG, MS and SPG:

Effect of season on sero prevalence of MG, MS and SPG was checked by determining the prevalence of these pathogens in the different months.

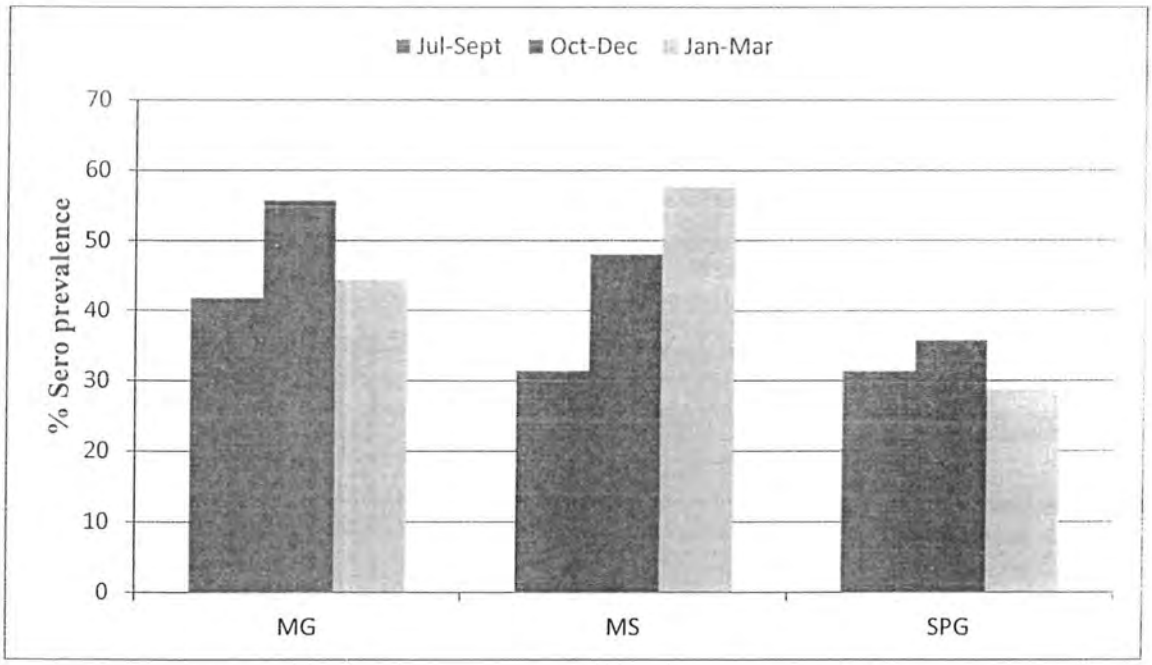

Fig. 4.9:Season wise sero prevalence in layer birds

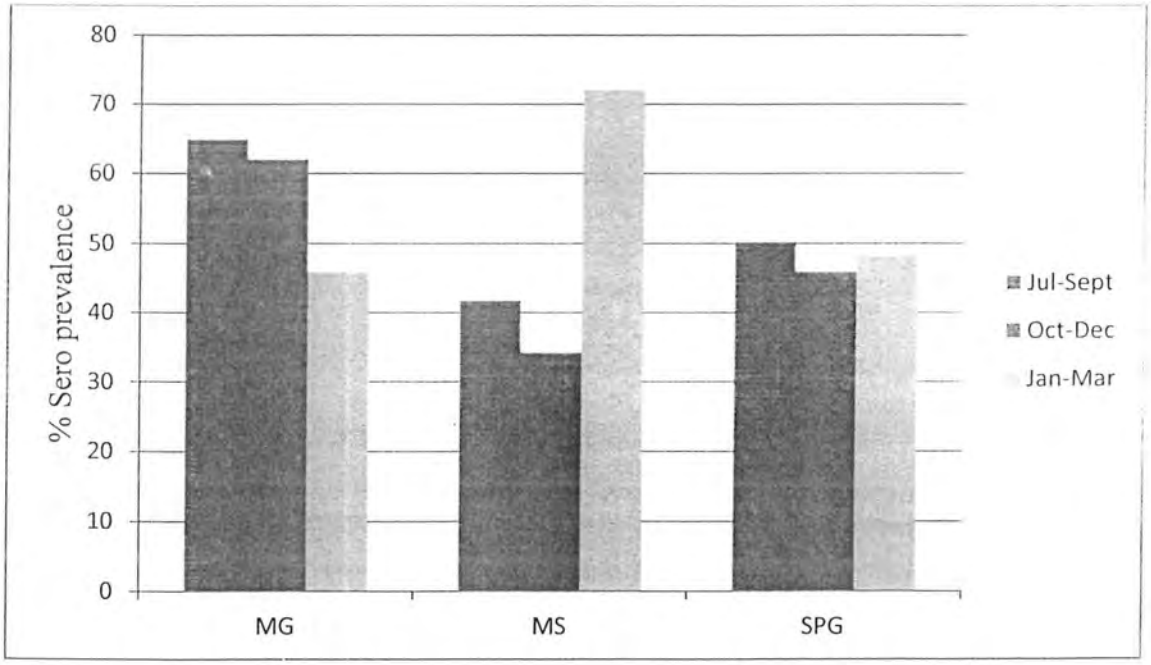

Fig. 4.10:Season wise sero prevalence in breeders

Result show that in case of layer birds, sero prevalence of MG and SPG is more in Oct-Dec and sero prevalence of MS was more in Jan-Mar. In case of broiler breeders, for MG and SPG highest sero prevalence is found in rainy season and for MS highest prevalence is found in the spring season.

CHAPTER-5:
DISCUSSION

5.2 Serological screening of MG, MS and SPG:

According to the present study the overall sero prevalence of MG in chicken on average was 45.96%. Earlier studies conducted show that over all sero prevalence of MG in chicken on average was 58% (Barua et al., 2006) and 57.1% (Prodhan, 2002). The overall sero prevalence of MG in chickens was found to be 69.90% (Heleili et al., 2012) which is higher than results of present study. In the flocks showing respiratory distress, 80% of the birds were sero positive for the MG (Siddique et al., 2012). The results of the present study are also supported by Rehman et al., (2013) who showed that there was significant difference in the sero prevalence of MG in the broiler, layers and breeders. The incidence of MG was highest in broiler breeders which was recorded 59.6%. The incidence in case of layers was 44.9% while lowest incidence was found in case of broiler which was 7.14%. According to the present study incidence of MG is higher in layer than broiler which is similar to results that incidence of MG in broiler is 49.5% and in layers is 66.5% (Barua et al., 2006).In the broiler chickens the incidence of MG is 65.91% (Heleili et al., 2012) which is much higher when compared with incidence shown in the present study. The sero prevalence of MG in broiler was shown to be 1.25% (Baruta et al., 2001) which is less than the incidence in present study, 49.5 % (Barua et al., 2006) and 30% (Aimeur et al., 2010) which are higher than the incidence in the present study. The sero prevalence of MG was found to be 31.25% in the broilers and 39.64% in the layer (Rehman et al., 2013). When compared with present study incidence is higher in broiler and almost similar in case of layers. To detect the antibodies against MG, the serum samples of the layer birds were checked by ELISA and SPA test and result showed that 69.9% and 58.3% samples were positive by ELISA and SPA respectively (Osman et al., 2009). The incidence of MG is higher when compared with the present study. Sero prevalence of MG in backyard and commercial layer was found to be 62.5% and 53.61% respectively (Islam et al., 2014). In the present study incidence of MG by SPA was found to be 44.9% which is less than 53.61%. Another study showed that incidence of the MG in the broiler breeder flocks was 56.86% (Skider et al., 2005) while the prevalence of MG in broiler breeder in the present study is 59.6%. The findings of the two studies are almost similar. The sero prevalence of MG infection in layers was shown to be 54.9% and 53% by

(Biswas et al., 2003) and (Zhang et al., 2001) which is higher than the incidence in the present study. The sero prevalence of MG in breeder flock is 58.9% (Sarkar et al., 2005) which is very similar to result of present study. In the breeder flocks sero prevalence was found to be 57.15% (Pradhan 2002) which is very close to incidence of MG in breeder in the present study (59.6%). However the incidence of MG in breeder was shown to be 52% (Dulali, 2003) which is less than the incidence in the present study. The incidence of MG in the broiler breeder was 43% (Rehman et al., 2013) which is less than the incidence in the present study. The incidence of MG in broiler breeder was shown to be 14.2% in 2002, 21.4% in 2003, 10.3% in 2004 and 3.9% in 2005 (Seifi and Shirzad, 2012). The study showed that there was variation in the incidence of MG in the different years. The variation in sero prevalence findings of MG in chicken in different studies may be due to difference in management practices, treatment regime and biosecurity measures (Barua et al., 2006).

The overall sero prevalence of MS in all types of chickens was found to be 40.43%. The overall sero prevalence of MS in chickens was found to be 66.33% (Heleili et al., 2012) which is higher than results of present study. The findings of Rehman et al., (2013) supports the results of current study that in case of broiler, layers and breeders the sero prevalence was significantly different. The incidence of the MS was highest in the broiler breeder (50.14%) and lowest in broiler (10%). while in case of layer incidence of MS was found to be 42.60%. The sero prevalence of MS in broiler was found to be 69.96% (Heleili et al., 2012) which is much higher when compared with results of present study. The prevalence of MS in the broiler is shown to be 6% (Feberwee et al., 2008) which is consistent with the finding of current study. The low prevalence of MS in broilers is may be due to short life span of broilers and extensive antibiotic treatment. The incidence of MS in case of broiler is 12.5% and in layers is 39.64% (Rehman et al., 2013). These results are very much similar and consistent with the findings of present study. The prevalence of MS in layers is found to be 73% by (Feberwee et al., 2008) and 78.6% by (Hagan et al., 2004). The prevalence is high when compared with the findings of current study. The prevalence of MS in layer birds might be due to poor biosecurity and multiple ages housing (Kleven, 2003). So layer birds may be source of perpetual threat to other types of poultry birds.

The sero prevalence of MS in the breeder birds is shown to be 26.46% (Luciano et al., 2011). The incidence is less than the incidence found in the current study. The incidence of MS in the breeder birds was shown to be 28% (Rehman et al., 2013) which is less than the incidence found

in the current study. Feberwee et al., (2008) found that sero prevalence of MS in the breeder flock was 35% while in the current study the incidence is found to be 50%.

The overall sero prevalence of SPG was found to be 41.8% in all types of chickens. The sero prevalence of SPG in case of broiler was very low i.e. 5.36%. The sero prevalence of SPG in broiler was two to four times less as compared to layers. This difference might be due to longer life of layers and breach in the managemental conditions during their longer life span (Rehman et al., 2013). High prevalence of salmonellosis in the layers in the Faisalabad region was reported by Majid et al., (1991). The highest incidence of SPG was recorded 51.32% in case of layers while incidence was 44.11% in case of breeders. The incidence of SPG was found to be 21.88% in broilers and 32.32% in case of layers (Rehman et al., 2013). When compared with present study the incidence was less in case of broilers and more in case of layers in the present study. The sero prevalence of SPG in the layer was found to be 43.4% (Islam et al., 2006) which is little lower than the present study. The incidence of SPG in layers was reported to be 64.2% (Ashenafi et al., 2003) and 63.5% (Rahman et al., 2003). The sero prevalence of SPG in the layer was found to be 65.9% (Jalil and Islam, 2011). This finding is discordant with the present study in which incidence of SPG in layers is 51.32%. Sero prevalence was found 25.3% (Hossain et al., 2010) in the layer birds which is less than the incidence in the present study. These reports show different incidence than current study and it may be due to difference in geographical variation and difference in management conditions.

The prevalence of *Salmonella* infection was reported to be 23.8% (Alam et al. 2003) and 22.7% (Sarker, 2004) in the different districts of Bangladesh. The present findings are higher than reports of Alam et al. 2003 and Sarker, 2004. The sero prevalence of SPG in model breeder poultry farms was found to be 23.46% (Skider et al., 2005) which is less than the finding of present study. The incidence of SPG in case of breeder was 35% (Rehman et al., 2013) while incidence in the current study is 44.11%.

Results of the present study show that the sero prevalence of all the three pathogens was more in case of birds which were older than 20. Sero prevalence of MG was found to be 57% in pullets and 53.5% in adult layers (Islam et al., 2014). The sero prevalence in case of adult birds is almost similar to present study while according to Islam et al., (2014) sero prevalence is more in case of pullets. Mukhtar et al., (2012) showed the highest sero prevalence of MG in case of pullets than adults and laying birds. Similar results are also reported by (Hossain et al., 2007).

(Skider et al., 2005) and (Sarkar et al., 2005). The sero prevalence of MG and MS was more in adult birds 75% and 95.83% respectively as compared to young birds (Heleili et al., 2012). Similar results were also reported by (Osman et al., 2009). These findings support the result of current study that incidence of MG and MS in case of layers is more in the adult birds as compared to young birds.

According to (Islam et al., 2006) seroprevalence of SPG was 19.1% in 9-20 weeks while 52% in the group 20 week and above. The findings of present study are similar to results of (Islam et al., 2006) that showed with the increase in age of birds sero prevalence of SPG increased in the layer birds. The results of present study are in concordant with the findings of (Jalil and Islam, 2011) who reported the highest ser prevalence of SPG in layer birds which were more than 56 week old. The sero prevalence of SPG in the layers increased with increase in the age of the birds (Truong and Tieuquang, 2003; Hossain et al., 2010). The sero prevalence of SPG in the layers was highest in adult birds as compared to growing birds and the pullets (Rahman et al., 2004).

According to findings of this study the sero prevalence of MG was more in birds which were less than 20 week old. The sero prevalence of MS and SPG was more in the birds which were more than 20 week old. In the model breeder poultry farms the sero prevalence of MG was found to be 71% at 18 week of age and 50% at 49 week of age (Skider et al., 2005). The findings of this study are in concordant with the results of present study. In both the studies sero prevalence of MG in the breeder decreased with increase in age. According to (Sarkar et al., 2005) the sero prevalence of MG in the breeders was decreased with increase in the age which was recorded 73% in the 20 week old birds and 60% in the 42 week old birds. These results are exactly similar to findings of the present study in which maximum sero prevalence of MG was recorded in birds 0-20 week of age. According to findings of (Skider et al., 2005) the sero prevalence of SPG was maximum at 46 week of age and current study also showed that sero prevalence of SPG was more in birds which were older than 20 weeks. Sero prevalence of MG was found to be 28% at 10 to 20 week of age and 3.4% above the 60 week of age in Iranian broiler breeders (Seifi and Shirzad, 2012). These findings simulate the results of present study that in case of broiler breeders, sero prevalence is more in young birds as compared to the older birds. Similar findings were reported by Talha (2003) who showed that prevalence in the breeders decreased with increase in the age. Higher prevalence in the younger birds might be due to the vertical transmission of the disease.

According to the results of present study, sero prevalence of MG was higher in female birds as compared to male birds while the sero prevalence in case of MS and SPG was higher in male birds as compared to female birds. The sero prevalence of MG (52%) and SPG (24%) was in case of female birds while prevalence of MG (46%) and SPG (15%) in case of male birds (Skider et al., 2005). So the prevalence was more in case of female birds than male birds. In the present study for MG prevalence is more in case of female while for SPG prevalence is more in case of male birds. According to (Sarkar et al., 2005) sero prevalence of MG was higher in female birds (60%) than in male birds (48.57%). Similar results were also reported by (Seifi and Shirzad, 2012).These results simulate the results of present study which shows that female birds are more susceptible to MG than male birds.

According to findings of present study, the sero prevalence of MG and SPG in case of layer birds was highest during October to December i.e. 55.73% and 35.78% respectively. This high incidence may be due to more pre disposition of birds to these pathogens when the temperature is low. Sero prevalence of MG in Faisalabad district was found to be 20% higher in the layers in the winter than summer (Rehman et al., 2013). The incidence of MG in layers in Faisalabad district was 61.8% in winter and 47.74% in the summer (Mukhtar et al., 2012). Sero prevalence of MG was more in winter season as compared to summer season (Seifi and Shirzad, 2012). The prevalence of MG infection was more in the winter (70%) as compared to 60% in summer (Heleili et al., 2012). Similar results were also reported by (Skider et al., 2005), (Sarkar et al., 2005), (Hossain et al., 2007) and (Thai et al., 2009).

Sero prevalence of MG was found to be 58% in winter and 48% in the summer in the layers (Islam et al., 2014). These findings are in concordant with present study in which highest prevalence is found in period from October to December. According to (Islam et al., 2014) in case of layer birds the sero prevalence was found to be more in winter season (60%) as compared to summer season (51%) which is in consistent with the findings of present study. The higher sero prevalence in winter might be due to sudden changes in temperature and cold stress. The results of current study support the findings of (Sarkar et al., 2005), (Hossain et al., 2007) and (Mukhtar et al., 2012).

In the present study the sero prevalence of MS is highest during January to March i.e.57.64%. The incidence of MS was found to be 46.69% in the winters which is in concordant with the present study (Heleili et al., 2012). Similar findings were also reported by (Arbelot et al., 1997).

In the case of broiler breeders the highest sero prevalence of MG and SPG was found during the July to September i.e. 64.91% and 50.22% respectively. The sero prevalence of MS was highest (72.02%) in the period from January to March. The sero prevalence of MG in the Iranian broiler breeder was more (19%) in winter as compared to summer (9%). It is due to the stress of cold weather (Seifi and Shirzad, 2012). In case of breeder the sero prevalce of MG was highest during winter season and the sero prevalence of SPG in the breeder during rainy season was more as compared to during winter season (Skider et al., 2005). These results are similar to findings of current study in case of SPG while in case of MG similar prevalence was observed in rainy and winter season in the present study. Sarkar et al., (2005) reported that sero prevalence of MG was more in winter (64%) as compared to summer (53%). These results are in agreement with findings of current study. Similar findings are also reported by (Pradhan et al. 2000), (Alam et al. 2003) and Talha (2003). Higher incidence in winter might be due to influence of cold weather.

Salmonella pullorum and *Salmonella gallinarium* both contains O antigen 9 and 12 and may also have O antigen 1. There is variation in O antigen 12_1, 12_2 and 12_3 in case of *Salmonella pullorum*. There is more concentration of 12_2 than 12_3 in variant forms while reverse is true for accurate form. In the case of *Salmonella gallinarium* there is no such variation (Brooks et al., 2008). In the diagnostic test polyvalent antigen should be used because of this variation. Same antigen is used for detection of *Salmonella pullorum* and *Salmonella gallinarium* but the results for *Salmonella gallinarium* may be poor (Proux et al., 2002). Serum plate agglutination test was used to detect the antibodies in the serum against Mycoplasmas and Salmonella. Sensitivity and specificity of SPA test was compared with culture, PCR technique and ELISA. It was shown that SPA test performed equally well as ELISA (Feberwee et al., 2005). Tests other than SPA available for sero monitoring of flocks are ELISA and HI. Sensitivity and specificity of SPA test are almost same as HI test and ELISA. However, ELISA is not feasible for sero monitoring because it is more time consuming and costly (Higgins and Whithear, 1986). A very high prevalence by SPA test may be due to false positive results which are because of cross reactivity, use of inactivated vaccine, contaminated sera and age of flock (Luciano et al., 2011). Major constraints in the use of SPA test for diagnosis are its low specificity and higher incidence of false positive results (Pourbakhsh et al., 2010). Serum plate agglutination test can be used for screening flocks but not for screening individual birds. For proper diagnosis and control,

programs based on sero conversion may be inadequate, so sero monitoring should be combined with culture and molecular techniques (Luciano et al., 2011). The accuracy of sero prevalence by SPA test is dependent on the number of farms which were sampled, birds per farm sampled and test characteristics such as sensitivity and specificity (Feberwee et al., 2008). The difference in the sero prevalence of MG, MS and SP between this study and previous studies might be due to different production system, management conditions, age and flock variation, geographical locations, vaccination status of flock and interaction with the other pathogens. A very high sero prevalence of MG, MS and SPG requires keeping a vigilant eye of respective authorities and very strict biosecurity measures for control of these pathogens.

CHAPTER-6:
CONCLUSION

Conclusion

Control of mycoplasmosis and salmonellosis is generally based on the elimination of these organisms from poultry flocks. Control of these avian diseases is based on detection and elimination of the infection in the flocks. It is only possible in those flocks where prevalence is low, like in grandparent flocks. In layers such approach is not feasible, medication and vaccination are the parts of control strategy of these diseases. Now a days in the different areas of the world there is wide spread development of the poultry industry. Very close location of the poultry farms, rearing of mixed avian species in close milieus, mixed commercial poultry farming and presence of wild birds in close proximity to poultry farms have made the control of these diseases very difficult. In such conditions it is very difficult or almost impossible to maintain *Mycoplasma* and *Salmonella* free flocks. As result of it there is re emergence of the mycoplasmosis and salmonellosis and it is dire need of time to re assess the methods and strategies used to manage *Mycoplasma* and *Salmonella*.

Establishment of infection free breeding flocks is required for the control of mycoplasmosis and Salmonellosis as both the diseases are transmitted by vertical method. Before adding to the flock poultry birds should be tested and breeding stock should be purchased from certified infection free sources. Poultry birds should be hatched and reared in a way to reduce the horizontal transmission by preventing the contact with infected flocks. The poultry equipments and premises should be disinfected and cleaned on regular basis. To eliminate the infection from flock, repeated testing and culling of carrier birds can be helpful. Compounds containing phenols and quaternary ammonium based compounds should be used for effective disinfection.

Losses caused by mycoplasmosis and salmonellosis can be reduced by quick diagnosis, serological monitoring, effective antibiotic treatment plan and implanting strict biosecurity measures. More over it is the need of hour to design country wide detailed studies for prevalence of *Mycoplasma* and *Salmonella* to know the current status of these diseases in Pakistan.

CHAPTER-7:
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