Isolation, Identification and Antibiotic Sensitivity of Mycoplasma Species from Commercial Poultry Flocks in the Rawalpindi Division

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In

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بسم الله الرحمن الرحيم

DECLARATION

I hereby declare that the work accomplished in this thesis is the result of my own research carried out in Microbiology lab, Department of Animal Sciences, Quaid-i-Azam University, Islamabad and in Biotechnology lab, Poultry Research Institute Rawalpindi.

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Hizran Khatoon

Dedication

I dedicated this effort & work to the

Holy Prophet ***HAZRAT MUHAMMAD (PBUH)****, and his* ***(PBUH) Family***

The greatest educationist of mankind

My Father

Who always encourages me to go forward

My Mother

Her prayers always paved the way of success for me

CERTIFICATE

This dissertation "Isolation, Identification and Antibiotic Sensitivity of *Mycoplasma* Species from Commercial Poultry Flocks in the Rawalpindi Division" submitted by **Ms. Hizran Khatoon,** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Animal Microbiology.

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ABBREVIATIONS

AI	Avian influenza
Bp	Base pair
CFU	Colony forming unit
CRD	Chronic respiratory disease
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GDP	Gross Domestic product
GSH	Glutathione
GI	Growth inhibition
GTS	Gene-targeted sequencing
HA	Hemagglutination
HI	Hemagglutination inhibition
IB	Infectious Bronchitis
IFA	Indirect fluorescent antibody
Kb	Kilobase pairs
KDa	Kilodalton
MBB	Mycoplasma broth base
MIC	Minimum Inhibitory Concentration
MG	*Mycoplasma galisepticum*
Mgc*2*	*Mycoplasma gallisepticum* cytadherence membrane 2
MS	*Mycoplasma synoviae*
NAD	Nictoinamide adenine dinucleotide
NCD	New castle disease
OIE	Office International des Epizooties
P52	Proteins *52*
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PH	Power of hydrogen
PMGA	Proteins of *Mycoplasma gallisepticum* A
PPLO	Pleuropneumonia like organism
PRI	Poultry research institute
Pvp	Phase-variable putative adhesion protein

RFLP	Restriction fragment length polymorphism
Rpm	Rounds per minute
RAPD	Restriction amplified polymorphic DNA
RSA	Rapid serum agglutination
SPA	Serum plate agglutination test
SOD	Superoxide dismutase
VlhA	Variable lipoprotein hemagglutinin
WHO	World health organization

ABSTRACT

Mycoplasma galisepticum (M. galisepticum) and Mycoplasma synoviae (M. synoviae) are important avian pathogens and cause economic losses to the poultry industry. Molecular biology techniques, such as PCR, are currently used for rapid detection of these pathogens and adoption of control measures of the diseases. The aim of this study was to know the incidence of *M. galisepticum* and *M. synoviae* in local commercial poultry, to evaluate the efficacy of a PCR to detect the *M. galisepticum* and *M. synoviae* and to analyse the antibiotic sensitivity of the *M. galisepticum* isolates. For this purpose, tracheal swabs and tracheal tissue were taken from birds with signs of respiratory distress from 424 selected commercial poultry flocks (broiler, layer and breeder). These flocks were from different districts of Rawalpindi Division, Punjab province, during July 2015 to June 2016. *M. gallisepticum* was isolated and confirmed by growth characteristics and PCR on genomic DNA from the isolates. At the same time genomic DNA from tissue samples and swabs taken from infected birds was extracted using PureLink Genomic DNA Extraction kit following manufacturer's protocol and PCR was performed for *M. galisepticum* and *M. synoviae* using primers given in the OIE's online manual. The results of PCR for *M. gallisepticum* correlated with the results obtained by isolation of *M. gallisepticum* indicating the tested PCR can be used for direct detection of *M. gallisepticum* in infected birds without the need for isolation of this organism. Lastly, antibiotic sensitivity was performed in PPLO broth by the broth microdilution method. Out of 424 suspected cases, a total of 50 flocks were found positive for *M. gallisepticum*. Thus overall incidence was 11.79%. For individual bird type the (incidence of *M. gallisepticum* was 12.90 %, 10.19% and 10.34% in broilers, layers and breeders respectively. Out of the 424 flocks, 90 flocks were also screened for *M. synoviae* through PCR on genomic DNA from tissues and swabs and 17 (18.88%) flocks were found to be positive. For individual bird type the incidence of *M. synoviae* was 19.04 %, 18.18% and 19.14% in broilers, layers and breeders respectively. Incidence of *M. gallisepticum* and *M. synoviae* was higher in winter season compared to the summer season as compared to those reared in summer. Results of antibiotic sensitivity of the 50 *M. galisepticum* isolates revealed that they were resistant to multiple drugs. Tylosin and Ceftifer were found to be highly effective drugs for the treatment of *Mycoplasma gallisepticim* isolates, while Oxytetracycline showed limited therapeutic value for treating Mycoplasmosis. Enrofloxacine, Colistin, Chlorotetracycline found to be least effective against *M. gallisepticum*.

INTRODUCTION

Poultry is the second largest industry of the Pakistan after textile. Poultry industry in Pakistan alleviates the poverty by offering enormous opportunities to millions of people in the country. 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). The contribution of livestock in the gross domestic product (GDP) is 11.9 %, out of which share of poultry is 1.3 % at constant cost factor. This sector produces 1074 tons of poultry meat and 15, 346 million eggs (Economics Survey, 2014-15). Despite all these figures and a huge infrastructure, availability of poultry meat in Pakistan per annum is 3.90 Kg per capita while it is 55 Kg per capita in Kuwait, 50 Kg per capita in USA and 12 Kg per capita at world level (Bootwala, 2007). Similar situation prevails regarding the consumption of eggs. The above mentioned data indicates that there is still room for development in the poultry sector of Pakistan. One of the factors that hamper the progress of poultry industry in Pakistan is infectious diseases that inflict great economic losses. In Pakistan, the main bacterial diseases that are prevailing are mycoplasmosis and salmonellosis (Salmonella gallinarum and Salmonella pullorum) (Rehman *et al.*, 2013).

There are more than hundred species of Mycoplasma that cause infections in animals. In chicken, the most important species of Mycoplasma that cause infections are *Mycoplasma gallisepticum* and *Mycoplasma synoviae* (Marois *et al.*, 2002). Though, birds of all ages are susceptible but young ones are more prone to mycoplasmosis (Seifi and Shirzad, 2012). Mycoplasmosis, transmitted via eggs and contaminated hatcheries, also play a role in dissemination of this economically devastating disease of chicken. Mycoplasmosis has a worldwide existence and causes decreased egg production, reduced growth rate and condemnation at slaughterhouses (Kleven *et al.*, 2008). About 10-20% losses in egg production occur in the flocks affected from Mycoplasmosis (Bradbury, 2001).

1.1 Mycoplasmosis

Many species of Mycoplasma cause Avian Mycoplasmosis (Quinn *et al.*, 2002) including *M. gallisepticum*, *M. synoviae*, *M. iowae*, *M. meleagridis*. Among all, *M. gallisepticum* is more prominent pathogen that cause disease in poultry as well as

in few other birds (Bradbury, 2001). Chronic respiratory disease (CRD) in domestic poultry birds is caused by *M. galisepticum*, when other respiratory pathogens are present in poultry flock and when there is an issue of management stress. The symptoms of disease are conjunctivitis, coryza, sneezing, and sinusitis. Which appear particularly in game birds and turkeys. The results of this disease appear as loss of production and decline of meat-type birds, loss of egg production. *M. synoviae* usually causes respiratory disease, may lead to silent infection and synovitis (Charles and Graham, 1989; OIE, 2012).

1.2 *M. gallisepticum* and *M. synoviae*

A. B. Frank in 1889 used the term Mycoplasma referring these *M. synoviae* as a fungus (Krass and Gardner, 1973). Mycoplasmas are free living, self-replicating which are known to have smallest genomes (Nicolas and Ayling, 2003). Cell wall is absent in Mycoplasmas and have low GC content of 23- 40 %. The cell membrane of these organisms, *M. synoviae,* is incorporated with sterols which differentiates these from other organism (Kleven, 2008). Based on 16S rRNA analysis Mycoplasma belong to phylum Firmicutes, class Mollicutes and family Mycoplasmataceae (Ley, 2006).

In birds, 22 Mycoplasma species are known, out of which four species have pathogenic characteristics such as *M. gallisepticum*, *M. melegridis, M. Synoviae,* and *M. iowae* (Bradbury, 2001). Of all avian Mycoplasma pathogens, *M. gallisepticum* and *M. synoviae* are very important pathogens (Kleven, 2008). The natural hosts of *M. gallisepticum*, other than chicken are turkeys, pheasants, partridges, pigeons and quails (Ley, 2006). *M. gallisepticum* causes chronic infections in both turkeys and chickens and is the most virulent of all Mycoplasmas species (Liu *et al.*, 2001). *M. gallisepticum* and *M. synoviae* causes respiratory disease in chicken and turkeys. *M. iowae* and *M. melegridis* causes diseases only in poultry (Fan, *et al.*, 1995).

1.3 Structure and Classification of Mycoplasmas

The Mollicutes have only the plasma membrane, in which proteins make approximately two-thirds of the mass, while the rest of these make lipids of membrane. It has been predicted by Motif analysis of genome of *M. gallisepticum* that a large range of membrane-associated proteins. The lipoproteins of membrane are

majorly the surface antigens of mycoplasma (Razin, S. 1994). Actually, mycoplasma lipids are found in the cell membrane and contain phospholipids, glycolipids and neutral lipids. The genome is comprised of 9964 bp with an overall 31% GC (Guanine + Cytosine) content. The organism stains well with Giemsa's stain, but weakly gram negative. It is generally coccoid, approximately (0.25-0.5 ìm) (Whitcomb, R. F.1984). The organism shows a filamentous or flask-shaped polarity of the cell body due to the presence of terminal organelles or bleb (Almanama, M. A. 2011). *M. gallisepticum* requires a protein-rich medium for their growth, containing 10-15% added animal serum (Frey, 1968).

Taxonomy of *M. gallisepticum* is shown in table (1.1)

Table 1.1 Taxonomy of *M. gallisepticum* (Maniloff and Morowitz, 1972)

Taxonomy ID	233150
Kingdom	Bacteria
Class	Firmicutes
Order	Mollicutes
Family	Mycoplasmataceae
Genus	Mycoplasma
Species	*M. galisepticum*

1.4 Chronic Respiratory Disease (CRD)

M. gallisepticum and *M. synoivae*, both cause CRD in all types of chickens (Kleven and Bradbury, 2008). The primary causative agent of CRD is *M. gallisepticum* 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 outbreaks that result in great economic losses worldwide (Evans *et al.*, 2005). *M. gallisepticum* primarily damages respiratory tract by colonizing it and then secondary bacteria like *E. coli* and viruses cause 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. An avian Mycoplasma species, *M. galisepticum,* is invasive in vitro. This is the reason why *M. gallisepticum* not only resists host defense and antibiotics but also enters the blood and causes systemic infection (Winner *et al.*, 2000).

1.5 Signs and Symptoms of CRD

Mycoplasmosis, caused by *M. galisepticum,* in turkeys is more severe than in chickens, and clinical signs include, severe coughing, nasal discharge reduced feed intake, emaciation, and difficult breathing and sometimes conjunctivitis is also seen in the infected birds (Ley, 2008). Depression and lacrimation is also observed in infected birds (Forrester *et al.*, 2012). Fatal encephalopathy, arthritis and salpangitis are sometime seen in *M. gallisepticum* infected birds (Much *et al.*, 2002). In case of infected broiler, breeder and commercial layer egg production is sharply decreased.

There is marked increase in embryo mortality in eggs of infected birds (Ley, 2006). The clinical sign in case of *M. synoviae* are somewhat similar to *M. gallisepticum*. *M. synoviae* causes subclinical upper respiratory tract infection and synovitis in chickens and turkeys is one of very important finding (Khan, 2003). Sub clinical infections of the respiratory tract and tenosynovitis or bursitis is caused by *M. synoviae* in turkeys and chicken (Moraes *et al.* 2013). *M. synoviae* disseminates very quickly after it is introduced at farm because horizontal spread of *M. synoviae* is quick both directly and between the cages (Kleven, 2008).

Major pathological finding in *M. gallisepticum* infection is the air sacculitis while in some bird upper respiratory tract infection may also be present (Hong *et al.*, 2005). Pathogenic mechanism of Mycoplasma is controlled by a number of factors which includes ability of pathogen to attach host cell, type of cell injury and ability to resist host immune

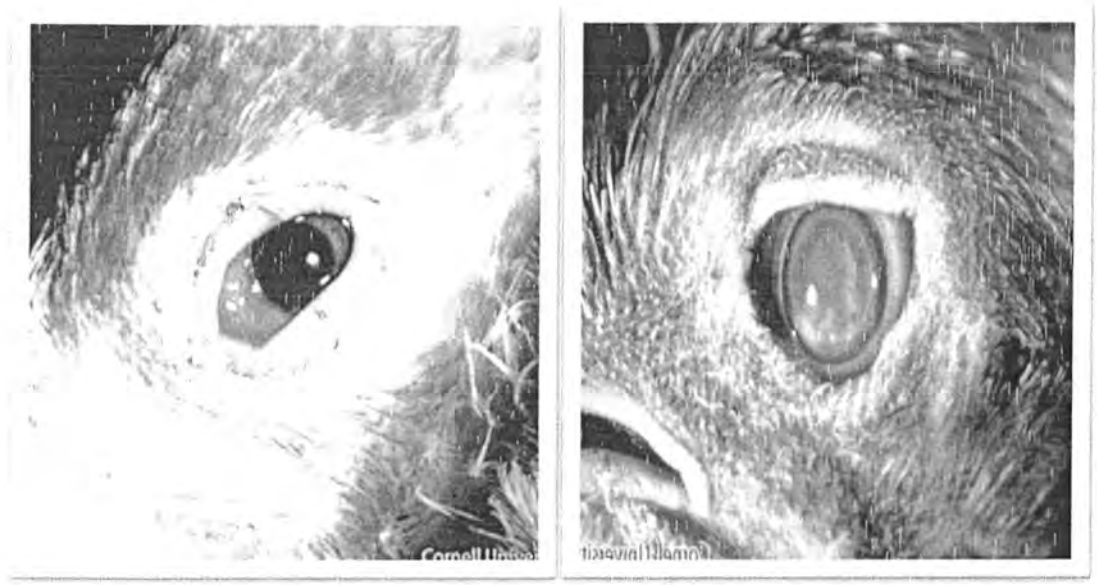


Fig.1.1 Conjunctivitis, edema (eyelid, periorbital), conjunctivitis, corneal opacity

Source: Atlas of Avian disease, Cornell University, 2002.

Fig.1.2 Advanced case of infectious sinusitis after exudates in one sinus was removed (Calnek *et al.*, 2014).

1.6 Pathogenesis

In the respiratory epithelium, the Sal glycoprotein receptors are required for the attachment of 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 Mycoplasmas, so for their survival Mycoplasmas need very close interaction with the host cell (Simecka *et al.*, 1993). Adherence mechanism of Mycoplasmas is very similar to adhesion of *M. pneumonia* which is better studied and adhesion is mediated with 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 of 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 chance of genetic alteration in host cell which may lead to auto immune disease (Bhandari & Asanai, 1989). Mycoplasma species produce hydrogen peroxide (H_2O_2) which plays a very important role in cell injury. Hydrogen peroxide released by Mycoplasma causes oxidative stress to host cell and may also causes hemolysis. During adherence process, Mycoplasmas produce H_2O_2 which causes damage to cell membrane and facilitates the entry of Mycoplasma. Nascent oxygen (O_2) is produced from hydrogen peroxide by catalase enzyme. This Nascent oxygen causes oxidative damage inside the host cell and is 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).

1.7 Transmission and Economic Losses

M. gallisepticum causes disease in birds of all ages but young birds are more susceptible to this pathogen (Nunoya *et al.*, 1995). In case of *M. gallisepticum* infection, the organism first colonizes the respiratory tract. In the different strains of *M. gallisepticum* the tissue tropism, cell injury, attachment and pathogenicity may

vary (Domanska *et al.*, 2008). *M. gallisepticum* causes great economic losses in all types of chickens and is also vertically transmitted. *M. gallisepticum* also causes drop in egg production and is hatchery disseminated. It is presumed that *M. gallisepticum* enters the respiratory tract by inhalation, aerosol or via the conjunctiva but it is not clear how it surmounts the bird's natural defense mechanism (Bradbury, 2001). The respiratory tract and lungs are frequent sites of infection (Quinn *et al.*, 2002). Decreased hatchability and low egg production count for the major economic losses caused by *M. gallisepticum*. Birds infected with *M. gallisepticum* show slower growth rate and produce low quality day old chicks. This results in increased medication and control procedure costs in poultry farming (Ley, 2006).

1.8 Diagnosis

For the diagnosis of Mycoplasmosis, a number of methods including serological, isolation and molecular identification of the causal organism have been used. Cultivation is the most reliable method for the confirmatory diagnosis of Mycoplasma (Ley, 2010). The limitations of diagnostic tests and similarities in the diseases 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 has been discussed below.

1.9 Isolation

Direct isolation and identification is not a 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. Mycoplasma requires 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 nonpathogenic Mycoplasma species and growth of other bacteria and fungi (Garcia *et al.*, 1995). Selective pressure on population of Mycoplasmas that differ substantially in vitro and in vivo are also an important factor. Pathogenic properties of the strain may be lost during passages in the culture media. Mycoplasma has very small genome and has little capacity for biosynthesis and is dependent on host cell for its requirements.

Mycoplasma is dependent on host for cholesterol, amino acid, fatty acid, vitamins, nucleotides and others nutrients. Therefore, in vitro growth of Mycoplasma is very difficult. Mycoplasmas do not have regulatory mechanism involved in changes in gene expression and cannot respond to fluctuating environmental conditions in vitro. It makes extremely demanding to work with this organism (Razin *et al.*, 1998). Once Mycoplasmas are isolated from their host they may die rapidly if they are not kept in suitable environment and proper medium (Zain & Bradbury, 1996). Handling of sample between collection and inoculation is very critical for isolation of Mycoplasma. Swabs, which were dipped in Mycoplasma broth, and kept on 4°C are more workable than dry swabs. Due to these reasons, Mycoplasma isolation is difficult, time consuming and costly. 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.

Complex media are used for the in vitro cultivation of Mycoplasmas for sake of overcoming the deficiencies of Mycoplasma. This media was initially termed by Frey *et al.*, (1968). Generally, this medium is composed of protein digest and meat infusion base. The medium is enhanced with horse or swine serum, yeast extract and glucose. To inhibit the growth of bacteria and fungi, antibiotics and bacterial inhibitors are added (Hong *et al.*, 2005). Major inhibitors of bacterial growth like Thallium acetate and ampicillin are added in the media. Mycoplasmas are resistant to Thallium acetate while TA prevents the development of gram positive and gram negative bacteria (OIE Terrestrial Manual, 2008).

Ampicillin inhibits the growth of bacteria by inhibiting the cell wall synthesis and cross linking of peptidoglycans. Ampicillin has no effect on Mycoplasma because cell wall is absent. Biochemical, physiological, and morphological characteristics of Mycoplasma are affected by composition of media and cultural condition. Lipid content of membrane, nutritional quality and osmotic strength of medium are basic factors which affect the morphology of Mycoplasma (Rodwell & Mitchell., 1979).

Mycoplasma is rapidly mutating organism as changes occur in short periods during growth. This ability of diversification plays important role in pathogenesis of disease caused by Mycoplasma.

During cultivation, pathogenic characteristics of Mycoplasma may be lost due to rapidly occurring mutations (Wise *et al.*, 1992). All these attributes should be kept in mind while attempting isolation and cultivation of Mycoplasma. Typical egg fried, small and clear colonies of Mycoplasma are observed on solid medium. Colonies are clear with central whitish raised parts (Kleven, 2008).

Pure colonies of *M. gallisepticum* are clear while pigmented colonies are indicative of bacterial contamination (OIE Terrestrial Manual, 2012). Both can be passed through 0.45µm filter if the contamination is seen early. Mycoplasma species are very flexible and due to absence of cell wall, these can pass through filter. Mycoplasma colonies can pass through

0.22 µm filter and can contaminate the cell culture. This disadvantage of contaminating the cell culture can be used as advantage in the cultivation of Mycoplasma. It is slow growing organism and cultures are kept for 21 days before discarding as negative. The colonies of Mycoplasma are visible under a stereomicroscope (Serena, 2013).

1.10 Detection by Serology

Many serological tests are used routinely for checking of flocks against Mycoplasma. Serological test are easy, provide fast detection and require less expertise. These tests include serum plate agglutination (SPA), heamagglutination inhibition (HI) and ELISA (Kleven, 1997). Though 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 Mycoplasmas 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 protein due to variations 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 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 the diagnosis are its low specificity and higher incidence of false positive results (Pourbaksh *et al.*, 2010).

SPA test can be used for screening flocks but not for screening individual birds. For proper diagnosis and control program, *M. synoviae* based on sero-conversion may be inadequate, hence, sero-monitoring should be combined with culture and molecular techniques (Luciano *et al.*, 2011). *M. gallisepctium* is shown to be cross reactive with closely related *M. imitans* that would also lead to aberrance in prevalence of specific Mycoplasma species (Bradbury *et al.*, 2001). This is because both *M. gallisepticum* and *M. imitans* 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, 2008).

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

Hassan *et al.*, (2014) carried out a study to see the sero-prevalence of *M. gallisepticum* and *M. synoviae* by SPA test. The results showed that 74.28% samples from turkeys were positive for *M. gallisepticum* and 25.71% for *M. synoviae*. In Baluchistan, overall prevalence was 8% and 15% by SPA and ELISA respectively (Atique *et al.*, 2012). The prevalence in case of broilers was 70% and in case of layers prevalence was 71% (Gharibeh and Roussan, 2008).

1.11 Molecular Diagnosis

For differentiation of Mycoplasma strains, several molecular techniques have been developed including Polymerase chain reaction (PCR), real-time PCR, Protein Profile Analysis, Ribotyping, Restriction Fragment Length Polymorphism (RFLP), and 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).

Due to high sensitivity and increase specificity of the polymerase chain reaction (PCR), it has become an invaluable device in the identification 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 reduces the need to isolate and culture the organism.

Since PCR is dependent on the target, its specificity is highly flexible. It is either species specific or strain specific by targeting unique gene in particular species or conserved region in the specific strain. PCR assays were developed in 1990s for four main disease causing Avian Mycoplasma species (Raviv & Kleven, 2009). Earlier PCR methods targeted 16S rDNA region but the recent PCR assays target the species specific regions and surface proteins (Liu *et al.*, 2001). PCR assays that target 16S rDNA region are less specific and may cross react with other avian Mycoplasmas because 16S rDNA region are highly conserved among phylogenetically related groups (Garcia *et al.*, 2005). Those PCR assays are less sensitive which target surface protein because of high levels of intraspecific genetic polymorphism (Ravia *et al.*, 2009).

Many PCR methods can be applied for detection of *M. galisepticum*, include Genekam Biotechnology AG, commercial kits produced by IDEXX laboratories, and others. PCR assays have been established to target different genes including pvpA, lipoprotein, *mgc*2, gapA, 16S rDNA gene, and 16S-23S intergenic spacer region (Domanska-Blicharz *et al.*, 2008). For the detection of *M. gallisepticum* mcg2 gene, PCR is highly specific and sensitive (Garcia *et al.*, 2005).

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

PCR assays are based on surface proteins which 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 the host cell, pathogenesis and host cell modifications occur (Goh *et al.*, 1998; Winner *et al.*, 2000). One of the important cytadhesins is encoded by *M. gallisepticum*c2 gene (Boguslavsky *et al.*, 2000). In *M. gallisepticumu mg*c2 gene is used for molecular detection of isolates as it is fairly conserved. Assay based on *M. gallisepticum m*cg2 gene is able to differentiate between field strain and 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 very fast, less expensive, effective and more reliable method.

1.12 Differential Diagnosis

In poultry, differential diagnosis involves respiratory diseases such as Newcastle disease (ND), Infectious bronchitis (IB), and avian influenza (AI). *Mycoplasma synoviae* infections and some others like *Pasteurella multocida* and *Haemophilus paragallinarum* should not be included. In turkeys, mixed infections of other pathogens involve avian pneumovirus, Chlamydia, *Pasteurella multocida*, and *M. synoviae* occur with *M. gallisepticum* (Gross *et al.*, 1990; David H. Ley, 2013).

1.13 Control of *M. gallisepticum* and *M. synoviae*

Major reason for ineffective control of Mycoplasma is its vertical transmission (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 those places where complete removal is problematic, live vaccines are used as substitute of control strategy. There are three commercially available vaccines for control of *M. gallisepticum* which include the F strain, Ts-11 and 6/85 strain (Liu *et al.*, 2001). The *M. gallisepticum*-F strain is a typical pathogen and naturally occurring strain and the advantage is that single dose is needed with this strain (Ley, 2008). The Ts-11 strain originated in Australia and 6/85 is of US origin (Ferraz & Danelli, 2003). Ts-11 as well as 6/85 are live vaccines and contain poorly transmitted strain, due to this reason these strains are considered to be safer than *M. gallisepticum*-F strain.

1.14 Treatment

Mycoplasmas do not possess cell wall (Siddique *et al.*, 2012). So, cell wall inhibitors like penicillin or other antibiotics are ineffective against the pathogen. Antibiotics that inhibit metabolic processes of microorganism, like fluoroquinolones, macrolides, pleuromutillins, tetracyclines and others are effective against Mycoplasma (Ley, 2008). Tylosine and gentamycin are effective against Mycoplasma species but higher dose of 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 by use of SPA, HI test and ELISA kits.

1.15 Mycoplasmosis in Pakistan

In Pakistan, serological evidence for the presence of Mycoplasma has been reported as early as 1964 (Mukhtar *et al.*, 2012). The mean prevalence of diseases caused by *M. gallisepticum* and *M. synoviae* was found to be 8.7% and 1.5% respectively in Punjab from June 2011 to July 2012 (Rehman *et al.*, 2013). The Incidence of Mycoplasma was found to be 12.69% in the flocks with respiratory problem in Faisalabad in 1997 (Tariq *et al.*, 1987). The increase in sero prevalence was noted in 2000 and prevalence was 90% and 15.5% respectively for *M. gallisepticum* and *M. synoviae* (Mukhtar *et al.*, 2012). The overall sero prevalence of *M. gallisepticum* in Baluchistan in broiler was 10% and 19.67% by SPA test 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 the sero prevalence of *M. gallisepticum* in the layers birds in the Faisalabad region was 49.38%. A study by Rehman *et al.*, (2013) in Punjab Provence of Pakistan showed that prevalence of *M. gallisepticum* was found 31%, 39%, and 45% for broiler, layer and breeder respectively. For *M. synoviae*, the sero-prevalence was found to be 12.4%, 36.3% and 34.1% for broilers, layers and breeders respectively. *M. gallisepticum* and *M. synoviae* both are responsible for massive economic losses to poultry industry of Pakistan (Rehman *et al.*, 2013).

Aims and Objectives

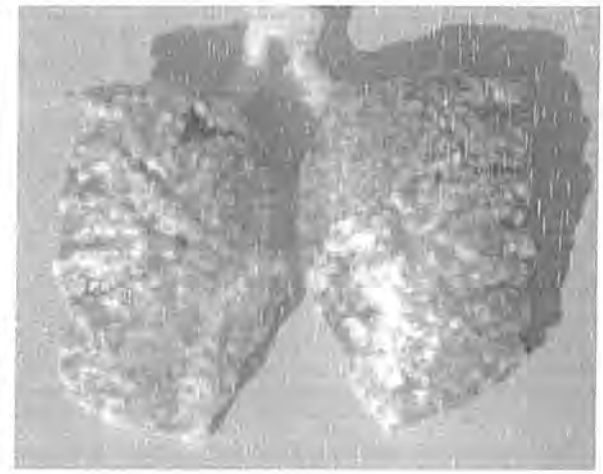
- To evaluate the efficacy of PCR suggested by the OIE in detecting *M. gallisepticum* and *M. synoviae* from commercial poultry flocks of various districts of Punjab Province.
- To investigate the relative involvement of *M. galisepticum* and *M. synoviae* in respiratory diseases of Avian *Mycoplasmosis.*
- To determine the incidence of Mycoplasmosis in broiler, layer and breeder.
- To determine MICs of different antibiotics of local *M. gallisepticum* isolates in order to devise proper treatment strategy.

Materials and Methods

2.1 Poultry Birds and Samples

This study was conducted in the Disease Diagnostic Section of Poultry Research Institute (PRI), Rawalpindi and at the department of Animal Sciences, Quaid-i-Azam University, Islamabad. The research was carried out from July 2015 to June 2016 on commercial poultry birds. The birds included in this study were suspected of having Mycoplasma infection on the basis of postmortem lesions and clinical signs. These birds were brought to PRI, from commercial poultry farms of Rawalpindi division, for postmortem examination. Out of 424 flocks selected for isolation of Mycoplasma, 248 flocks were Broilers, 118 layers and 58 breeders.

Samples comprising of pieces of trachea, air sacs, lungs and tracheal swabs were collected from the suspected cases of Mycoplasma.



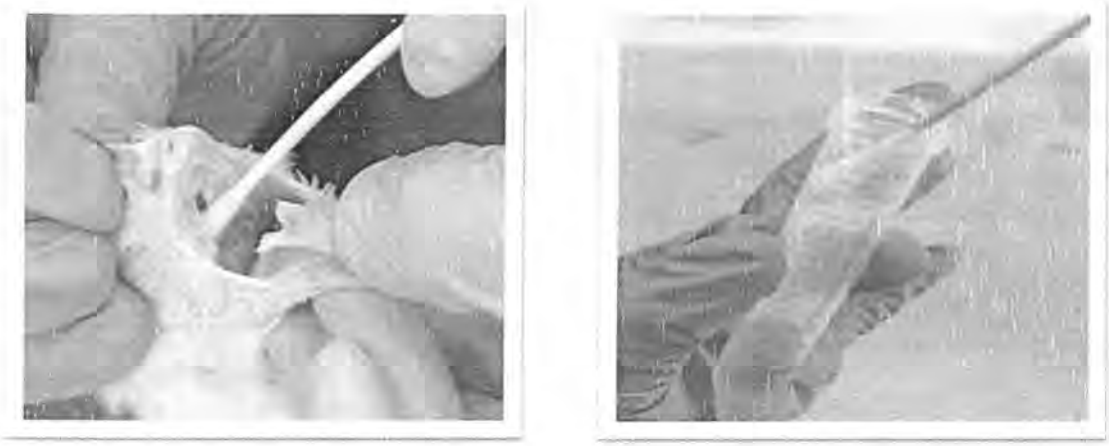


Fig 2.1 Tissues affected by Mycoplasma (a) Trachea, (b) lungs, (c and d) Swabs.

After postmortem examination suspected samples were shifted to biotechnology lab of Disease Section.

2.2 DNA Extraction from tissue and swabs

2.2.1 Lysate Preparation from tissue

Extraction of genomic DNA was done from tissue samples using PureLink Genomic DNA Extraction kit (Invitrogen Company, Cat No. K1820-01) following manufacturer's protocol. Since the structure of Mycoplasma is similar to that of eukaryotic cells, the extracted DNA was expected to contain Mycoplasma DNA in addition to chicken tissue DNA. Briefly 30 mg tissue was kept in a 1.5 ml microcentrifuge tube and 180 µl of PureLink Genomic Digestion Buffer then 20 µl Proteinase K was added. After this the homogenate was shifted to bath for 1 to 4 hours and the temperature of bath was 55°C with occasional vortexing until lysis was complete then centrifugation of lysate was performed at maximum speed at room temperature for 3 minutes. Then the supernatant was poured into a new 1.5 ml microcentrifuge tube and RNase A (20 µl) was added, followed by vortexing for 5 seconds and incubated for 2 minutes at room temperature. Then, PureLink™ Genomic Lysis or Binding Buffer (200 µl) was added and mixed by vortexing in order to make homogenous solution. Then, 200 µl of ethanol (96-100%) was added to lysate and mixed, for 5 minutes, by vortexing. Approximately, 640 µl of lysate was produced.

2.2.2 Binding DNA

In a collection tube, a PureLink Column was placed and then lysate (640 µl) was poured into this spin column then centrifuged (10,000x g) at room temperature for 1 minute. Then the collection tube was emptied and transferred this spin column to a new collection tube. Left over sample was applied to the spin column with same method.

2.2.3 Washing DNA

By adding 500 µl of Wash buffer 1, adsorbed DNA was washed and this was followed by centrifugation at 10,000x g at room temperature for 1 minute. Then, spin column was kept in another collection tube. Wash Buffer 2 (500 µl) was added to lysate, then centrifuged for 3 minutes at maximum speed (18,000 rpm) at room temperature and kept into a new collection tube.

2.2.4 Eluting DNA

The spin column was placed in a sterile 1.5 ml microcentrifuge tube. For the elution of DNA from the spin column PureLink™ Genomic Elution Buffer (100 µl) was added. Then, it was incubated for 5 minutes at room temperature. The collection of DNA was done by the centrifugation of spin column which was inserted into a collection tube at 18,000 rpm for 1 minute. This step of elution of DNA was repeated and eluted DNA fraction united. This labelled DNA was stored at -20 °C.

2.3 PCR amplification of *M. gallisepticum* and *M. Synoviae*

The confirmation of positive *M. gallisepticum* and *M. Synoviae* isolates was done by PCR using Species Specific Primers. For each primer set, the primers sequences and cycling conditions are given in the table 2.1

Table 2.1 Mycoplasma species, product, conditions and PCR primer pairs used for amplification.

Multiplex primer	Primer sequence	Product	Cycling Conditions	REFERNCES
MG-14F MG-13R	5' GAGCTAATCTGTAAAGTTG GTC 3' 5 'GCTTCCTTGCGGTTAGCAA C 3'	185	Denaturation; 94°C, 30 sec. Annealing; 55°C, 30 sec. Extention; 72°C, 1 min. Final Ex;72°C,5 min Cycles; 40	OIE Terrestrial Manual 2008
MS–F MS–R	5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3' 5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'	207	Denaturation; 94°C, 30 sec. Annealing; 55°C, 30 sec. Extention; 72°C, 1 min. Final Ex;72°C, 5 min Cycles; 40	OIE Terrestrial Manual 2008

Mixture of total 50µL volume was prepared for each reaction of PCR amplification, containing 5µl of 10x PCR buffer, 1 µM forward primer similarly 1 µM reverse

primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5µl Taq DNA Polymerase, 5µl genomic DNA and 22.5 µl nuclease free water.

PCR reaction parameters include initial denaturation at 95°C for 5 min, then 35-40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension for 1 min at 72°C. The final extension for 5 minutes at 72°C. Then, electrophoresed the PCR products in ethidium bromide stained 1% agarose gel. Then DNA bands were visualized and pictures were taken in DNA gel documentation system (Gene Genius, Syngene, UK). A negative control PCR, in which the DNA was replaced with water was also included in PCR for both *M. gallisepticum* and *M. synoviae*.

2.5 Isolation and culture of *M. gallisepticum*

2.5.1 Preparation of culture media

Mycoplasma agar and broth were prepared as per OIE's manual. Mycoplasma broth (for broth) or agar (for agar) base, yeast extract glucose, phenol red and thallium acetate (part 1) were added in distilled water and autoclaved. Horse serum, cysteine hydrochloride and penicillin (part 2) were filter sterilized by using 0.2µm and 4.5µm membrane filters. Autoclaved part 1 was cooled down to 50 °C and was aseptically added into filter sterilized part 2. The media were sterilized by autoclaving and filtration and stored at 4 °C until use.

2.5.2 PPLO broth medium

Initially, tissue and swab samples were inoculated in PPLO broth medium for isolation of *M. gallisepticum*. Following ingredients mentioned in table 2.2 were mixed to prepare PPLO broth medium according to Kleven and Yoder Jr (1998).

2.5.3 Modified Frey's solid media

Tissue and swab samples were streaked on Frey's solid medium for isolation of *M. galisepticum*. Ingredients mentioned in table 2.2 were mixed to prepare Frey's solid medium (Kleven and Yoder Jr 1998)

2.5.4 Isolation of *M. gallisepticum*:

Isolation of *M. gallisepticum* was carried out following OIE's manual and procedure used by Quinn *et al* (2002).

For isolation of Mycoplasma same tissue samples were used that were used for DNA extraction. Tissue samples of lungs, air sacs, and trachea of each individual bird were crushed separately using pestle and mortar. Then, homogenous tissue suspension was passed through sterile sieve and filter paper, the filtrate was passed into sterile plastic test tubes and centrifuged at 5000 rpm for 20 minutes. Using sterile syringe (20 ml), the supernatant was passed through sterile membrane filter of 0.45 μm pore size. Finally, the filtrate was dispensed (inoculated) on plates of PPLO agar and broth media and at 37°C incubation with 5% CO_2 was done.

Plates were examined daily for any visible colony growth under a stereomicroscope (25X). Prominent and visible distinctive *M. gallisepticum* (round colonies with central nipple) colonies were marked under stereomicroscope. Then, inside safety cabinet, marked colonies were incised including the agar with special scalpel and sub-cultured on to Mycoplasma agar by striking and swabbing and also the sub-cultured *M. gallisepticum* into an air tight (to prevent dehydration) glass tube of *Mycoplasma* broth.

Agar was incubated at 37°C in 5% CO_2 incubator and the broth at 37 °C without CO_2. After primary isolation and sub-culturing on to Mycoplasma agar and in broth, further sub culturing 10 to 12 times was conducted from agar to agar, agar to broth and broth to broth (after passing a Millipore of 0.45μm membrane filter) to obtain pure *M. gallisepticum* colonies.

After obtaining pure culture, DNA was extracted from bacterial colonies and PCR was done to confirm the isolates as *M. gallisepticum*.

In case of *M. synoviae* isolation on laboratory media was not attempted, because the cost of growth medium for *M. synoviae* was not affordable. Therefore, for *M. synoviae* only PCR on DNA extracted from tissues and swabs was performed.

Table 2.2 Formulations of two commonly used media for the isolation and propagation of Avian Mycoplasmas.

Medium	Constituent	Amount
Frey's medium	Mycoplasma broth base (BBL, Cockeysville, MD)	22.5 g
Glucose		3 g
Swine serum		120 ml
Fresh yeast extract		100 ml
Cysteine hydrochloride*		0.1 g
NAD*		0.1 g
Phenol red (1%)		2.5 ml
Thallium acetate (10%)		2.5 to 5 ml
Penicillin G potassium		106 units
Distilled water q.s.		1000 ml
Adjust pH to 7.8 with 20% NaOH and filter sterilize		
PPLO broth	**PPLO broth without crystal violet (Difco)**	**14.7g**
Glucose		10 g
Fresh yeast extract		100 ml
Swine serum		150 ml

Cysteine hydrochloride*	0.1 g
NAD*	0.1 g
Phenol red (1%)	2.5 ml
Thallium acetate (10%)	2.5 to 5 ml
Penicillin G potassium	106 units
Distilled water q.s.	1000 ml

Adjust pH to 7.8 with 20% NaOH and filter sterilize

(Source: Charles and Graham, 1989).

2.6 Antibiotic sensitivity testing

A total of 50 isolates of Mycoplasma confirmed by PCR and culturing isolation were subjected to antibiotic sensitivity testing. Antibiotic sensitivity was performed in PPLO broth by the broth microdilution method (Whithear, Bowtell *et al.* 1983). In Frey broth medium of each Mycoplasma isolate, 24-h broth culture was used as inoculum. Then, in 4 ml of Frey broth, a 1 ml aliquot was added and incubated at 37 until the color of medium changed from dark red to orange (pH 6.8–7.0). A 2 ml amount of this broth was added to 18 ml of Frey broth and homogenized. This gave sufficient inoculum to set up two replicate plates.

In 50µl volumes of Frey broth, replicate doubling dilutions of antibiotics were made in sterile tissue with culture quality 96-well U-bottomed microtiter plates. When the dilution for each antibiotic finished, 150µl of broth with pH 7.8, having the required density of organisms was inoculated into each well with the help of a multichannel dispenser. Culture controls in Frey broth plus bacteria without antibiotic were involved in all tests. Then, plates were covered with plastic wrap and then incubated at 37°C. Lowest concentration of antibiotic was regarded as the minimum inhibitory concentration (MIC) this is concentration of drug that completely checks the color change in the medium. When the phenol red indicator in the culture control turned orange then the MIC was read. MIC was determined by comparison with the color of sterile Frey broth that was adjusted to pH 7.0. Control of density of inoculum was attained by viable count in the Frey broth. Densities of inoculum in the range of 10^3 to 10^5 CFU/ml were deliberated suitable.

Table 2.3 List of Drug and their Concentration

Serial Number	Drugs	Symbol	Class of Antibiotics	Concentration of Antibiotics Used mg/ml
1	Tylosin	TYL	Macrolide	200
2	Colistin	CO	Polypeptide	200
3	Chlortetracycline	CT	Tetracyclines	200
4	Enrofloxacin	ENR	Quinolones	200
5	Oxytetracycline	OT	Tetracyclines	200
6	Ceftiofur	EFT	Beta-lactams	200

Results

Post mortem examination

Clinically sick chickens of commercial poultry flocks were showing signs of respiratory distress (dyspnea), tracheal rales, nasal discharge, depression, facial swelling, mouth breathing, and with history of reduced appetite.

Fig. 3.1 Clinically sick chicken

Mucoid material, observed in trachea, lungs and air sacs of the birds affected by Mycoplasma, was consolidated, bloody, light yellow in appearance and copious caseous exudates were accumulated as shown in fig.3.2

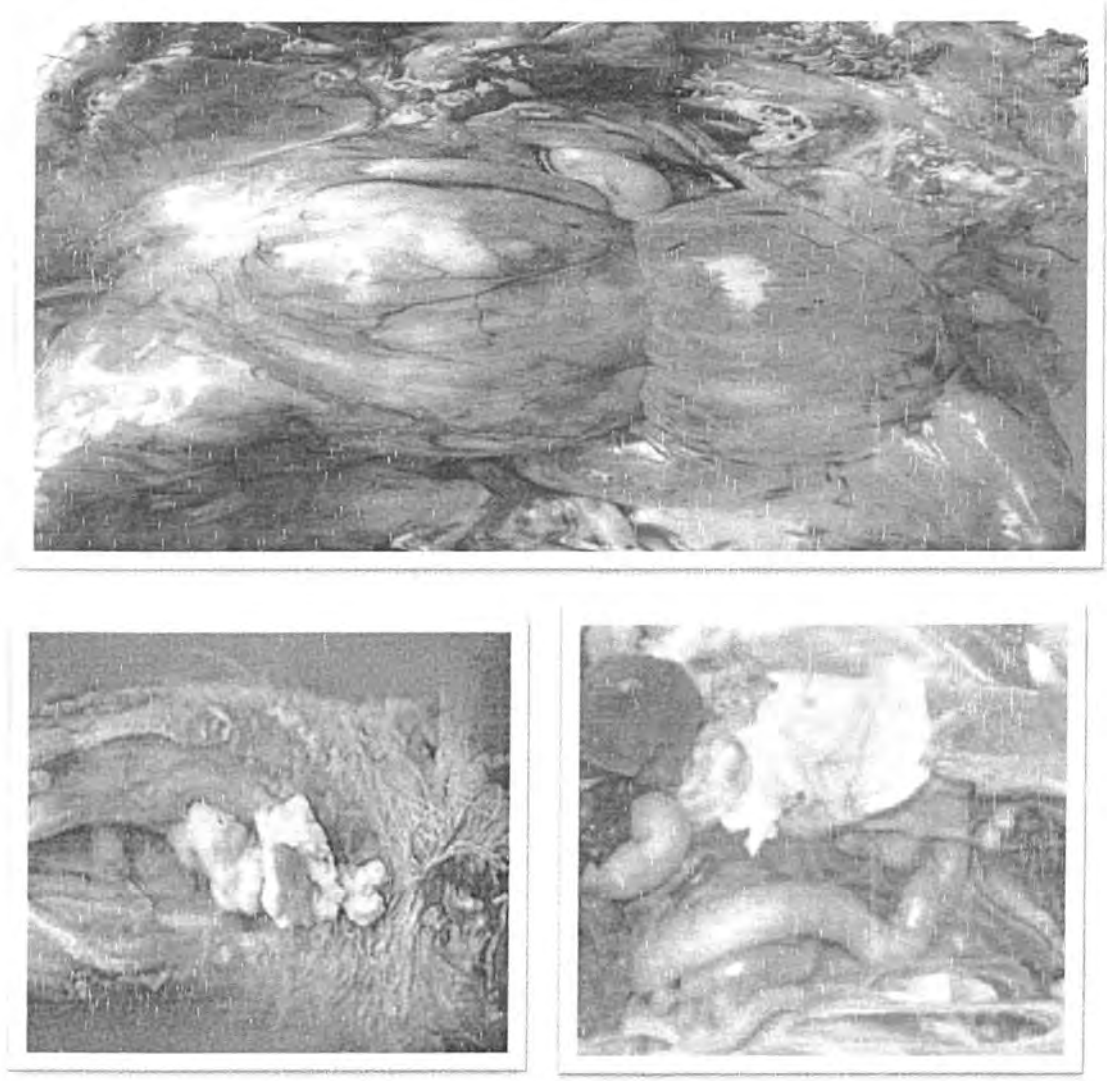


Fig. 3.2 Trachea, Lungs and Air sacs of birds affected by Mycoplasma

Results of PCR for *M. gallisepticum* on Genomic DNA Extracted from tissues and swab

In current study, by PCR a total of 50 birds (1 bird representing 1 flock) out of 424 were found *M. gallisepticum*-positive. The results of PCR using primer pair MG-14F and MG-13R, showing a band of 185bp are shown in Fig.3.3.

The second PCR using set of primer pair MS–F and MS–R that confirmed *M. synoviae,* amplified a product of 211bp as shown in Fig.3.4.

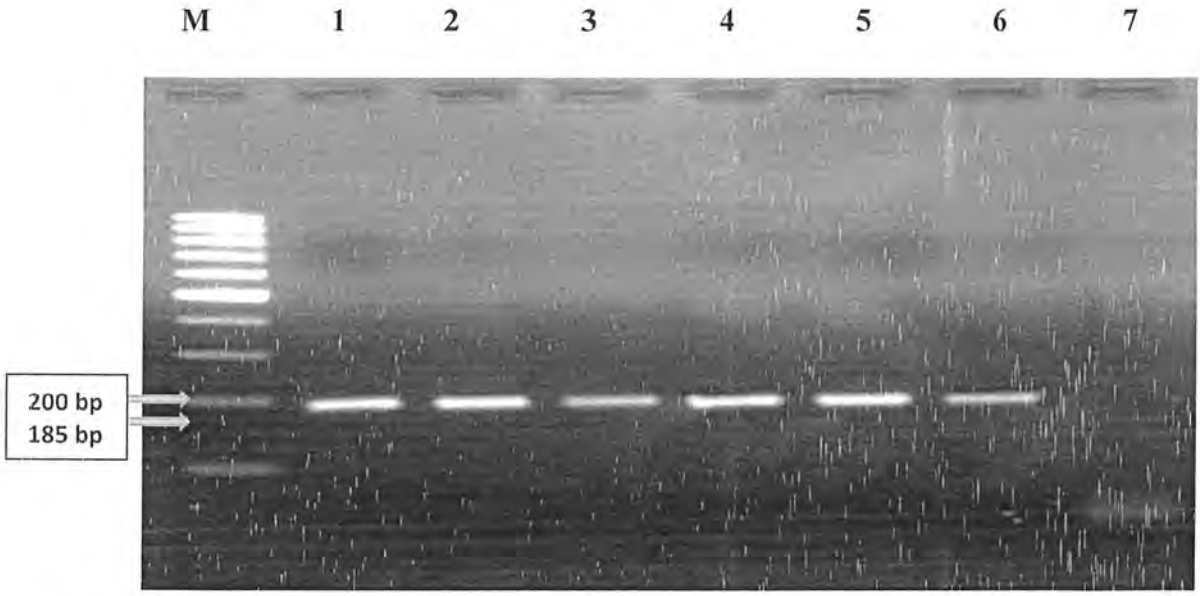


Fig. 3.3 Represents the Agarose gel electrophoresis of PCR product of 185 bp. From left: lane M represents a molecular weight marker of 100bp, Lane 1-5 represents *M. gallisepticum isolates,* Lane 6 represents positive control of Mycoplasma, Lane 7 represents Negative control.

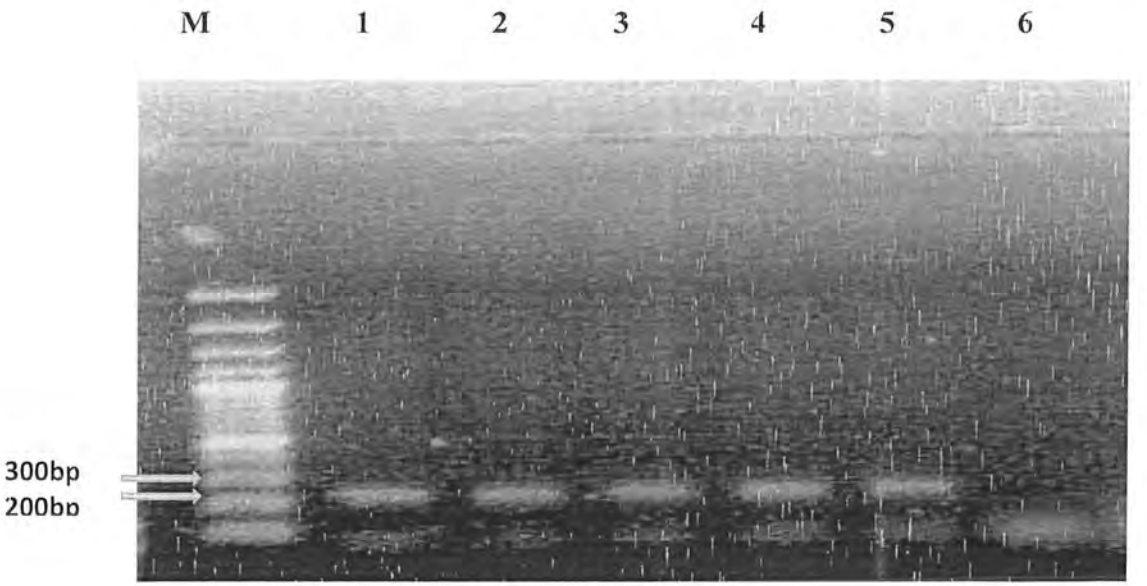


Fig. 3.4 Represents the Agarose gel electrophoresis of PCR product of 211 bp. From left: lane M represents a molecular weight marker of 100bp, Lane 1-5 represents *M. synoviae* isolates, Lane 6 represents Negative control of Mycoplasma.

Culture and isolation of *M. gallisepticum* on the agar and PPLO broth.

Mycoplasma was identified on the basis of culture and isolation. The organism was cultured on PPLO agar media. Pure fried egg colonies were produced and the organism was confirmed as *M. gallisepticum* in Figure 3.5. Then fried egg colonies were transferred to PPLLO broth. The dark red color of broth turned to orange that confirmed the growth of *M. gallisepticum* as shown in Figure 3.6.

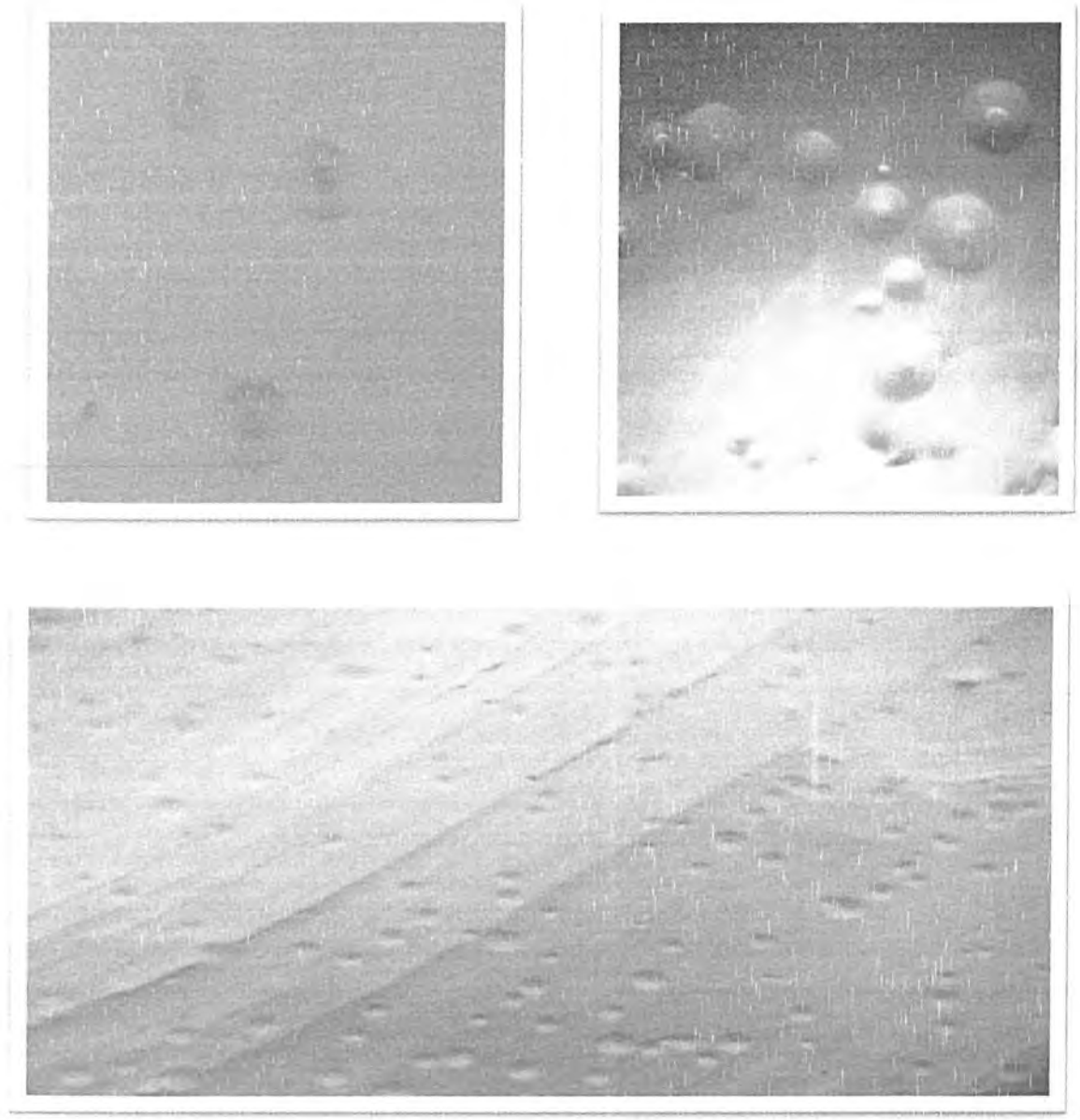


Fig. 3.5 shows Pure Mycoplasma micro colonies on PPLO agar with characteristic round and central nipple (25xUnder stereomicroscope).

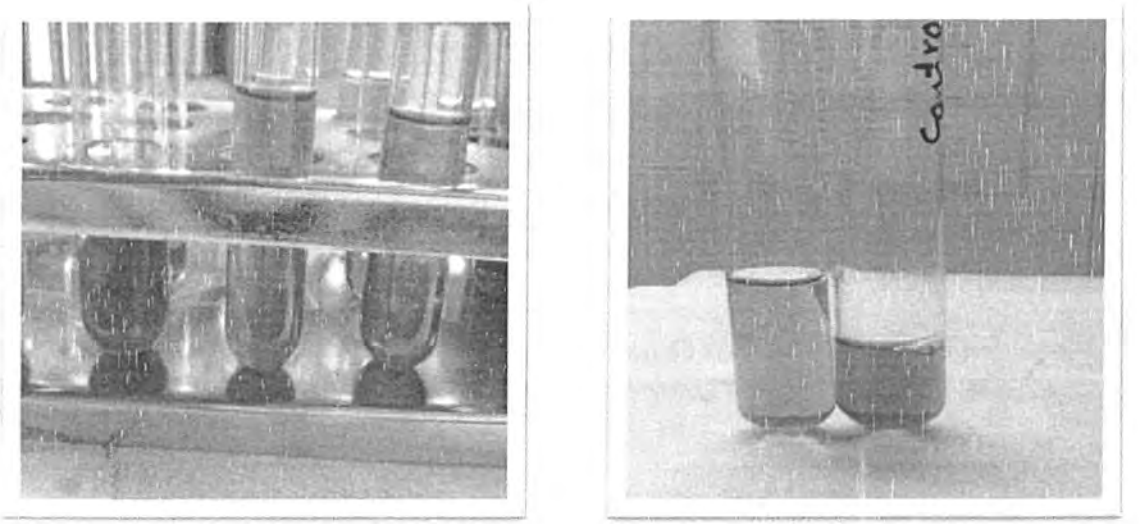


Fig. 3.6 Change in colour of broth from dark red to orange shows Mycoplasma growth.

PCR on Genomic DNA Extracted from Colonies to confirm *M. galisepticum*

For confirmation of *M.gallisepticum* DNA extracted from both solid and liquid culture media were subjected to PCR. On DNA gel electrophoresis 185 bp band confirmed the isolate as *M. gallisepticum* Fig. 3.7.

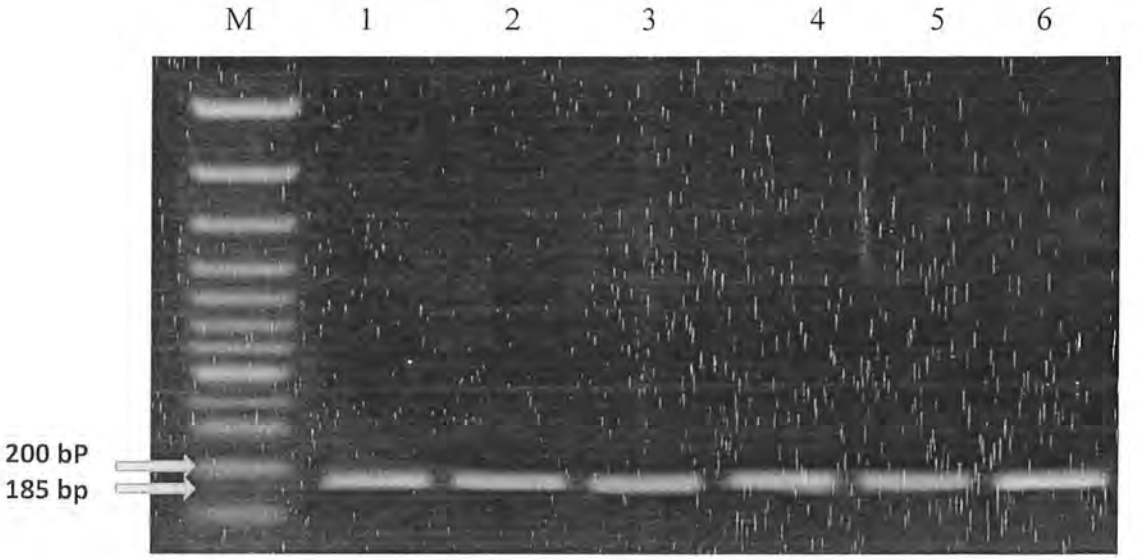


Fig. 3.7 Represents the Agarose gel electrophoresis of PCR product of 185 bp. From left: lane M represents a molecular weight marker of 100bp, Lane 1- 6 represents Re confirmed *M. gallisepticum.*

All the 50 samples that were found positive for *M. gallisepticum* through PCR were also positive for isolation of *M. gallisepticum* on culture media. The isolates were reconfirmed using PCR technique, all were found positive. These results show that the PCR for *M. gallisepticum* tested in this study can be used to detect the *M. galliseptcum* infection by extracting DNA from organs of birds suspected of having infection without the need to isolate the organism and confirm it via biochemical tests.

Determination of MIC Antibiotic Sensetivity

Antibiotic susceptibility of 50 isolates of *M. gallisepticum* was tested against 6 antibiotics, tylosin, enrofloxacine, oxytetracycline, chlortetracycline, colistin, and ceftifer by broth micro dilution method (Whithear *et al.* 1983).

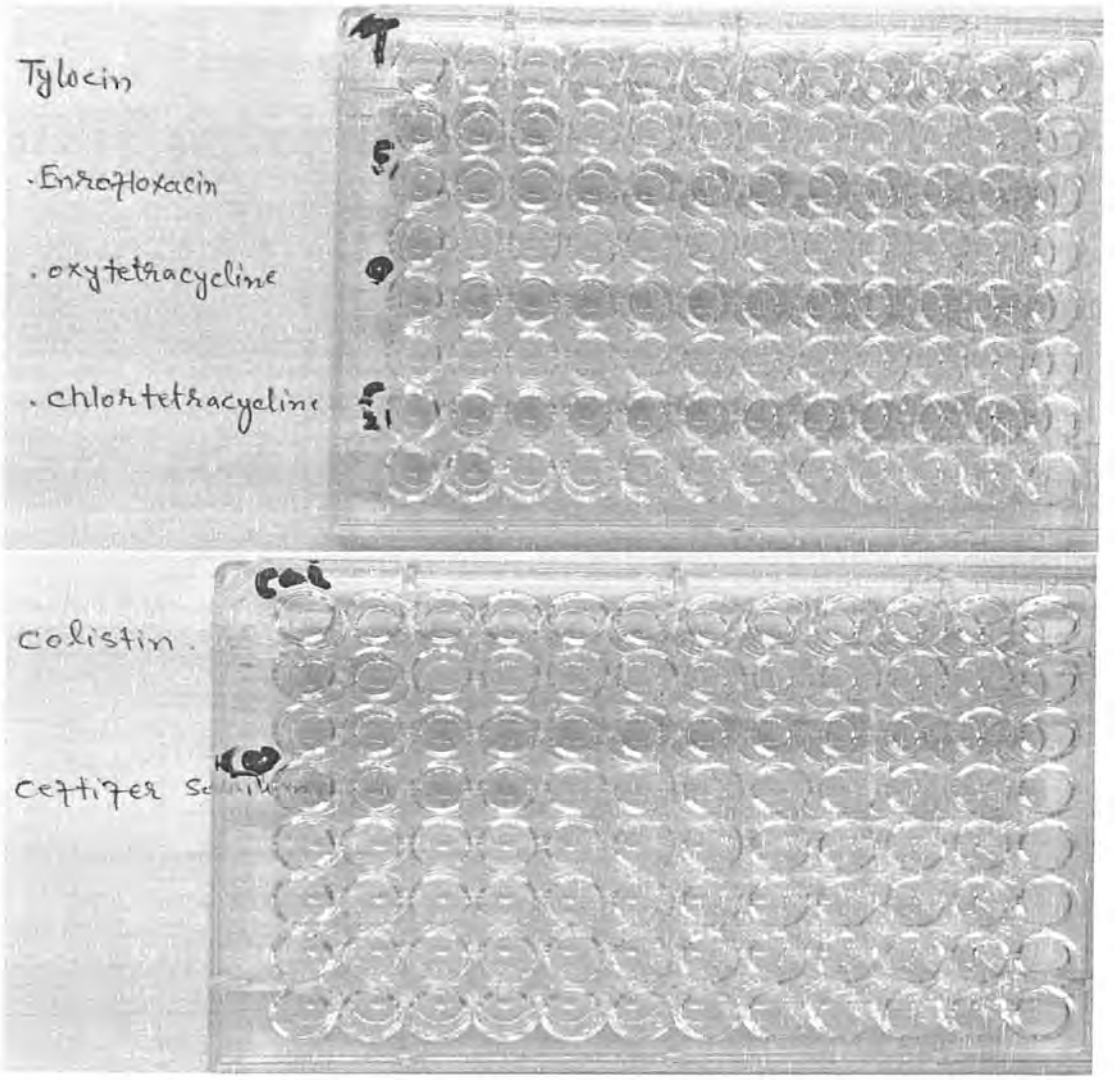


Fig.3.8 MICs of various antibiotics showing growth patterns of *M.gallisepticum*

On the basis of color change of antibiotic from red to orange, the susceptibility of *M. gallisepticum* was declared. The isolates of *M. galisepticum* were interpreted to be either sensitive, intermediate or resistant.

Table 3.1 shows the results of MIC of different antibiotics used against strains of *M. gallisepticum*. Tylosin and Ceftifer were found most efficacious antibiotics having mean MIC values of 12 ug/ml each. Oxytetracycline found to be intermediate in efficiency against *M. gallisepticum* with mean MIC value of 48.8 ug/ml while Enrofloxacine, Colistin, Chlorotetracycline are found to be least effective against *M. gallisepticum* with mean MIC value of 98.6 ug/ml each.

Table 3.1 showing MIC (Mean and Range) of Antibiotics

Antibiotics used	Mean MIC (ug/ml)	MIC Range (ug/ml)
Tylocin	12	6-48.8
Enrofloxacine	98.6	24.4-195.3
Colistin	98.6	24.4-195.3
Chlortetracycline	98.6	24.4-195.3
Oxytetracycline	48.8	24.4-195.3
Ceftiofer	12	6-24.4

Incidence of Mycoplasma

Tissue samples were collected from affected birds of selected farms and processed by PCR. After confirmation remaining birds showing morbidity were considered positive. Farmers were asked to give information about sick and dead birds and results were interpreted.

Out of 424 flocks, 50 flocks were *M. gallisepticum* positive. Out of 50, Broiler, Layer and Breeder were 32, 12, and 6 respectively. The incidence of *M. gallisepticum* was 11.79%. The incidence of *M. galisepticum* in broilers was 12.90%,

in layer 10.19% and in breeder 10.34%. Similarly, from the above mentioned 424 flocks, 90 flocke were subjected to PCR for *M. synoviae*. Out of 90 flocks 17 were positive by PCR. The incidence of *M. synoviae* was 18.88%. The incidence of *M. synoviae* in broilers was 19.04%, in layer 18.18 % and in breeder 19.44%.

Table 3.2 Incidence of *M. gallisepticum*

Overall incidence of Mycoplasma in Poultry in Rawalpindi Division	**Incidence in Broilers %**	**Incidence in Layers %**	**Incidence in Breeders %**
11.79%	12.90%	10.19%	10.34%

Table 3.3 Incidence of *M. synoviae*

Overall incidence of Mycoplasma in Poultry in Rawalpindi Division	**Incidence in Broilers %**	**Incidence in Layers %**	**Incidence in Breeders %**
18.88%	19.04%	18.18%	19.44%

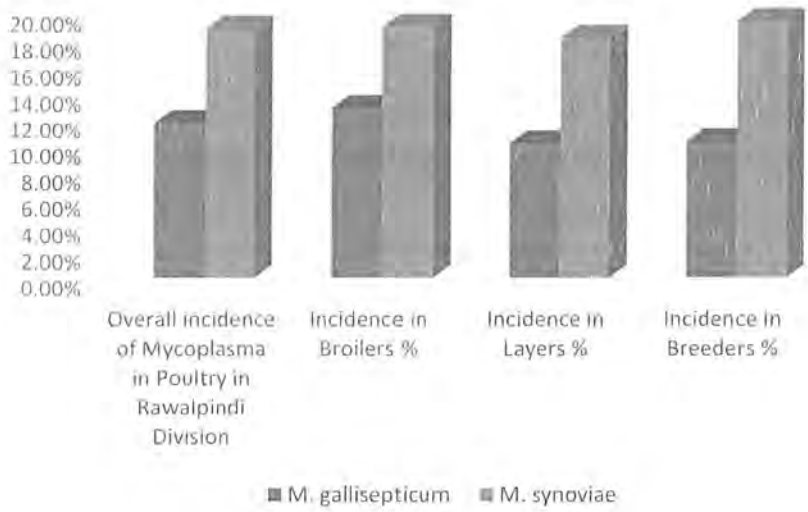


Fig 3.9 Incidence of *M. galisepticum* and *M. synoviae*

In season-wise incidence of *M. gallisepticum*, a higher incidence rate (13.65%) was recorded in winter season as compared to summer (8.49%) (Table 3.4).

Table 3.4 Season wise incidence of *M. galisepticum* among commercial Broiler, Layer and Breeders in district Rawalpindi, Pakistan

Type of Birds	No. of flocks tested	*M. galisepticum*					
		(Oct-March 2015-16)			(April -Sept 2016)		
		total	+ ive	Incidence (%age)	total	+ive	Incidence (%age)
Broiler	248	149	23	15.44	99	09	9.09
Layer	118	77	09	11.60	41	03	7.3
Breeder	58	45	05	11.11	13	01	7.69
Total	424	271	37	13.65%	153	13	8.49%

In season-wise incidence of *M. synoviae*, a higher incidence rate (20.75%) was recorded in winter season as compared to summer (16.21%) (Table 3.5).

Table 3.5 Season wise incidence of *M. synoviae* among commercial Broiler, Layer and Breeders in district Rawalpindi, Pakistan

Type of Birds	No. of flocks tested	*M. synoviae*					
		(Oct-March)			(April -Sept)		
		total	+ive	Incidence (%age)	total	+ive	Incidence (%age)
Broiler	21	13	03	21.4	08	1	12.5
Layer	33	21	04	19	12	2	16.6
Breeder	36	19	04	21	17	3	17.6
Total	90	53	11	20.75%	37	6	16.21%

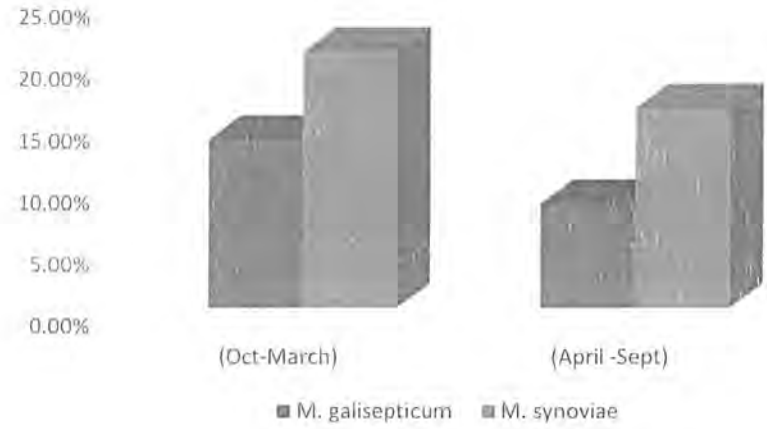


Fig 3.10 Season wise incidence of *M. galisepticum* and *M. synoviae* among commercial Broiler, Layer and Breeders in district Rawalpindi, Pakistan

Table 3.6 Poultry flocks tested for *M. gallisepticum* from July 2015-June 2016

S.NO	Types of birds	No of Flocks tested	*M. gallisepticum* positive	% of *M. gallisepticum* positive flocks	Organ used for DNA isolation
1	Broiler	248	32	12.90	Tissue/swab
2	Layer	118	12	10.10	Tissue/swab
3	Breeders	58	6	10.34	Tissue/swab
	Total	424	50	11.79	

Table 3.7 Poultry flocks tested for *M. synoviae* from July 2015-June 2016

S.NO	Types of birds	No of Flocks tested	*M. synoviae* positive	% of *M. synoviae* positive flocks	Organ used for DNA isolation
1	Broiler	21	04	19.04	Tissue/swab
2	Layer	33	06	18.18	Tissue/swab
4	Breeders	36	07	19.44	Tissue/swab
5	Total	90	17	18.88	

Discussion

M. gallisepticum and *M. synoviae* were considered as significant poultry respiratory pathogens that are responsible for huge economic losses in Pakistan and worldwide (Kleven, 2008). Ante mortem and post mortem examination of birds were conducted in Disease Diagnostic Section, Poultry Research Institute, Rawalpindi. The sign and symptoms and gross lesions were carefully observed. Infected birds exhibited depression with a ruffled feather, severe conjunctivitis, coughing and sneezing with nasal discharge. (Roussan *et al.*, 2008) reported similar findings in experimental and field infections of Mycoplasma in poultry.

Immunological methods are used to detect mycoplasmosis. These include direct immunofluorescence, Indirect Fluorescent, Antibody (IFA) test, Immunoperoxidase (IP) test, Growth inhibition (GI) test, Metabolism inhibition (MI) test, Hemagglutination Inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA). These are not easy to interpret because antibodies are present in both apparently healthy carriers and in infected birds. These tests are time-consuming and are less specific and sensitive (Ley, 2003; Yoder, 1991; Kelvin *et al.*, 1988). Screening programs based on seroconversion may be inadequate for Mycoplasmosis diagnosis and control. Some authors suggest the adoption of other techniques to confirm the presence of the agent (*M. synoviae)* such as DNA detection by PCR because antibodies based tests were not informative about the active infection (Euuing *et al.*, 1998).

In current study, PCR was used for the detection of *M. gallisepticum* and *M. synovae* in organs (trachea, lungs, and tracheal swab) of infected birds as described by (Santha *et al.*, 1990). PCR represents a rapid and sensitive alternative to traditional Mycoplasma culture methods, which requires specialized media, reagents for serotyping isolates and are time-consuming (Kempf, 1998; Levisohn and Kelvin, 2000). Salish *et al.*, (1998) concluded that a commercial PCR-based kit for the detection of MG was specific and at least as sensitive as identification by culturing. Haque *et al.*, (2011) compared conventional isolation techniques with PCR for the identification of *M. synoviae* infected birds. Molecular detection was of significantly higher efficacy than culture isolation for early detection of *M. gallisepticum* and *M.*

synoviae in Pakistan (Haque *et al.*, 2012; Saddique *et al.*, 2012).

Several methods were cited by Kempf and, in addition, a manual published by Lauerman that contain a validated PCR assay for *M. gallisepticum* and *M. synoviae* and other avian mycoplasmas based on unique sequences contained in the 16S rRNA gene. In the USA, a PCR based on the mgc2 gene of *M. gallisepticum* or the vlhA gene of *M. synoviae* has become more widely used. Because preliminary strain identification can be made by sequencing of the PCR product; it must be remembered that unrelated strains may occasionally share the same sequence (Dohms *et al.*, 1993; Farnandez *et al.*, 1993).

Primers recommended by OIE terrestrial manual 2008 were used in current study. PCR amplicon product of 185 base pairs and 211 base pair for *M. gallisepticum* and *M. synoviae* respectively visualized in agarose gel revealed that *M. gallisepticum* and *M. synoviae* primers had successfully targeted the corresponding gene. PCR targeted the mgc2 gene of *M. gallisepticum* isolates by using MG-f and MG-r primers and 185 base pair amplicon was obtained after gel electrophoresis. Similarly, PCR targeted the vlhA gene of *M. synoviae* isolates by using MS-f and MS-r primers and 211 base pair amplicon was obtained after gel electrophoresis. The results that have been declared by M. Rauf *et al.*, (2013) in district Lahore of Punjab, Pakistan. According to the authors, the *M. gallisepticum* primers amplified the target gene effectively. Therefore, their findings were in line with our present study. A study by Arshad *et al.*, (2013) revealed similar results for *M. synoviae*.

Among 50 samples (1 sample represent 1 flock) were found strong positive for *M. gallisepticum* as they gave 185 bp products and 17 samples were found strong positive for *M. synoviae* as they gave 211 bp products (Figure 3.3 and 3.4). Due to lack of differentiation in colony characteristics of different Mycoplasma species and time-consuming nature of cultural techniques, PCR was applied for diagnosis of *M. gallisepticum* and *M. synoviae* using specific primers. However, in this study both isolation and molecular identification was done and the isolate of *M. gallisepticum* from numerous *M. gallisepticum* positive flocks grew well in PPLO broth within 48 hours at 37 °C with 10% CO2 and showed 10^3 colony forming units (CFU) per ml of the medium that gave 1.5% packed cell volume (PCV) in the Hopkins tube.

It is recommended that Mycoplasma free flock must be maintained through early detection of respiratory pathogens particularly the *M. gallisepticum* and *M. synoviae* status which can assessed easily through PCR.

M. gallisepticum on the basis of in vitro and in vivo trials have shown sensitivity to different antimicrobial agents including macrolides (Jordan and Horrocks, 1996) tetracyclines and quinolones (Bébéar *et al.*, 1999; Wu *et al.*, 2000). As Mycoplasma lack the cell wall, so they are resistant to penicillin and other cell wall inhibiting antimicrobial agents. Many other antimicrobial agents like oxytetracycline, aminoglycosides, lincosamides, fluoroquinolones and tiamulin have been found to possess different levels of efficacy against different Mycoplasmas (Bradbury *et al.*, 1994; Hannan *et al.*, 1997a). But it has been reported in animals and humans that Mycoplasma has resistance against tetracyclines (Hannan *et al.* 1997a), macrolides (Bradbury *et al.*, 1994; Hannan *et al.*, 1997a; Gautier-Bouchardon *et al.*, 2002) and quinolones (Bébéar *et al.*, 1999; Wu *et al.*, 2000).

In current study all the isolates of *M. gallisepticum* were tested and observed to be sensitive to various different antibiotics at varying levels. Tylosin, Enrofloxacin, Oxytetracycline, Chlortetracycline, Colistin, and Ceftiofur were the six antibiotics tested against *M. gallisepticum* by broth microdilution

method (Whithear *et al.*, 1983) and this is a convenient method to find the sensitivity of Mycoplasma to antibiotics. Results of this study shows that the best mean minimum inhibitory concentration (MIC) value and MIC Range (ug/ml) were given by two antibiotics, Tylosin and Ceftifer, which were 12 mg/ml and 6-48.8% ug/ml each respectively. Tylosin belongs to macrolides group of antibiotics, (Bébéar *et al.*, 1999; Wu *et al.*, 2000). Despite of the fact that it has been used extensively in many countries and regions, very little resistance has been reported against it to date. In our study similar results were found, all 50 isolates of *M. gallisepticum* susceptible to Tylosin. No *M. gallisepticum* isolate in this study was found resistant to it. Tylosin is already used extensively in field. Based on the MIC results of this study it was further dcecribed to the flocks onditions. All the isloates collected from Rawalpindi, Pakistan were treated using Tylosin. Same is the case with Ceftifer (Cephlosporins group), as both Tylosin and Ceftifer are found to be most effective for the treatment of *M.*

gallisepticum infection. Both of these antibiotics destroys the microbial protein synthesis. Tylosin destroys the 50s ribosomal subunit while Ceftiofur like β-lactams, interfers with Pencillin binding proteins. Susceptibilty to tetracyclines has been reported to be decreased according to the study conducted years ago (Kleven *et al.* 1971; Jordan *et al.* 1989) and same was observed for the one member of this group, Chlortetracycline, whose mean MIC value was 98.6 ug/ml along with Enrofloxacin and Colistin having the same mean MIC value and considered as least susceptible. Enrofloxacine (Flouroquinolone group), Cholretetracycline (Tetracycline group) and Colistin (Polymexin Class) are found to be least effective against *M. gallisepticum* in this study (Table 3.5). Although Oxytetracycline (Tetracycline group) and Cholretetracycline (Tetracycline group) belongs to the same group of antibiotics having the same mode of action by blocking the 30s ribosomal subunit at aminoacyl tRNA but Oxytetracycline was found to be intermediate in efficiency & sensitivity to *M. gallisepticum* having mean MIC value of 48.8 ug/ml. As indicated by the findings of present study drug resistance must be taken into account while treating mycoplasmosis. Fifty isolates of *M. gallisepticum* were tested and two antibiotics Tylosin & Ceftiofur showed highest effectiveness followed by oxytetracycline.

In current study, 424 flocks from Rawalpindi division were selected for the study of the incidence of *Mycoplasma* from July 2015 to June 2016. The overall incidence of *M. gallisepticum* in chicken was 11.79%. The factors which contribute the mycoplasma infection are exposure to air, moist litter, lack of control on movement of personnel and biosecurity measures taken (Dulali, 2003).

The incidence of *M. gallisepticum* in layers was 39.64% reported by (Rehman *et al.*, 2013), which was higher than the present study. In one study in India conducted on breeder flocks, the incidence of *M. gallisepticum* was found to be 57.15% and 59.6% respectively (Sarkar *et al.*, 2005; Pradhan, 2002).

According to the findings in our study, the incidence of *M. gallisepticum* was highest in broilers (12.09%). The incidence in the case of layers was 10.19% while lowest incidence was found in the case of breeders which was 10.34%. Aimeur *et al.*, 2010 and Rehman *et al.*, 2013 reported

30%, 31.25% incidence in broilers respectively which were in line with the present report. The incidence in broiler was reported as 1.25% by (Baruta *et al.*, 2001) which was less than the incidence in the present study.

The study also showed that there was variation in the incidence of *M. gallisepticum* in different parts of the year. Present findings of the study showed a higher incidence of *M. gallisepticum* in winter (13.65%) as compared to the summer season (8.49%). The incidence of M. *gallisepticum* in Faisalabad district was 61.8% in winter and 47.74% in the summer (Mukhtar *et al.*, 2012). The incidence of *M. gallisepticum* was more in winter season as compared to summer season (Seifi and Shirazd, 2012). Heleili *et al.*, 2012 reported 70% incidence of M. *gallisepticum* in winter compared to in summer (60%). Similar results were also reported by other author's (Skider *et al.*, 2005; Sarkar *et al.*, 2005; Hossain *et al.*, 2007; Tahla *et al.*, 2009). The reason for such a high seasonal variation might be the sudden change in temperature and cold stress on the birds.

The overall incidence of *M. synoviae* in all types of chicken was found to be 18.88%. The incidence of *M. synoviae* was higher in breeder (19.44%) and brioler (19.04%) as compared to layer (18.18%). The incidence of *M. synoviae* in broiler was 69.96% which was reported by Heleili *et al.*, 2012. This percentage was considerably greater than present study. The incidence of *M. synoviae* in broiler was 6% which was reported by Feberwee *et al.*, 2008 which is less than the current results. The possible reason behind low incidence of *M. synoviae* in broilers was the short life span of broilers and extensive antibiotic treatment. The incidence of *M. synoviae* in broiler was found to be 12.5% and in layers 39.64% by Rehman *et al.*, 2013. The results for boilers were somewhat similar to our results but the incidence in layers does not match with the results of Rehman *et al* 2013. The incidence of *M. synoviae* in layers was found to be 73% by Feberwee *et al.*, 2008 and 78.6% by Hagan *et al.*, 2004. The incidence of *M.synoviae* in layer birds might be due to poor biosecurity and multiple age housing (Kelvin, 2003) so layer bird may be a source of infection to other types of poultry birds.

The incidence of *M. synoviae* in breeder birds was shown to be 26.46% (Luciano *et al.*, 2011) which was almost closer to this study. The incidence of *M.*

synoviae in breeder birds was 28% by Rehman *et al.*, 2013 which was in agreement with current research. Feberwee *et al.*, 2008 found the incidence of *M.synoviae* in the breeder flocks was 35% while in the current study the incidence was found to be 19.44%.

In current study, seasonal variation in the incidence of *M. synoviae* infection was observed. The incidence of *M. synoviae* was highest in winter and lowest in summer which was 20.75% and 16.21% respectively. Heleili *et al.*, 2012 found in incidence of *M. synoviae* was found to be 46.69% in the winter which was concordant with the current study. Similar findings were also reported by Arbelot *et al.*, 1997.

Conclusion

It is concluded that *M. galisepticum* and *M. synoviae* have a significant prevailence in commercial poultry flocks, including broiler, layer and breeder, in various districts of Punjab Province, Pakistan. In current study, incidence rate of *M. galisepticum* and *M. synoviae* in 424 commercial poultry flocks in Rawalpindi division was found to be 11.79% and 18.88% respectively. Isolation and culturing are laborious and time consuming and fail to detect Mycoplasma species from medicated birds and is thus less efficacious than PCR. On the other hand, PCR is a more reliable, rapid, sensitive and accurate method. Thus PCR is highly suitable for detection of Mycoplasmosis in Pakistan and the causal organism can be detected directly through PCR done on DNA from tissues and swab samples of affected birds without performing conventional methods of isolation and identification. To the best of our knowledge, this is the first report on molecular detection of *M. synoviae* in poultry in Pakistan. Antibiogram of Mycoplasma isolates showed that there is high prevalence of resistance against several antibiotics, which is an alarming situation. Tylosin and ceftifur sodium were found to be highly sensitive drugs for the treatment of *M. gallisepticim*, while Oxytetracycline showed limited therapeutic value for treating Mycoplasmosis. Enrofloxacine, Colistin, Chlorotetracycline were found to be least effective against *M. gallisepticum*. Continuous surveillance is required to evaluate antibiotics susceptibility because the antibiotic resistance pattern of microbes changes with the passage of time.

References

Abdul-Rahman A. M., Molteno, A. C. & Bevin, T. H. (2011). The epidemiology of giant cell arteritis in Otago, New Zealand: a 9-year analysis. *The New Zealand Medical Journal (Online), 124*(1329).

Abdul-Wahab, O. M., Ross, G. & Bradbury, J. M. (1996). Pathogenicity and cytadherence of *Mycoplasma imitans* in chicken and duck embryo tracheal organ cultures. *Infection and immunity, 64*(2): 563-568.

Aimeur, R., Bouaziz, O., Kabouia, R., & Bererhi, E. H. (2010). Prevalence de *Mycoplasma gallisepticum* and *Mycoplasma synoviae* dans les élevages avicoles de l'Est Algérien. *Revue de Medecine Veterinaire, 161*(3), 141.

Almanama, M. A. (2011). Prevalence of *Mycoplasma gallisepticum* in the Ten licensed Hatcheries in Gaza strip, Palestine (Doctoral dissertation, Islamic University-Gaza).

Arbelot, B., Dayon, J. F., Mamis, D., Gueye, D., Tall, J. C. F. & Samb, H. (1997). Seroprevalence survey of dominant avian diseases in Senegal: Mycoplasmosis, Fowl typhoid and Pullorum disease, Newcastle, infectious bursal and infectious bronchitis diseases. *Revue delevage et de Medecine Veterinaire des Pays Tropicaux, 50*(3): 197-203.

Atique, M. A., Abbas, F., Awan, M. A., Tariq, M. M., Ahmed, Z., Ali, I. & Alam, M. A. (2012). Identification of avian Mycoplasma species in commercial broilers and layers with respiratory symptoms in Balochistan. *African Journal of Biotechnology, 11*(100): 16557.

Atique, M. A., Abbas, F., Awan, M. A., Tariq, M. M., Ahmed, Z., Ali, I. & Alam, M. A. (2012). Identification of Avian Mycoplasma species in commercial broilers and layers with respiratory symptoms in Balochistan. *African Journal of Biotechnology, 11*(100): 16557.

Awad, F., Forrester, A., Baylis, M., Ganapathy, K., Lierz, M., Heffels-Redmann, U., Kaleta, E. F. & Heckmann, J. (2012). Detection of multiple poultry respiratory pathogens from FTA card. *International symposium on avian corona-and pneumoviruses and complicating pathogens, 18:* 136-138.

Barua, S. R., Prodhan, A. M., Islam, S. & Chowdhury, S. (2006). Study on *Mycoplasma gallisepticum* in chickens in selected areas of Bangladesh. *Bangladesh Journal of Veterinary Medicine, 4*(2): 141-142.

Bebear, C. M., Renaudin,J., Charron, A., Renaudin, H. De-Barbeyrac, B., Schaeverbeke T. & Bebear, C.(1999). Mutations in the gyrA, parC and par E genes associated with fluoroquinolones resistance in clinical isolates of *Mycoplasma hominis*. Antimicrobial Agents Chemotherapy, *43*: 954-956.

Bencina, D., Drobnic-Valic, M., Horvat, S., Narat, M., Kleven, S.H. & Dovc, P. (2001). Molecular basis of the length variation in the N-terminal part of *Mycoplasma synoviae* hemagglutinin. *FEMS Microbiology Letters*, *203*(1), 115-123.

Bhandari, S. & Asnani, P. (1989). Characterization of phospholipase A2 of mycoplasma species. *Folia microbiologica*, *34*(4): 294-301.

Bhatti, B. M. (2007). International Poultry Congress and Exhibition. Nov, 9-10, Islamabad Pakistan

Biswas, P. K., Rahman, M. A., Biswas, D. & Ahmed, S. (2003). A longitudinal study on the prevalence of endemic diseases affecting semi-scavenging poultry reared under PLDP area. In *ninth BSVER Conference*, *24*, 24-25.

Boguslavsky, S., Menaker, D., Lysnyansky, I., Liu, T., Levisohn, S., Rosengarten, R. & Yogev, D. (2000). Molecular characterization of the Mycoplasma gallisepticum pvpA gene which encodes a putative variable cytadhesin protein. *Infection and immunity*, *68*(7): 3956-3964.

Bootwala, S. (2007). Poultry development in the subcontinent, Present and future development and chalanges. Monthly Veterinary observer J. Sept: 4-5.

Bradbury, J. M. (2001). Avian mycoplasmosis. *Poultry diseases*, *5*: 178-193.

Bradbury, J. M., Yavari, C. A. & Giles, C. J. (1994). In vitro evaluation of various antimicrobial against *Mycoplasma gallisepticum* and Mycoplasma synoviae by the micro broth method and comparison with a commercially prepared system. Avian Pathalogy, *23*: 105-115.

Bradbury, J. M., Yavari, C.A. & Dare, C. M., (2001). Mycoplasmas and respiratory disease in pheasants and partridges. *Avian Pathology*, *30*(4): 391-396.

Calnek, B. W. (2014). Avian Diseases: The Creation and Evolution of P. Philip Levine's Enduring Gift. *Avian diseases*, *59*(1): 1-6.

Charles, H. D. & Graham, H. P. 1989): Mycoplasmosis. A laboratory manual for the isolation and identification of avian pathogens, 3rdedition, Kendall/Hunt publishing com. University of Pennsylvania. 57-62.

David H. Ley Mycoplasma gallisepticum Infection in Poultry (2013).

Domanska-Blicharz, K., Tomczyk, G. & Minta, Z. (2008). Comparison of different molecular methods for detection of *Mycoplasma gallisepticum*. *Bull Vet Inst Pulawy*, *52*: 529-532.

Economic Survey Report, 2012-13. Economic Affair Division, Ministry of Finance, GOP Islamabad.

Evans, J. D., Leigh, S. A., Branton, S. L., Collier, S. D., Pharr, G.T. & Bearson, S. M. D. (2005). *Mycoplasma gallisepticum*: Current and developing means to control the avian pathogen. *The Journal of Applied Poultry Research*, *14*(4): 757-763.

Fan, H. H., Kleven, S. H. & Jackwood, M. W. (1995). Application of polymerase chain reaction with arbitrary primers to strain identification of *Mycoplasma gallisepticum*. *Avian Diseases*, 729-735.

Ferguson, N. M., Hepp, D., Sun, S., Ikuta, N., Levisohn, S., Kleven, S. H. & Garcia, M. (2005). Use of molecular diversity of *Mycoplasma gallisepticum* by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. *Microbiology*, *151*(6): 1883-1893.

Ferraz, P. N. & Danelli, M. (2003). Phenotypic and antigenic variation of *Mycoplasma gallisepticum* vaccine strains. *Brazilian Journal of Microbiology*, *34*(3): 238-241.

Ferraz, P. N. & Danelli, M. M. (2003). Phenotypic and antigenic variation of *Mycoplasma gallisepticum* vaccine strains. *Brazilian Journal of Microbiology*, *34*(3): 238-241.

Forrester, C. A., Bradbury, J. M., Dare, C. M., Domangue, R. J., Windsor, H., Tasker, J. B. & Mockett, A. A. (2012). *Mycoplasma gallisepticum* in pheasants and the efficacy of tylvalosin to treat the disease. *Avian Pathology*, *40*(6): 581-586.

Frey, M. L. (1968). A medium for the isolation of avian mycoplasmas. *Am J Vet Res*, *29*: 2163-217.

Garcia, M., Jackwood, M. W., Levisohn, S. & Kleven, S. H. (1995). Detection of *Mycoplasma gallisepticum, M. synoviae, and M. iowae* by multi-species polymerase chain reaction and restriction fragment length polymorphism. *Avian Diseases*, (1995): 606-616.

García, M., Ikuta, N., Levisohn, S. & Kleven, S. H. (2005). Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. *Avian diseases, 49*(1): 125-132.

Gautier-Bouchardon, A.V., Reinhardt, A. K., Kobisch M. & Kempf, I. (2002).*In vitro* development of resistance to enrofloxacin, tylosin, tiamulin and oxytetracycline in *Mycoplasma gallisepticum*, *Mycoplasma iowae* and *Mycoplasma synoviea*. Veterinary Microbiology, *88*: 47-58.

Goh, M. S., Gorton, T. S., Forsyth, M. H., Troy, K. E. & Geary, S. J. (1998). Molecular and biochemical analysis of a 105 kDa *Mycoplasma gallisepticum* cytadhesin (GapA). *Microbiology*, *144*(11): 2971-2978.

Habib-ur-Rehman, S., Khan, H., Khan, S., Ahmad, N. & Bhatti, W. M. (2003). Incidence and gross pathology of salmonellosis in chicken in Hyderabad. *Journal of Animal and Veterinary Advances*, *2*(10): 581-584.

Hannan, P.T.C., Windsor, G. D., de Jong, A. N., Schmeer Kleven, S. H. & Anderson, D. P. (1971). In vitro activity of M.Stegemann, 1997a. Comparative susceptibilities of various animal to fluoroquinolones. Antimicrobial Agents Chemotherapy, *41*: 2037-2040.

Hassan, S., Mukhtar, N., Ur Rahman, S. & Ali Mian, A. (2014). Molecular Epidemiology of *Mycoplasma gallisepticum* in Different Types of Chickens. *International Journal of Agriculture & Biology, 16*(1).

Heleili, N., Ayachi, A., Mamache, B. & Chelihi, A. J. (2012). Seroprevalence of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* at Batna Commercial poultry farms in Algeria. *Veterinary World, 5*(12).

Higgins, P. & K. Whithear (1986). Detection and differentiation of *Mycoplasma gallisepticum* and *M. synoviae* antibodies in chicken serum using enzyme-linked immunosorbent assay. *Avian disease,* 160-168.

Hong, Y., Garcia, M., Levisohn, S., Savelkoul, P., Leiting, V., Lysnyansky, I. & Kleven, S. H. (2005). Differentiation of *Mycoplasma gallisepticum* strains using amplified fragment length polymorphism and other DNA-based typing methods. *Avian diseases*, *49*(1), 43-49.

Hossain, K. M. M., Ali, M. Y. & Haque, M. I. (2007). Seroprevalence of *Mycoplasma gallisepticum* infection in chicken in the greater Rajshahi district of Bangladesh. *Bangladesh Journal of Veterinary Medicine*, *5*(1): 9-14.

Hossain, K. M. M., Hossain, M. T. & Yamato, I. (2010). Seroprevalence of *Salmonella* and *Mycoplasma gallisepticum* infection in chickens in Rajshahi and surrounding districts of Bangladesh. *International Journal of Biology*, *2*(2): 74.

Islam, M., Hassan, J. & Khan, M. S. R. (2014). Seroprevalence of *Mycoplasma gallisepticum* infection in backyard and commercial layer chickens in Bhola district, Bangladesh. *Journal of Advanced Veterinary and Animal Research*, *1*(1): 11-15.

Jordan, F. T. W., Gilbert, S.,Knigth, D. L. & Yavari, C. A. (1989). Effects of Baytril, tylosin and tiamulin on avian mycoplasmas.Avian Pathology. *18*: 659–663.

Kempf, I., Blanchard, A., Gesbert, F., Guittet, M. & Bennejean, G. (1993). The polymerase chain reaction for *Mycoplasma gallisepticum* detection. *Avian Pathology*, *22*(4): 739-750.

Kempf, I., Blanchard, A., Gesbert, F., Guittet, M. & Bennejean, G. (1993). The polymerase chain reaction for *Mycoplasma gallisepticum* detection. *Avian Pathology*, *22*(4): 739-750.

Khan, M. I., Fadl, A. A. & Venkitanarayanan, K. S. (2003). Reducing colonization of *Salmonella Enteritidis* in chicken by targeting outer membrane proteins. *Journal of applied microbiology*, *95*(1): 142-145.

Kleven, S. H. & Anderson, D. P. (1971).In vitro activity of various antibiotics against *Mycoplasma synoviae*. Avian Diseases, *15*:551–557.

Kleven, S. H. & Bradbury, J. M. (2008). Avian mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae*). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth ed. *World Organization for Animal Health, Paris*, 482-496.

Kleven, S. H. & Yoder, H. (1998). Mycoplasmosis. A laboratory manual for the isolation and identification of avian pathogens, 4th ed. DE Swayne, JR Glisson, MW Jackwood, JE Pearson, and WM Reed, eds. American Association of Avian Pathologists, Kennett Square, PA: 74-80.

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

Kleven, S. H., Rowland, G. N. & Olson, N.O. (1997). *Mycoplasma synoviae* infection. *Diseases of poultry, 10*: 220-227.

Krass, C. J. & Gardner, M. W. (1973). Etymology of the term Mycoplasma. *International Journal of Systematic and Evolutionary Microbiology, 23*(1): 62-64.

Lauerman L. H. (1998). *Mycoplasma* PCR Assays: Nucleic Acid Amplification Assays for diagnosis of animal diseases. Department of Agriculture and Industries, Alabama. 150.

Levisohn, S., Glisson. J. R. & Kleven, S. H. (1985). In ovo pathogenicity of *Mycoplasma gallisepticum* strains in the presence and absence of maternal antibody. *Avian diseases*: 188-197.

Ley, D. H., Marusak, R. A., Vivas, E. J., Barnes, H. J. & Fletcher, O. J. (2010). *Mycoplasma iowae* associated with chondrodystrophy in commercial turkeys. *Avian Pathology, 39*(2): 87-93.

Ley, D. H., Sheaffer, D.S. & Dhondt, A. A. (2006). Further western spread of *Mycoplasma gallisepticum* infection of house finches. *Journal of Wildlife Diseases, 42*(2): 429-431.

Liu, T., Garcıa, M., Levisohn, S., Yogev, D. and Kleven, S. H. (2001). Molecular Variability of the Adhesin-Encoding GenepvpA among *Mycoplasma gallisepticum* strains and Its Application in Diagnosis. *Journal of clinical microbiology, 39*(5), 1882-1888.

Luciano, R. L., Cardoso, A. L. S. P., Stoppa, G. F. Z., Kanashiro, A. M. I., De Castro, A. G. M. & Tessari, E. N. C. (2011). Comparative study of serological tests for *Mycoplasma synoviae* diagnosis in commercial poultry breeders. *Veterinary medicine international,* 2011.

Lysnyansky, I., Garcia, M. & Levisohn, S. (2005). Use of mgc2-polymerase chain reaction-restriction fragment length polymorphism for rapid differentiation between field isolates and vaccine strains of *Mycoplasma gallisepticum* in Israel. *Avian diseases, 49*(2): 238-245.

Maniloff, J. K. & Morowitz, H. J. (1972). Cell biology of the mycoplasmas. *Bacteriological reviews, 36*(3): 263.

Marois, C., Dufour-Gesbert, F. & Kempf, I. (2002). Polymerase chain reaction for detection of *Mycoplasma gallisepticum* in environmental samples. *Avian Pathology, 31*(2): 163-168.

Moraes, M. E., Pereira, G. B., Astolfi-Ferreira, C. S. & Ferreira, A. P. (2013). Infecçao experimental por *Mycoplasma gallisepticum Escherichia coli* em perus. *Pesquisa Veterinária Brasileira, 33*(8): 975-978. Ye kon c zuban he

Much, P., Winner, F., Stipkovits, L., Rosengarten, R. & Citti, C. (2002). *Mycoplasma gallisepticum:* influence of cell invasiveness on the outcome of experimental infection in chickens. *FEMS Immunology & Medical Microbiology, 34*(3): 181-186.

Mukhtar, M., Awais, M. M., Anwar, M. I., Hussain, Z., Bhatti, N. & Ali, S. (2012). Seroprevalence of *Mycoplasma gallisepticum* among commercial layers in Faisalabad, Pakistan. *Journal of Basic & Applied Sciences, 8*(1).

Nascimento E. R., Yamamoto R., Herrick K. R. & Tait R. C. (1991). Polymerase chain reaction for detection of *Mycoplasma gallisepticum.* Avian Diseases. *35*: 62-69.

Nicholas, R. A. J. and Ayling, R. D. (2003). *Mycoplasma bovis*: disease, diagnosis, and control. *Research in veterinary science, 74*(2): 105-112.

Nunoya, T., Yagihashi, T., Tajima, M. & Nagasawa, Y. (1995). Occurrence of keratoconjunctivitis apparently caused by Mycoplasma gallisepticum in layer chickens. *Veterinary Pathology Online, 32*(1): 11-18.

OIE Terrestrial Manual 2008. Avain mycoplasmosis (*M. galisepticum* and *M. synoviae)* .Viewed 15 May 2012, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm

OIE, (2012). Avian Mycoplasmosis, In Manual of diagnostic tests and vaccines for terrestrial animals, OIE, France.

Papazisi, L., Frasca Jr, S., Gladd, M., Liao, X., Yogev, D. & Geary, S. J. (2002). GapA and CrmA coexpression is essential for *Mycoplasma gallisepticum* cytadherence and virulence. *Infection and immunity, 70*(12): 6839-6845.

Pourbakhsh, S. A., Shokri, G. R., Banani, M., Elhamnia, F. & Ashtari, A. S. (2010). Detection of *Mycoplasma synoviae* infection in broiler breeder farms of Tehran province using PCR and culture methods. *Archives of Razi, 65*(2): 75-81.

Pradhan, M. A. (2002). Studies on avian mycoplasmosis: Prevalence, isolation, characterization and antigenic properties (Doctoral dissertation, PhD Thesis Submitted to the Dept. of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh).

Quinn, C. P., Semenova, V. A., Elie, C. M., Romero-Steiner, S., Greene, C., Li, H., Stamey, K., Steward-Clark, E., Schmidt, D.S., Mothershed, E. and Pruckler, J., (2002). Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin protective antigen (Bioterrorism-Related Anthrax). *Emerging infectious diseases*, *8*(10): 1103-1111.

Quinn, P., Markey, B. (2002). "Mycoplasma." Veterinary Microbiology and Microbial Disease. Blackwell Science Ltd. English: 189-195.

Rauf, M., Chaudhary, Z. I., Younus, M., Anjum, A. A., Ali, M. A., Ahmad, A. N. & Khan, M. R. (2013). Identification of *Mycoplasma gallisepticum* by Polymerase Chain Reaction and Conventional Diagnostics from White Leghorn Layer Flocks. *Journal of Animal and Plant Sciences*, *23*(2): 393-397.

Raviv, Z. & Kleven, S. H. (2009). The development of diagnostic real-time TaqMan PCRs for the four pathogenic Avian Mycoplasmas. *Avian diseases*, *53*(1): 103-107.

Razin, S. (1994). DNA probes and PCR in diagnosis of mycoplasma infections. *Molecular and cellular probes*, *8*(6): 497-511.

Razin, S. (2006). The genus Mycoplasma and related genera (class Mollicutes). *The Prokaryotes, Springer*, 836-904.

Razin, S., Yogev, D. & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews*, *62*(4): 1094-1156.

Rehman, S. R., Muhammad, K., Yaqub, T., Khan, M. S., Hanif, K. & Yasmeen, R. (2013). Antimicrobial activity of mentofin and its effect on antibody response of broilers to Newcastle disease virus vaccine. *Journal of Animal and Plant Sci*ences, *23*(4): 1008-1011.

Rodwell, A. W. & Mitchell, A. (1979). The Mycoplasmas. I. Cell Biology.

Whitcomb, R. F. & K. J. Hackett (1989). Why are there so many species of mollicutes? An essay on prokaryote diversity. *Biotic Diversity and Germplasm Preservation, Global Imperatives*, Springer, 205-240.

Whithear, K. G., Bowtell, D. D., Ghiocas, E. & Hughes, K. L. (1983). Evaluation and use of a microbroth dilution procedure for testing sensitivity of fermentative avian mycoplasmas to antibiotics. *Avian Diseases*. 4: 937–949.

Whithear, K., Bowtell, D. (1983). Evaluation and use of a micro-broth dilution procedure for testing sensitivity of fermentative avian mycoplasmas to antibiotics. *Avian diseases*, 937-949.

Winner, F., Rosengarten, R. & Citti, C. (2000). In vitro cell invasion of *Mycoplasma gallisepticum*. *Infection and immunity*, *68*(7): 4238-4244.

Wise, K. S., Yogev, D. & Rosengarten, R. (1992). Antigenic variation. *Mycoplasmas*: molecular biology and pathogenesis. *American Society for Microbiology*, 473-489.

Wu, C.C., Shryock, T. R., Lin, T. L. & Faderan, M. (2000). Antimicrobial susceptibility of *Mycoplasma hyorhinis*. *Veterinary Microbiology*, *76*: 25-30.

Zain, Z. M. & Bradbury, J. M. (1996). Optimising the conditions for isolation of *Mycoplasma gallisepticum* collected on applicator swabs. *Veterinary microbiology*, *49*(1): 45-57.

Zhang, J., Guo, Y., Hu, S., Bi, D., Wang, X., Xiao, Y. & Xu, Q. (2011). An adhesion molecule-based colloidal gold immunochromatography assay strip for rapidly and specifically detecting chicken antibodies against *Mycoplasma gallisepticum*. *Research in veterinary science*, *90*(3): 379-384.