DEVELOPMENT AND EVALUATION OF WHOLE CELL AND MEMBRANE PROTEIN VACCINES AGAINST *Mycoplasma gallisepticum*

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DECLARATION

I hereby declare that the thesis, entitled **"DEVELOPMENT AND EVALUATION OF WHOLE CELL AND MEMBRANE PROTEIN VACCINES AGAINST *Mycoplasma gallisepticum*"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that the thesis entitled **"DEVELOPMENT AND EVALUATION OF WHOLE CELL AND MEMBRANE PROTEIN VACCINES AGAINST *Mycoplasma gallisepticum*"** is a record of research work done independently by SURYA SANKAR, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Introduction

INTRODUCTION

Poultry farming has emerged as one of the fully viable and self sufficient industries, contributing significantly to the Indian economy in terms of its products, employment generated and the resultant improvement in the standard of living of the people involved. The growth of poultry industry into a major agro based industry was aided by the advancements in breeding, rearing and by the advent of molecular biology in the field of diagnosis and vaccines. However, with the evolution of superior genetic stock of poultry, intensive rearing and selective breeding policy for higher production, the birds are put to stress levels beyond their physiological tolerance, making them vulnerable to diseases. These problems are compounded by constraints, such as emergence and re-emergence of many diseases, less sensitive diagnostics and non availability of effective control strategies.

Among the prevailing diseases, avian mycoplasmosis has become one of the most serious and frequently reported diseases affecting poultry industry throughout the world, causing considerable economic losses. The organisms most commonly associated with mycoplasmosis in poultry are *Mycoplasma gallisepticum (M. gallisepticum), Mycoplasma synoviae (M. synoviae), Mycoplasma meleagridis (M. meleagridis)* and *Mycoplasma iowae (M. iowae*). *Mycoplasma gallisepticum* is responsible for chronic respiratory disease (CRD) in chicken and infectious sinusitis in turkey. Although the mortality from this disease is not very high, it causes substantial losses as a result of downgrading and condemnation of carcasses, decreased egg production, poor hatchability, reduced feed conversion and retarded growth, as well as aggravation of various other disease conditions and also the high cost incurred during control programme (Jordan, 1975; Kleven, 1998). The severity of the disease is greatly influenced by the degree of secondary infection with Newcastle disease and Infectious Bronchitis viruses and / or bacteria such as *Escherichia coli*.

The most reliable diagnosis of mycoplasmosis is the isolation and identification of pathogens from infected birds, but this is laborious, time consuming and therefore far from a routine procedure (Zain and Bradbury, 1996). Polymerase

Chain Reaction (PCR) has been regarded as a valuable tool under practical diagnostic conditions and may offer similar or even higher sensitivity than isolation (Salisch *et al*., 1998; Mekkes and Feberwee, 2005). Other genetic methods for the identification and characterization of *Mycoplasma* include ribosomal RNA (rRNA) sequence analysis, Random amplification of polymeric DNA (RAPD) fingerprinting and DNA-DNA hybridization. Serological monitoring system for the detection of antibodies against *M. gallisepticum* is widely used in flock screening. The most commonly used tests are the rapid serum agglutination (RSA), enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition (HI) tests. Other detection methods in use include indirect fluorescent antibody (IFA) and immunoperoxidase (IP) techniques. Growth inhibition (GI) and metabolism inhibition (MI) are the tests commonly used for the identification of the isolates.

Two types of vaccines are available for the control of infections due to *M. gallisepticum*. They are mild to avirulent *M. gallisepticum* strains used as live vaccines, or inactivated oil-emulsion bacterins. Regardless of the antigenic variation seen among *M. gallisepticum* strains, vaccination with a single strain seems to be effective. There are currently three live *M. gallisepticum* vaccines approved and commercially available (F strain, 6/85 and ts-11).

The F strain of *M. gallisepticum* has been the most commonly used vaccine strain. It is a naturally occurring strain of mild to moderate virulence for chicken. Vaccinated chicken are permanent carriers, so a single dose is adequate. When administered to healthy chicken via the upper respiratory tract, little or no respiratory reaction is observed. However, in the presence of other respiratory disease agents, such as Newcastle disease or Infectious Bronchitis viruses, respiratory signs and airsacculitis may result. Use of F strain vaccine in each replacement flock on a multi-age site will eventually result in displacement of the field strain with the vaccine strain (Levisohn and Dykstra, 1987; Mohammed *et al*., 1987; Branton *et al*., 1988). Vaccination with F strain did not prevent colonization by the challenge strain of *M. gallisepticum* (Levisohn and Dykstra, 1987). The F strain can be transmitted both vertically and horizontally. It was found that vaccinal F strain is pathogenic in turkeys following experimental infection (Lin and Kleven, 1982a), and it has been associated with *M. gallisepticum* outbreaks in meat and breeder turkeys under field conditions (Ley *et al*., 1993).

The live vaccines, ts-11 and 6/85, have been introduced recently. These vaccines have been reported to possess little or no virulence for chickens and turkeys (Whithear *et al*., 1990; Evans and Hafez, 1992; Abd-el-Motelib and Kleven,1993; Ley *et al*., 1997).The strain ts-11 is transmissible from vaccinates when birds are in direct contact with each other and/or share a common feeder and waterer, but transmission is unlikely with indirect contact (Ley and Yoder, 1997).The ts-11 vaccine strain persists in the chicken for long periods, but the 6/85- strain vaccine does not persist and may be difficult to recover the strain after a few weeks (Ley *et al*., 1997). Both the strains are inherently safer than F strain, but the level of protection may be somewhat less. Hence, they may be useful as the primary vaccine strains on a multi-age site or as a secondary vaccine on sites previously using F strain vaccine. They may also be preferred in situations where inadvertent exposure of neighboring poultry flocks is of concern. The F strain displaces wild-type *M. gallisepticum* more efficiently than either ts-11 or 6/85, but ts-11 has been used to eradicate F strain *M. gallisepticum* from a commercial egg-production site (Turner and Kleven, 1998). Sites where strain 6/85 is consistently used often test *M. gallisepticum* negative, suggesting that it has displaced the wild-type strain.

Thus, in long term programmes, in view of the fact that live vaccines may overtake the field strains, use of inactivated vaccines should be emphasized. The application of *M. gallisepticum* bacterins and subunit vaccines so far developed are limited due to costs associated with vaccination protocols and less effectiveness (Ley, 2003). Aluminium hydroxide as an adjuvant in bacterin vaccine contributes to the production of a good antibody response but it has little capacity to stimulate cellular immune response (Petrovsky and Aguilar, 2004). Studies on cell-mediated immunity (CMI) in *Mycoplasma* infections in several species have indicated that CMI also plays a major role in the development of immunity to mycoplasmal respiratory tract diseases (Roberts, 1973; Biberfeld *et al*., 1974). Information on the effects of currently available *M. gallisepticum* bacterin vaccines on cell mediated immune system is scanty. Saponin as an adjuvant has the unique ability to stimulate the cell mediated immune system as well as to enhance antibody production and only a low dose is needed for adjuvant activity (Oda *et al*., 2000). Hence, a combination of aluminium hydroxide-saponin might be a promising adjuvant for *M. gallisepticum* bacterin vaccines as it might be able to stimulate and prolong both the humoral and

cell mediated immune responses. Hence, the present study is designed to make vaccines with an adjuvant that is able to stimulate both humoral and CMI responses.

Iron is an essential metal for almost all living systems since it serves as a cofactor or as prosthetic group for essential enzymes that are involved in basic cellular functions. Iron deprivation by sequestering the available iron in iron binding proteins like transferrin and lactoferrin is a prominent feature of the host defense mechanism to control microbial infections. Bacteria have evolved several mechanisms for iron acquisition from the host system, which involve various transcriptional regulators in genes involved in iron uptake. The regulation of iron-acquisition genes linked with many virulence factors allows the bacteria for cell invasion and possibly cell death, thereby increasing the iron available for uptake by the bacteria. During iron depletion, bacteria up regulate and down regulate many bacterial proteins and may also produce new immunogenic proteins (Salyers and Whitt, 2002). The antibodies produced against these proteins may offer better protection to host against natural infections.

Keeping in view with the available information, the present study was designed with the following objectives.

1) Assessment of the immunogenicity of whole cell and membrane proteins of *Mycoplasma gallisepticum* grown under different growth conditions.
2) Development of vaccines from the above proteins, using saponin alone and saponin plus aluminium hydroxide.
3) Evaluation of the comparative efficacy of the developed vaccines with commercially available vaccine.

Review of literature

REVIEW OF LITERATURE

Avian mycoplasmosis is a frequently reported infectious disease which can cause considerable economic losses in poultry, especially in chicken and turkeys, all over the world. Among the different species of *Mycoplasma* affecting poultry, *M. gallisepticum* is of greater concern as it causes CRD and decreased egg production in chicken and infectious sinusitis in turkey (Jordan, 1975; Ley and Yoder, 1997). Besides decrease in egg production, *M. gallisepticum* infection in chickens can also cause decreased growth rate, hatchability rates and significant downgrading of carcasses at slaughter due to air sacculitis and arthritic lesions, thus causing severe economic losses (Jordan, 1975; Kleven, 1997). Apart from chicken and turkey, other important hosts of *M. gallisepticum* include pheasants, partridges and song birds. It has also been recognized as a cause of conjunctivitis in house finches in North America (Ley *et al*., 1996; Luttrell *et al*., 1996).

2.1 Classification

Avian mycoplasmas are members of the division *Tenericutes*, class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae* and genus *Mycoplasma*. *Mollicutes* are characterized by the absence of cell wall, presence of tri-laminar membrane, small size, possession of fewer cellular organelle, particularly those for metabolism and reproduction, and a small genome of less than 600bp. The small genome size places a restriction on the number of proteins that can be coded for and as a consequence, *Mollicutes* possess limited metabolic activities and are dependent on a vast array of nutrients from their environment. The resultant nutritional fastidiousness is a major barrier to work with the *Mollicutes*. They are widely distributed as pathogens or commensal organisms of a wide range of plant and animal hosts, including insects.

In the 1950's the increasing association of mycoplasmas with poultry disease and the development of improved media for *Mycoplasma* isolation stimulated a considerable amount of investigative work.

Edward and Freundt (1956) proposed a system for the classification and nomenclature of organisms of the pleuropneumonia group, according to which all

species were arranged into the genus *Mycoplasma*, family *Mycoplasmataceae*, and order *Mycoplasmatales*.

Adler *et al*. (1958) described different strains of pleuropneumonia like organisms of avian origin, which are now designated as different species of *Mycoplasma*.

Edward and Kanavek (1960) made the species designation *Mycoplasma gallisepticum*.

Yamamoto and Adler (1958) described five serotypes, Kleckner (1960) described eight serotypes, Yoder and Hofstad (1964) characterized twelve serotypes and Dierks *et al*. (1967) described nineteen serotypes of *Mycoplasma*. So far, 23 avian *Mycoplasma* species have described by various authors but only *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are associated with significant economic losses in chickens.

Edward and Freundt (1967) proposed *Mollecutes* as a name of the class for the order *Mycoplasmatales*.

Two-hundred and two strains of avian *Mycoplasma* species belonging to 10 biotypes were typed serologically by Asnani and Agarwal (1974) by employing disk growth inhibition (DGI) and indirect haemagglutination (IHA) tests. These could be placed into eight serotypes, namely A, B, C, E, L, P, 1 and R. A close relationship was observed between DGI and IHA tests. The IHA test, however, was more sensitive and specific. It was also noticed that biochemically identical biotypes, namely E and G, as well as biotypes B and M biotypes were also found identical in serotyping, thus confirming the biochemical identity. In view of these facts, the strains of biotypes M and G were grouped under serotypes B and E, respectively.

Bradbury *et al*. (1993) differentiated mycoplasmas with phenotypic and antigenic similarities to *M. gallisepticum* by molecular techniques and designated them as *Mycoplasma imitans*.

Mycoplasma gallisepticum was first classified and differentiated from other avian *Mycoplasma* by serotyping and was commonly designated as serotype A (Ley, 2003).

2.2 History

Nelson (1936) isolated avian *Mycoplasma* for the first time and described it as cocco-baccillary bodies in coryza of chicken.

Delaplane and Stuart (1943) cultivated the agents isolated from chicken with CRD and turkeys with sinusitis, in embryos.

Markham and Wong (1952) and Van Rockel and Olesiuk (1953) reported the successful cultivation of these organisms and suggested that they belonged to pleuropnuemonia group, which opened an era of active research on avian mycoplasmosis.

Olson *et al*. (1954) were the first to describe association of infectious synovitis with *Mycoplasma*.

Jordan (1975) had reported the importance of *M. gallisepticum* as a cause of respiratory disease and decreased egg production in chicken and turkeys. He had also formulated suitable culture media for the isolation of *Mycoplasma*.

Bencina *et al*. (1987) reported that the chicken remained as *M. gallisepticum* carriers for many months after the acute phase of infection and they showed no signs of the disease.

Occurrence of kerato-conjuctivitis in chicken associated with *M. gallisepticum* was reported by Nunoya *et al*. (1995).

Kleven (1998) described egg transmission of avian *Mycoplasma* and opined that *M. gallisepticum s*howed wide variation in virulence, tissue tropism and antigen make-up, posing lot of difficulties in control and eradication programmes.

2.3 Host range

The infection by *M. gallisepticum* naturally occurs primarily in gallinaceous birds particularly in chicken and turkeys in commercial production.

The organism was isolated from naturally occurring infections in bobwhite quail and Japanese quail by Tiong (1978) and from chicken, turkeys, pheasants, chukar partridge and peafowl by Cookson and Shivaprasad (1994).

The organism was also isolated from duck (Amin and Jordan, 1979; Bencina *et al*., 1988a); geese (Buntz *et al*., 1986; Bencina *et al*., 1988b); house finches (Ley *et al.,* 1996; Luttrell *et al*., 2001), from a golden pheasant (Reece *et al*., 1986) and from a yellow-naped Amazon parrot (Bozeman *et al*., 1984).

Experimental infection had been produced in captive-reared wild turkeys by Rocke *et al*. (1988).

Mycoplasma gallisepticum was isolated from a blue jay that developed conjunctivitis after being housed in a cage previously occupied by affected house finches (Ley *et al*., 1996).

There are reports of *M. gallisepticum* detection in sera and isolations from wild turkeys (Davidson *et al*., 1982; Cobb *et al*., 1992; Fritz *et al*., 1992; Peterson *et al*., 2002) and various other free-flying birds including sparrows (Lin *et al*., 1996) but the significance of the presence of *M. gallisepticum* in these species have not been clearly established.

An outbreak of conjunctivitis caused by *M. gallisepticum* in evening gross beaks and pine gross beaks was reported by Mikaelian *et al*. (2001).

2.4 Incidence of *Mycoplasma gallisepticum* infection

2.4.1 Abroad

The *M. gallisepticum* infection appeared to be worldwide in distribution. Prevalence of the disease in chicken was studied and reported from many parts of the world.

A survey of *M. gallisepticum* was carried out on chickens reared in three different husbandry systems around Zaria, Nigeria by serum plate agglutination test. The overall prevalence was 47.54 per cent (Abdu *et al*., 1983).

Mohammed *et al*. (1986) studied the prevalence of *M. gallisepticum* in commercial pullet and layer flocks in southern and central California, USA in 1984, by testing serum and egg-yolk samples through ELISA. The estimated true prevalence of *M. gallisepticum* was found to be 73 per cent and 3 per cent in southern California and central California, respectively

The infections by *M. gallisepticum* had become an emerging problem for commercial chicken flocks, especially those reared in a continuous production site. However, there is some evidence that *M. gallisepticum* is also present in small back yard poultry flocks (McBride *et al*., 1991).

Kelly *et al*. (1994) conducted a study in Zimbabwe, on backyard chicken by ELISA and the result showed that the prevalence of *M. gallisepticum* and *M. synoviae* was 33 per cent.

Observations of *M. gallisepticum* infection in village chicken from other countries include Zimbabwe (Kelly *et al*., 1994), Benin (Chrysostome *et al*., 1995), Malaysia (Shah-Majid, 1996), Senegal (Arbelot *et al*., 1997), Zambia (Pandey and Hasegawa, 1998) and Botswana (Mushi *et al*., 1999). The overall prevalence in these investigations ranged from 26 per cent (Malaysia) to 62 per cent (Benin).

Investigation of Zhang *et al*. (1995) in Gansu, China showed that *M. gallisepticum* is significantly present in study areas.

Shah-Majid (1996) conducted a seroprevalence study on village chicken in Malaysia using ELISA and 26 per cent samples were found to be positive to *M. gallisepticum*.

The *M. gallisepticum* infection appears to be worldwide in distribution (Ley and Yoder, 1997).

Saif-Edin (1997) found that the seroprevalence of *M. gallisepticum* was significantly evident in Egyptian chicken in the Middle East region.

The prevalence of mycoplasmal conjunctivitis in house finches was studied by Dhondt *et al*. (1998) and found that the disease seemed to fluctuate seasonally with increase in the fall, probably as a result of dispersing juveniles. The finch population decreased throughout winter season in areas with cold winter and high conjunctivitis prevalence, suggesting significant mortality associated with the disease.

Zelenika *et al*. (1999) reported that the prevalence of *M. gallisepticum* in heavy hen hybrids was 10 per cent in 1997 and 13 per cent in 1998 from Croatia.

A prevalence study was conducted in layer chickens in Aragua State, Venezuela by HI test. Based on the result of the study (59 per cent, 22 per cent and 66 per cent prevalence in three municipalities), it was suggested that control programmes for mycoplasmosis must be established to reduce its incidence (Godoy *et al*., 2001).

Zhang *et al*. (2001) studied the prevalence and pathogenicity of *M. gallisepticum* in broiler chickens in Mongolia. Serum plate agglutination (SPA) test was used and 53 per cent of the birds were positive to *M. gallisepticum*.

The sero-prevalence of *M. gallisepticum* infection of chickens in selected Model Breeder Poultry Farms in Bangladesh was determined by Sarkar *et al*. (2005). A total of 382 sera samples were collected. Serum Plate Agglutination test was

performed using commercial *M. gallisepticum* antigen to detect the presence of antibodies against *M. gallisepticum*. The overall sero-prevalence was 58.90 per cent in the study area. The highest prevalence of infection was 62.44 per cent infection in winter season followed by 53.10 per cent in summer season. The results further revealed that the infection was higher (59.94 per cent) in female birds than in male birds (48.57 per cent).

2.4.2 India

There is no or scanty literature in India, on the studies of various biological properties including serotyping of avian *Mycoplasma* species, defining the prevalence of particular biotype or *Mycoplasma* species.

Three hundred and seventy nine tracheal swabs were collected from healthy and sick live birds, representing various states of the country were investigated by Asnani and Agarwal (1974). One hundred and fifty nine strains of *Mycoplasma* species (41.9 per cent) were isolated. These and 58 strains obtained from other sources were identified on the basis of their biological properties into 10 biotypes. The incidence of each biotype was recorded as follows; A (36.9 per cent), B (22.9 per cent), C (1.38 per cent), E (10.6 per cent), G (5.06 per cent), I (0.92 per cent), L (5.99 per cent), M (11.05 per cent), P (1.84 percent) and R (7.39 per cent).

Singh and Singh (1967) conducted cultural and serological examinations for pleuropnuemonia like organisms (PPLO) infection on 1546 and 1623 poultry samples, respectively from different parts of the country. The percentage of isolation and serology was 49 and 58 respectively.

Asnani and Pathak (1975) isolated 22 strains of avian *Mycoplasma* from trachea of 63 live, normal and healthy white leghorn and desi birds.

Katoch and Chandiramani (1984) obtained 37 isolates from the respiratory tract of 202 chickens.

Incidence of avian mycoplasmosis in India based on isolation and serology had been reported in Haryana by Mahajan *et al*. (1994), in Namakkal by Srithar *et al*. (1997); and in West Bengal by Chakraborthy *et al*. (2001).

Manohar (2001) isolated three *M. gallisepticum* and four *M. inus* isolates from ailing and dead birds.

Dipu (2006) conducted a study for the detection and isolation of three significantly pathogenic mycoplasmas of chicken namely *M. gallisepticum*, *M. synoviae* and *M. iowae*. Samples were collected from the poultry farms in different parts of Kerala. Five isolates of *M. gallisepticum* were obtained in the study.

2.5 Transmission

Mycoplasma gallisepticum infection is transmitted both horizontally and vertically.

M. gallisepticum can be transmitted through the chicken or turkey hatching egg to the offspring. It has been isolated from the oviduct of infected chickens and semen of infected roosters (Yoder and Hofstad, 1964).

Jain *et al*. (1971) reported that wild birds harboured *M. gallisepticum* and played an important role in the flock-to- flock transmission of this disease.

Kleven and Fletcher (1983), on the basis of serological surveys and experimental infections, have suggested that house sparrows might act as mechanical carriers of *M. gallisepticum*.

Vertical transmission of *M gallisepticum* has been successfully produced following experimental infection of susceptible chickens (Glisson and Kleven, 1984; Yoder and Hofstad, 1965; Yoder and Hopkins, 1985)

The infection can spread through direct contact of susceptible birds with infected chickens or turkeys. Spread may also occur by contaminated airborne dust, droplets, or feathers (Ley and Yoder, 1997).

Naturally occurring mycoplasmal conjunctivitis was reported among wild-caught and initially sero-negative house finches maintained in captivity for 12 weeks (Luttrell *et al.,* 1998). They opined that infected birds without lesions act as asymptomatic carriers in the wild.

Hartup *et al*. (1998) opined that the feeders might be significant in the transmission of house finch conjunctivitis caused by *M. gallisepticum*.

Stallknecht *et al*. (1998) found that chickens became infected with *M. gallisepticum* via direct contact with naturally infected house finches.

The studies conducted by Hartup and Kollias (1999) suggested that pseudo vertical transmission of *M. gallisepticum* between the breeding adults and their dependent offspring as the main mode of transmission of infection and there was no evidence supporting the transovarian transmission of *M. gallisepticum* in these house finches.

2.6 Predisposing factors

Presence of other pathogens like Newcastle disease and Infectious Bronchitis viruses and/or *Escherichia coli* may exacerbate the disease in chicken (Omuro *et al*., 1971; Gross, 1990). Other pathogens reported to act synergistically with *M. gallisepticum* in chicken are *Haemophilus paragallinarum*, Reovirus, Adenovirus, and Infectious Bursal disease virus (Rampin *et al*., 1977; Carpenter *et al*., 1981; Uchida *et al*., 1990). Various other factors such as increased environmental ammonia, high levels of dust, poor nutrition, immunosuppressive agents and social stresses associated with intensive management play an important role in *M. gallisepticum* infection (Bradbury, 2001).

2.7 Clinical signs and lesions

Under natural conditions, the incubation period may vary considerably from 3 to 38 weeks (Jordan, 1975).

Reece *et al*. (1986) described conjunctivitis, sinusitis, mucoid tracheitis and air sacculitis in infectious sinusitis caused by *M. gallisepticum* in Japanese quails, chukar patridges and golden pheasants.

In flocks, infected through eggs, clinical signs might develop at the age of three to six weeks or only near the onset of egg production. In the case of flocks hatched from eggs dipped in antibiotic solutions to control *M. gallisepticum*, in good hygienic conditions, signs might not appear until some associated disease or stress factors occurred. The most common clinical signs were nasal discharge, tracheal rales, coughing, sneezing and swelling of one or both infra-orbital sinuses (mostly in turkeys) and mild conjunctivitis. Appetite remained near normal as long as the birds could eat. Sometimes ataxia, lameness, swelling of the hock and enlargement of the eyeballs were observed. Non-specific signs such as reduction in growth rate and egg production and decreased feed conversion efficiency were common (Yoder, 1991).

Natural infection of *M. gallisepticum* in pheasants, chukar patridges and pea fowl, characterized by foamy eyes, swollen infra orbital sinuses, respiratory distress and death, was described by Cookson and Shivaprasad (1994).

Mc Martin *et al*. (1996) reported an outbreak of conjunctivitis and severe respiratory disease caused by *M. gallisepticum* in integrated chukar partridge facility in San Diego County, California leading to severe drop in egg production and increased culling rates.

Clinical symptoms were generally more severe in males than in females and turkeys appeared more diseased than chicken. Morbidity varied depending on age (young birds were more severely affected than old ones) and on ambient temperature (in cold climates, the disease was more severe and of longer duration). Complicated CRD (*i.e.* air sac disease due to other agents, such as *E.coli*) was encountered more commonly in the field. The mortality could be low in uncomplicated outbreaks (Ley, 2003).

Lesions include an excess of mucus, catarrhal exudates in nares, sinuses, trachea, bronchi and lungs, air sacculitis, oedema of the air sac walls and caseous exudates in the air sacs and in the oviduct. In complicated cases, pericarditis, perihepatitis and sometimes, swelling and oedema of peri-articular tissue, excess joint fluid, erosion of the articular surface (arthritis), inflammation of tendovaginal sheaths, bursae and the synovial membrane (synovitis) and pale areas in the cerebrum (vasculitis) may be observed (Salami *et al*., 1992).

2.8 Isolation and characterization

The most reliable method of diagnosis of *M. gallisepticum* is isolation and identification of pathogen from the disease (Takahata *et al*., 1997). It can be identified by immunological and molecular tests after isolation in *Mycoplasma* media or the presence of its DNA can be demonstrated in field samples or cultures. However, isolation and identification of *Mycoplasma* organisms were difficult and time consuming. Therefore, serological assays had been used routinely (Bradley *et al*., 1988). Further, the serological procedures were useful for flock monitoring in *Mycoplasma* control programme and to aid in diagnosis when infection was suspected. A positive serological test, together with history and signs typical of the *M.*

gallisepticum infection, allowed a presumptive diagnosis, pending isolation and or identification of the organism (Kleven, 1981).

2.8.1 Isolation

Most media formulation for the isolation of *Mycoplasma* has as base components meat infusion (usually beef heart) and tryptone and/or peptone. Many variations and supplements to the basic formulation have been shown to be efficacious or essential for propagation of some *Mycoplasma* species. Apart from different types of media, the organism can also be propagated in embryonated eggs.

Avian *Mycoplasma* was first isolated by Nelson (1936).

Delaplane and Stuart (1943) cultivated an agent associated with CRD of chicken in embryonated chicken eggs.

A PPLO was isolated in artificial medium by Van Herick and Eaton (1945), although they did not associate the organisms with respiratory disease of chicken.

The common media formulations for the isolation of mycoplasmas have as base components, meat-infusion (usually beef heart), tryptone and/or peptone, yeast extract, glucose and 10 to 20 per cent horse or swine serum (Edward, 1947).

Markham and Wong (1952) used a medium that contained beef heart infusion and yeast extract, enriched with equine serum, for isolation of *M. gallisepticum*.

Grumble *et al*. (1953) described a medium composed of fermentable carbohydrates and phenol red broth base enriched with bovine serum fractions for the isolation of avian *Mycoplasma.*

Adler *et al*. (1954) suggested a medium composed of 10 per cent blood agar slope overlaid with PPLO broth with 20 per cent horse serum to be more satisfactory for the isolation of PPLO than several other media tested.

Lecce and Sperling (1954) proposed heart infusion agar with thallium acetate as a selective medium suitable for the growth of avian strains of PPLO and the trachea of a chicken with CRD seemed to be more likely to harbor PPLO than lungs or air sacs. They concluded that isolation of PPLO in chickens with respiratory symptoms might be used as a diagnostic aid for CRD.

Taylor and Fabricant (1957) reported that the primary isolation of PPLO from tracheal exudates was most successfully accomplished by cultivation in the modified Grumble's medium.

Fabricant (1958) reported that chick embryo passage was superior to commercial media for the isolation and detection of avian *Mycoplasma*. He also opined that, media generally in use were not satisfactory because of their inability to support adequate growth of many pathogenic strains of PPLO.

A culture media containing avian meat infusion, turkey serum, yeast extract, and penicillin and thallous acetate as bacterial inhibitors was employed by Yoder and Hofstad (1964) for the isolation of avian mycoplasmas.

Frey *et al*. (1968) compared the mycoplasmal media bases prepared from commercially available ingredients with commercial media bases and fresh meat medium for the isolation of avian mycoplasmas. They concluded that French medium 4th formula supplemented with 15 per cent human plasma supported good growth of all three pathogenic species of avian mycoplasmas, *viz*., *M. gallisepticum*, *M. synoviae* and *M. meleagridis*.

Pathogenic strains of avian *Mycoplasma* were isolated using Difco *Mycoplasma* base with swine serum, yeast extract, arginine, glucose and nicotinamide adenine dinucleotide (Power and Jordan, 1976).

The isolation rate of *Mycoplasma* was significantly improved after the addition of bacterial inhibitors like thallium and penicillin in *Mycoplasma* media base (Branton *et al*., 1984).

Majid (1986) reported that heavy contamination was detected when specimens from fowl were directly inoculated on to *Mycoplasma* agar plates for the isolation of *M. gallisepticum* isolation.

Kleven (1994) modified the Frey's medium for the isolation of *M. gallisepticum* by direct plating of exudates or tissue swabs onto agar medium and opined that initial enrichment in broth was generally a more sensitive method.

Though Branton *et al*. (1991) observed that wetting of swab prior to swabbing did not improve the recovery of *M. gallisepticum* from commercial layers, the finding was contradicted by the research findings of Zain and Bradbury (1995 and 1996), who reported that cotton swabs could retain a large per cent of the total numbers of colony forming units of *M. gallisepticum,* even after thorough agitation. The experiment revealed that wet swabs yielded significantly greater number of mycoplasmas than dry swabs.

The discrepancies between isolation and PCR results are often encountered in *Mycoplasma* isolation studies. The reason could be attributed to the detection of non-viable mycoplasmas in PCR (Kempf *et al*., 1994; Moalic *et al*., 1997).

The solid medium was found to be more effective than liquid medium for the isolation of avian *Mycoplasma* by Ronglian *et al*. (1996).

Kempf *et al*. (1997) used a transportation medium for avian *Mycoplasma* with peptone, glycerine and penicillin.

Over growth by faster growing saprophytic species of *Mycoplasma* was a frequently encountered problem during isolation (Kempf *et al*., 1997).

Luttrell *et al*. (1998) used SP4 broth and Frey's medium with swine serum agar for the isolation of *M. gallisepticum* from captive flock of house finches with conjunctivitis.

Kleven (1998) described the use of choanal cleft swabs for inoculation in Frey's media.

Branton *et al*. (1999) conducted an experiment to determine the effects of age at inoculation and induced moult on the re-isolation of *M. gallisepticum* and observed a significant decrease in the *M. gallisepticum* isolations from post moult swabs, compared with the pre-moult swabs.

Increased humidity and CO_2 tension between five to ten per cent were found to enhance the growth of *Mycoplasma* (OIE, 2008).

Manohar (2001) compared six media for isolation of *M. gallisepticum* and found that Buffalo heart infusion with ten per cent freshly prepared yeast extract, 20 per cent horse serum, and two per cent glucose could provide better growth of avian mycoplasmas.

Buffalo heart infusion broth was found to be ideal for the isolation and propagation of avian mycoplasmas George (2003).

2.8.2 Growth of bacteria under iron restricted conditions

Iron is an essential metal for almost all living systems and serves as a cofactor or as a prosthetic group for many essential enzymes involved in basic cellular functions. Iron is normally bound to specific proteins in host to prevent its

precipitation in aqueous systems and to protect the cell from damaging UV light and lipid peroxidation reactions. In this way, iron is sequestered for use by the host and invading pathogens is prevented as part of the innate immune defence. Bacteria have evolved several mechanisms for the uptake of iron from host cells which include the regulation of various genes in the bacterial genome, which in turn may result in the up or down regulation of various immunogenic proteins (Salyers and Whitt, 2002).

Jacques *et al*. (1994) found that addition of the iron chelators 2, 2'dipyridyl, desferoxamine mesylate or apotransferrin to culture media affected the composition and morphology of *Pasteurella multocida* cells. Cells grown under iron restricted conditions expressed iron regulated proteins and, in addition, iron deprivation markedly reduced the amount of capsular material covering *Pasteurella multocida*.

The outer membrane protein profile of *Actinobacillus pleuropneumoniae* grown under iron restricted conditions was studied by polyacrylamide gel electrophoresis and immunoblotting by Harry and Potter (1989). They found that a virulent serotype synthesized a novel protein and increased synthesis of another protein under iron restricted conditions.

Bacteria have evolved mechanisms for iron acquisition that are tightly controlled by the level and availability of iron in the environment (Ratledge and Dover, 2000).

Madsen *et al*. (2006) conducted a transcriptional profiling of *Mycoplasma hyopneumoniae* during iron depletion (2,2′-dipyridyl at 1 mg/ml used for iron chelation) using micro arrays and identified that about 27 genes were either up or down regulated in response to low-iron growth conditions. These included genes encoding transport proteins, enzymes involved in energy metabolism, and components of translation process.

2.8.3 Characterization

The various tests used for the characterization of *Mycoplasma* isolates includes morphological characters, biochemical tests, metabolic and growth inhibition tests, agar gel precipitation tests, immunofluorescence and immunoperoxidase tests, molecular and serological tests. Edward and Freundt (1969) stated that for characterization, biochemical and serological (Poveda, 1998)

procedures should be used together, as the independent use of either might not reveal mixed cultures.

2.8.3.1 Colony morphology and biochemical characteristics

Adler *et al*. (1954) isolated several serological and pathological variants of PPLO of avian origin and found that some of the pathogenic types failed to grow on agar whereas some did not ferment carbohydrates.

Yamamoto and Adler (1958) characterized avian strains of PPLO according to morphological, physiological and antigenic characteristics.

Diene's stain was taken up by *Mycoplasma* and bacterial L-forms. But only colonies of *Mycoplasma* retain the stain, while other get decolourised in 15-30 min (Dienes and Weinberger, 1951). This could be used for the confirmation of *Mycoplasma* isolates (Timms, 1967).

Mycoplasma isolates on agar produced typical-fried egg appearance on the *Mycoplasma* agar surface, with a dark blue depressed centre and a light coloured raised periphery (Madoff, 1960).

Harry (1964) observed marked difference in the size of colonies of different isolates of *M. gallisepticum*. He also found irregularities in carbohydrate fermenting ability among the isolates, probably due to differences in media, isolates and interpretation of the colour of the phenol red indicator. He observed that tetrazolium reduction and haemolysis were shown only by some isolates.

Fermentation of specific carbohydrates like glucose and fructose, non-fermentation of galactose, mannose, xylose, cellobiose, hydrolysis of arginine and reduction of tetrazolium could help in the preliminary identification of *M. gallisepticum* isolates (Fabricant, 1969; Aluotto *et al*., 1970; Barber and Fabricant, 1971).

2.8.3.2 Growth and Metabolic inhibition tests

In Growth Inhibition test, anti-serum impregnated sterile filter paper discs were applied onto the surface of the agar plates and the surface area adjacent to each disc was examined microscopically for zones of inhibition, after incubation at 37°C under 5-10 per cent CO_2 tension for four days (Clyde, 1964; Stanbridge and Hayflick, 1967).

Dierk's *et al*. (1967) suggested that agglutination and growth inhibition studies were the most meaningful in the characterization of isolates.

Growth inhibition test using species specific anti-sera was found to be a highly specific method for the identification of unknown isolates (Domermuth and Gourlay, 1967; Davies and Read, 1968; Ogra and Bohl, 1970).

In growth inhibition studies of avian *Mycoplasma* using specific antiserum, inhibition zone around the disc seemed to depend on the potency of specific antiserum, concentration of the test culture and moisture content of the agar. Immunodiffusion and growth inhibition appeared to correlate fairly well in the antigenic analysis of avian *Mycoplasma* (Aycardi *et al*., 1971).

In the metabolic inhibition test with specific antiserum, used for characterization of *M. gallisepticum,* the zones of complete or almost complete inhibition were wider with the shallower media (Jordan, 1973).

Woode and Mc Martin (1973) reported that in metabolic inhibition test using high dilution of antiserum, the lag phase of growth of *M. gallisepticum* was significantly prolonged, but eventually growth commenced and proceeded normally. It appears probable that limited growth of organisms did occur during the lag phase, which was sufficient to absorb the low level of antibody present. The failure to detect this growth could have been due to counting errors arising from agglutination of organisms. The growth was permanently inhibited by using low dilutions of antiserum.

2.8.3.3 Agar gel precipitin test

Agar gel precipitin test has been used to identify the cultures of *M. gallisepticum* by Aycardi *et al*. (1971) and Nonomura and Yoder (1977).

2.8.3.4 Immunoflourescence test

Studies conducted by Corstvet and Sadler (1964) indicated that the application of immunoflouroscence for the identification of PPLO in smears, urine sediments, broth and agar cultures, tissue cultures and tissue biopsies using specific anti-sera would provide a rapid and efficient means of their identification.

Immunofluorescene and Immunoperoxidase techniques were the most suitable and sensitive methods for the identification of *Mycoplasma* species in mixed cultures on primary isolation plates (Del Guidice *et al*., 1967; Polak-Vogelzang *et al*., 1979).

Bradbury (1982) prepared antiserum from 12 of the recognized avian *Mycoplasma* species including *M. gallisepticum* and *M. synoviae*, in chickens by giving intravenous inoculation of a concentrated suspension of live organisms. These sera proved to be suitable for use in Fluorescent antibody test (FAT).

Bencina and Dorrer (1984) demonstrated *M. gallisepticum* in smear prepared from tracheal mucus by indirect FAT.

Morse *et al*. (1986) detected *M. gallisepticum* by direct immunofluorescence using a species-specific monoclonal antibody (MAb). Laboratory strains of avian *Mycoplasma* serotypes were classified by direct immunofluorescence.

May *et al*. (1994) isolated broth cultures (24h old) of high passage strains of *M. gallisepticum* and *M. synoviae* by immunofluorescence and flowcytometry, using homologous and heterologous anti-sera. High fluorescent per cent and intensities were observed for M. gallisepticum (93.7 per cent) and *M. synoviae* (84.9 per cent) and non-specific staining was low (10.3 per cent and 12.3 per cent respectively).

Direct immunoflourescence employing colonies on the surface of agar plates, or colony imprints, had been very effective for culture identification (Talkington and Kleven, 1983; 1984; Morse *et al*., 1986). These techniques and their modifications were particularly useful for identification of *M. gallisepticum* in cultures containing other *Mycoplasma* species (Morse *et al*., 1986; Bencina and Bradbury, 1992).

2.8.3.5 Immunoperoxidase test

Studies conducted by Quinn *et al*. (1981) indicated that Immunoperoxidase test was not only as simple as the Fluorescent antibody test and as specific as the Growth inhibition test, but also more sensitive than these tests.

Colonies of type strains of 22 *Mycoplasma* species, three *Acholeplasma* species, and three *Ureaplasma diversum* serogroups were identified by indirect immunoperoxidase with high sensitivity and specificity by Imada *et al*. (1987).

Immunobinding based on indirect immunoperoxidase test of *Mycoplasma* cells blotted on a nitrocellulose paper was found to be a useful method for rapid

identification of mycoplasmas in broth medium and clinical specimens by Kotani and McGarrity (1989).

Bencina and Bradbury (1992) used a combination of FAT and immunoperoxidase tests for simultaneous identification of *M. gallisepticum* and *M. synoviae* and concluded that this procedure offered an improvement over existing methods in terms of both speed and analytical sensitivity.

Radi *et al*. (2000) developed an avidin-biotin immunoperoxidase diagnosis test to facilitate rapid identification of *M. gallisepticum* and *M. synoviae* in respiratory tissue of turkey. This test was significantly sensitive to detect *M. gallisepticum* antigen at one, two, three and four weeks post inoculation. However, weak to moderate staining was observed in tracheas of turkeys inoculated with *M. synoviae*.

2.8.3.6 Molecular methods of detection

Various molecular methods used for the detection of *Mycoplasma* include Random amplification of polymorphic DNA (RAPD), PCR, Restriction fragment length polymorphism (RFLP), Amplification fragment length polymorphism (AFLP), use of DNA and RNA probes and Gene targeted sequencing.

2.8.3.6.1 Random amplification of polymorphic DNA

Random amplification of polymorphic DNA is a PCR based method of DNA fingerprinting. It has been used to study heterogeneity in closely related organisms. This method detects difference in DNA sequence at sites in the genome that are defined by the primer used. Sequence variation is revealed by the number and length of amplified products which may be phylogenetically conserved.

Geary *et al*. (1994) demonstrated that the RAPD finger printing method distinguished genetically different strains of *M. gallisepticum* and indicated that it should be reliable for monitoring transmission of this pathogen.

Charlton *et al*. (1999) used RAPD to invest the molecular epidemiology of 26 *M. gallisepticum* isolates from turkeys located in the central valley of California. The *M. gallisepticum* isolates were recovered from five different companies and 13 ranches. Similar RAPD banding pattern of isolates from different ranches within the same company suggested horizontal spread of *M. gallisepticum* between the ranches.

2.8.3.6.2 Polymerase chain reaction

Polymerase chain reaction technique has been proven to be a very specific and sensitive method for amplifying low amounts of nucleic acid to a level that cannot be easily detected by other methods (Silveira *et al*., 1996; Salisch *et al*., 1998; Mekkes and Feberwee, 2005). In the PCR technique, a thermo stable DNA polymerase, specific oligo primers, deoxynucleotide triphosphates, and a suitable template nucleic acid sequence are reacted in a mechanical thermal cycler to greatly increase the quantity of a desired DNA template target sequence, allowing for ready detection.

Nascimento *et al*. (1991) developed a *M. gallisepticum* specific PCR, based on the sequence of a 760 bp fragment isolated from *M. gallisepticum* genomic library. The product amplification was specific to *M. gallisepticum* amplifying a 732 bp product, but did not amplify DNA of *E. coli* or 16 other species of avian mycoplasmas. As low as 6-10 pg of *M. gallisepticum* DNA was detected following amplification by PCR.

Van Kuppeveld *et al*. (1994) elucidated species specific sequences of *Mycoplasma collis* by asymmetric amplification and dideoxynucleotide sequencing of variable regions, using primers complementary to conserved regions of 16s rRNA. They developed a highly sensitive PCR assay on the basis of high copy number of rRNA and opined that the nucleic acid content equivalent to a single organism could be detected.

Mycoplasma gallisepticum F-vaccine strain polymerase chain reaction (MGF-PCR) was developed and standardized by Nascimento *et al*. (1993). The amplified product of 524 bp was directed at F-strain related *M. gallisepticum* only. None of the other 16 avian mycoplasmas that were tested yielded MGF-PCR product. The MGF-PCR was found to be 1000 to 10, 000 times more sensitive than dot-blot assays using two MG-F strain specific probes.

Kempf *et al*. (1993) developed a PCR for the detection of *M. gallisepticum* by choosing a pair of primers based on the alignment of 16s rRNA sequences of *mollicutes*. The primers detected a specific amplification of all *M. gallisepticum* strains, yielding an expected 330bp product, but there was no amplification when other mollicutes or *E. coli* were used as PCR templates.

Lauerman *et al*. (1993) selected *M. synoviae* species specific primers from the 16s rRNA sequence. The *M. synoviae* PCR had detected 100 colony forming

units (CFU) of *M. synoviae* and had 100 per cent specificity and sensitivity. Analysis of 122 flock data sets indicated a sensitivity of 82 per cent and a specificity of 100 per cent for the *M. synoviae* PCR test, as determined in comparison with culture, serology, epizootiology and history.

Zhao and Yamamoto (1993) synthesized a pair of 25 base primers to develop a MS PCR, based on partial sequencing of approximately 1.1 kbp length MS species specific recombinant clone, Pms 156-20. The primers amplified the target DNA of approximately 1.1 kbp and had an excellent specificity over a range of annealing temperature from 60°C to 68°C. The primers amplified 100 pg of 26 strains or isolates of *M. synoviae*, but did not amplify three strains of *M. gallisepticum*, 15 other avian *Mycoplasma* species and pUC8 plasmid.

Garcia *et al*. (1995) designed a single set of oligonucleotide primers from known 16s rRNA sequences of *M. gallisepticum*, *M. synoviae* and *M. iowae*. This set of primers selectively amplified a 780 bp DNA fragment within the 16s rRNA gene of *M. gallisepticum*, *M. synoviae* and *M. iowae*, but did not amplify other avian mycoplasmas or other bacteria. The detection limit of the multispecies PCR was approximately 100 *Mycoplasma* (*M. gallisepticum*, *M. synoviae* and *M. iowae*) CFU per PCR reaction.

Lauerman *et al*. (1995) employed 16s rRNA region based PCR to generate amplicon from nine avian *Mycoplasma* species which were reacted with 24 restriction enzymes. The electrophoretic patterns of RFLP were evaluated for differences among *Mycoplasma* species. Four (*DraI, MseI, RsaI, Tsp5091*) of the 24 restriction enzymes cut the PCR amplicon of all nine *Mycoplasma* species. The nine avian *Mycoplasma* species could be distinctly differentiated using the RFLP analysis of the PCR amplicon.

Kiss *et al*. (1997) used a primer pair complementary to the 16s rRNA gene, designed for the detection of human and rodent mycoplasmal species and they examined its ability to detect the most important avian mycoplasmas. They found that this primer pair could detect *M. synoviae*, *M. meleagridis* and *M. iowae* by PCR and further distinction could be made among them by RFLP with two restriction enzymes (*Bam HI* and *RsaI*). They concluded that, for the detection of *M. gallisepticum*, species specific primers were required.

Kempf (1998) described the DNA amplification methods for the diagnosis and epidemiological investigation of avian mycoplasmosis. The isolates were differentiated based on RFLP patterns. He opined that arbitrarily primed-PCR could be used to characterize the isolates below the species level.

Marois *et al*. (2000) described the successful detection of *M. synoviae* by culture and PCR from samples collected in the environment of experimentally infected chickens and turkeys or under field conditions. In the experimental infection, 10/96 and 46/96 samples of food, drinking water, feathers, droppings or dust were positive by culture and *Mycoplasma* PCR respectively. In field conditions, the number of positive results for environmental samples were 7/28 and 17/28 respectively.

Bradbury (2001) reported three approaches to diagnose *M. gallisepticum*, which were isolation and identification of the organism, detection of its DNA and lastly, detection of specific antibodies.

Liu *et al*. (2001) developed a polymerase chain reaction method for the specific detection of *M. gallisepticum* strains, based on the difference in adhesion encoding gene *pvpA* in commercial poultry.

Marois *et al*. (2002a) used PCR to detect *M. gallisepticum* in samples collected from the environment of experimentally or naturally infected poultry. Culture was also used in the experimental infections. Of 160 samples of food, drinking water, feathers, droppings or dust collected during experimental infection, 103 were positive using a *M. gallisepticum*-specific PCR and 57 were positive using a PCR (*Mycoplasma*-PCR) that detects all species of the genera *Mycoplasma*, *Spiroplasma*, *Acholeplasma* and *Ureaplasma*. Six of these samples were also positive by culture. In environmental samples collected on a depopulated *M. gallisepticum*-positive turkey farm, three and two out of a total of 12 were positive by *Mycoplasma*-PCR and *M. gallisepticum*-PCR, respectively. These results indicated the disseminating capacity of this *Mycoplasma* and the possible use of PCR methods for epidemiological analyses and control of farm decontamination before the introduction of new birds.

Marois *et al*. (2002b) used PCR to detect *M. gallisepticum* in samples collected from the environment of experimentally or naturally infected poultry and the possible use of PCR methods for epidemiological analysis and control of farm

decontamination before the introduction of new birds. In order to study horizontal transmission of *M. synoviae*, Reverse Transcriptase PCR (RT-PCR) based on 16S rRNA was developed to detect viable *M. synoviae* in the environment. Results showed that MS 16S rRNA was stable up to 23 h after cell death and that RT-PCR was useful in checking the efficiency of biosecurity measures and in improving the cleaning and disinfection protocols.

Hong *et al*. (2004) validated and applied PCR and DNA sequence analysis of the N-terminal end of the haemagglutinin encoding gene *vlhA* as an alternative for the detection and initial typing of field strains of *M. synoviae* in commercial poultry. The *vlhA* gene targeted PCR assay was highly specific in identification of *M. synoviae,* with a detection limit of 4.7×10^2 CFU/ml. They conducted DNA sequence analysis of the amplified product to validate the potential for typing *M. synoviae* strains using the N-terminal region of the *vlhA* gene.

Among three approaches available to diagnose *M. gallisepticum*, *viz*., isolation and identification of the organism, detection of its DNA and detection of specific antibodies, OIE (2008) described the PCR as one of the best techniques for the identification and confirmation of *Mycoplasma*, based on 16s rRNA gene.

Garcia *et al*. (2005) compared a panel of four genomic *M. gallisepticum* PCRs *viz*., 16s rRNA PCR, *mgc2*, *nLP* and *ngapA* for analytical sensitivity and specificity of *M. gallisepticum* detection from tracheal swabs. All PCR methods were able to detect the vaccine strains and the challenge strain R directly from the tracheal swabs. They concluded that, among the four PCR methods evaluated, the *mgc2* PCR had a faster turnaround time and was the method of choice for further validation in the field.

Mardassi *et al*. (2005) developed a duplex PCR assay targeting the haemagglutinin multigene families, *vlhA* and pMGA of *M. synoviae* and *M. gallisepticum* respectively. The assay proved to be specific and sensitive enough to justify its use for the simultaneous detection of two major avian *Mycoplasma* species.

Ramirez *et al*. (2006) validated a PCR with one primer based on the intergenic spacer region (IGSR) for the detection of *M. synoviae*. The IGSR primer was paired with a general primer from within the 23s rRNA genes. The PCR primers were tested with 22 other recognized avian *Mycoplasma* species to check the specificity and with 21 field isolates of *M. synoviae* from various hosts and countries, and with several swab samples. They concluded the PCR to be specific and sensitive.

A study was undertaken for the detection of *Mycoplasma* DNA and to differentiate the three pathogenic avian mycoplasmas namely *M. gallisepticum*, *M. synoviae* and *M. iowae* from other less pathogenic ones by PCR. Out of 225 birds subjected for the study, 30 samples were positive for the presence of avian *Mycoplasma* by genus-specific PCR and five of them were found positive for *M. gallisepticum* PCR (Dipu, 2006).

Raviv *et al*. (2007) designed a polymerase chain reaction based on the 16s-23s intergenic spacer region (IGSR) sequence and *M. gallisepticum* IGSR PCR was tested on 18 avian mollicute species and was confirmed as *M. gallisepticum* specific. They found that sequencing of *M. gallisepticum* IGSR appeared to be a valuable single-locus sequence typing (SLST) tool for differentiation of *M. gallisepticum* isolates in diagnosis and epizootiological studies.

2.8.3.6.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism is a variation in the DNA sequence of a genome that can be detected by breaking the DNA into pieces with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis. It is the sequence that makes DNA from different sources, and RFLP analysis is a technique that can identify some differences in sequence (when they occur in a restriction site). Analysis of RFLP variation is an important tool in genome mapping**,** localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing.

Kleven *et al*. (1988) and Santha *et al*. (1988) opined that RFLP could be used as a tool for the differentiation of *M. gallisepticum* strains.

Liu *et al*. (2001) evaluated the features of *M. gallisepticum* -specific gene encoding a phase variable putative adhesion protein (*pvpA*) as the target for molecular typing, accomplished using a *pvpA* PCR-RFLP assay. They found that this method could be used for rapid differentiation of vaccine strain from field isolates by amplification directly from clinical samples. Also molecular epidemiology of *M. gallisepticum* outbreaks could be performed using RFLP and / or sequence analysis of the *pvpA* gene.

2.8.3.6.4 Amplification fragment length polymorphism

Amplification fragment length polymorphism is a selective restriction fragment amplification technique based on the ligation of adapters to a digest of total genomic DNA followed by a PCR based amplification with adapter-specific primers. This allows simultaneous sampling of multiple loci distributed throughout the entire genome, allowing the researcher to control the number of bands generated by using increasingly specific primer sets. It can generate consistent and reproducible banding patterns covering a large number of loci with a single amplification.

Hong *et al*. (2005) used AFLP to type 34 strains of *M. gallisepticum* including vaccine strains ts-11/ 6/85 and F. The AFLP method was able to identify and differentiate both *M. gallisepticum* field strains from recent outbreaks and those that were epidemiologically related. Amplified Fragment Length Polymorphism discrimination potential was compared to other molecular type technology such as Gene targeted sequencing (GTS) and RAPD assay on the same *M. gallisepticum* isolates and they found that three assays correlated with one another, with AFLP analysis having a much higher discrimination power and reproducibility.

2.8.3.6.5 Nucleic acid probes

Hybridization probe is a fragment of DNA or RNA of variable length, which is used to detect in DNA or RNA samples the presence of nucleotide sequences (the DNA target) that are complementary to the sequence in the probe. The probe thereby hybridizes to single-stranded nucleic acid (DNA or RNA) whose base sequence allows probe-target base pairing due to complementarity between the probe and target. The labeled probe is first denatured into single DNA strands and then hybridized to the target DNA (Southern blotting) or RNA (northern blotting) immobilized on a membrane or *in situ*. To detect hybridization of the probe to its target sequence, the probe is labeled with a molecular marker, commonly used markers are ^{32}P or digoxigenin, which is a non-radioactive antibody-based marker.

The DNA and ribosomal RNA probes were used by Khan and Kleven (1993), Khan *et al*. (1987a), Yogev *et al*. (1988), Hyman *et al*. (1989), Nascimento *et al*. (1991) and Dohms *et al*. (1993) to speciate *M. gallisepticum* and identify vaccine strains .

2.8.3.6.6 Gene targeted sequencing

Ferguson *et al*. (2005) characterized a total of 67 *M. gallisepticum* field isolates by gene-targeted sequencing (GTS) analysis of portions of the putative cytadhesin *pvpA* gene, the cytadhesin *gapA* gene, the cytadhesin *mgc2* gene and uncharacterized lipoprotein encoding gene. The regions of the surface protein encoding genes targeted in this analysis were found to be stable within a strain. Gene Targeted Sequence analysis based on the nucleotide sequence identities of individual and multiple genes correlated with epidemiologically linked isolates and with RAPD analysis. They also found that GTS of multiple surface-protein encoding genes showed better discriminatory power than RAPD analysis and opined that this was a sensitive and reproducible typing method and would allow rapid global comparisons between laboratories.

2.8.3.6.7 Whole cell and membrane protein separation

Cell membranes of *Mycoplasma* were disrupted by an osmotic lysis and the membrane proteins were separated electrophoretically in polyacrylamide gels. The electrophoretic patterns of membrane proteins were highly specific for the different *Mycoplasma* strains examined and the use of this method could prove the identity or dissimilarity of *Mycoplasma* strains (Razin and Rottem, 1967).

Shirvan *et al*. (1982) described a method for the separation of membrane proteins of *M. gallisepticum* by an alkaline lysis method and found it to be free of cytoplasmic proteins.

2.8.3.6.8 Estimation of protein concentration

A standard method for estimation of protein concentration was described by Lowry *et al*. (1951). The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The method was found to be very sensitive for low concentrations of proteins.

2.8.3.6.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The standard protocols for deducing the protein profile of the samples had been described by Laemmli (1970).

Gel electrophoresis was used as early as 1968 for speciation of avian mycoplasmas. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was found to be a useful procedure in epidemiological and other studies where minor but unique difference in protein patterns may be used to identify a particular strain of *M. gallisepticum*.

Polyacrylamide gel electrophoresis of whole cell proteins was first used for the identification of *Mycoplasma* species by Razin and Rottem (1967).

Daniels and Meddins (1973) showed that SDS-PAGE yielded much sharper separation of protein bands than the acidic gel system.

Strains of *M. gallisepticum* isolated from chicken flocks in which the response to infection was considered atypical were compared with selected reference by polyacrylamide gel electrophoresis. The electrophorectic patterns were similar substantiating the serotypic identity of the strains. No significance could be attached to the minor variations observed (Rhoades *et al*. 1974).

Khan *et al*. (1987b) observed major differences in protein banding pattern between three of mycoplasmas studied namely *M. iowae* strain 695, *M. meleagridis* strain A529 and *M. gallisepticum* strain S6 and A 5969.Various strains of *M. gallisepticum* were examined by SDS-PAGE. Minor but distinct and reproducible differences in protein banding patterns were detected between strains, which included the vaccine F strain from various sources, an atypical (variant strain) and the standard (A5969) strains. The protein banding patterns in the lowest part of the gel (approximately below 65 kilo Dalton (kDa) level) of all strains of *M. gallisepticum* resembled each other closely and these could be used to identify this species. On the other hand minor but distinct variation among *M. gallisepticum* strains were detected, particularly around the 68 kDa level.

In one dimensional SDS-PAGE, considerable antigen profile variability has been reported amongst mycoplasmas belonging to the same species (Andersen *et al*., 1987; Stadtlander *et al*., 1991; Avakian *et al*., 1991; Stadtlander and Watson, 1992).

Strains of *M. gallisepticum* differed in their SDS-PAGE profiles (Thomas and Sharp, 1988).

SDS-PAGE and RFLP methods were especially useful for identification of vaccine strains of *M. gallisepticum* and for epidemiological investigations of *M. gallisepticum* outbreaks (Khan *et al*., 1987b; Thomas *et al*., 1991; Ley and Yoder, 1997).

The poly acrylamide gel electrophoresis was an effective technique which allowed direct comparisons to be made between different protein samples run on the same gel (Duffy *et al.*, 1998).

Mycoplasma gallisepticum strains (wild type S6 (208) and a vaccine type F-810) grown in Frey's and Hayflick's media were analyzed on SDS-PAGE by Ferraz *et al*. (2000).No visual change in the protein profile of these strains were observed regardless of media composition used although polyacrylamide gel electrophoretograms showed minor differences when densitometer traces of the gel were compared. Both strains were easily differentiated on SDS-PAGE analysis by a peptide band of p75 specific for *M. gallisepticum* F-K810 strains used as vaccine.

Pulsed-field gel electrophoresis (PFGE) and RAPD analysis were used to compare 21 *M. gallisepticum* strains and five *M. imitans* strains. Each strain of *M. gallisepticum* typed by PFGE and RAPD methods was genetically quite unique and RAPD and PFGE fingerprinting enabled strain characterization. Thus, PFGE and RAPD could be used to investigate disease outbreaks in vaccinated flocks or for epidemiological tracking. For *M. gallisepticum*, the *in vitro*, *in-ovo* and *in-vivo* reproducibility of RAPD and PFGE was 100 per cent. The RAPD drawback was the inconsistent band intensity complicating the interpretation of patterns, while the PFGE limit was its low typability (86 per cent). Thus, these two molecular typing methods seemed complementary for *M. gallisepticum* epidemiological studies (Marois *et al*., 2001).

Mycoplasma gallisepticum isolates were analyzed by Triton X-114 phase partitioning and SDS-PAGE to determine phenotypic profiles by Papazisi *et al*. (2002).

Studies conducted by Ferraz and Danielli (2003) showed that SDS-PAGE gel profiles of vaccine and wild type *M. gallisepticum* strains were similar. A 64 kDa

peptide was observed in all strains of *M. gallisepticum*. They also found that the major difference among the strains tested was due to the detection of a 75 kDa band exclusively in *M. gallisepticum*-F but not in *M. gallisepticum* S6 or weakly detected in *M. gallisepticum* Ts-11. These results confirmed the phenotypic diversity between F and S6 *M. gallisepticum* strains.

2.8.3.6.10 Immunoblot

Pooled chicken antisera from 33 and 77 days post *M. gallisepticum* strain R contact-exposure were reacted with cell protein of 19 *M. gallisepticum* strains. These pooled antisera reacted with more protein and with greater intensity to reference strains (R, PG31, S6, and A5969) and nine field strains than they did with six other field strains including three (503,703 and730) that have been described as serological variants. Following extraction with Triton X-114 majority of immunogenic *M. gallisepticum* partitioned into detergent phase indicating that they are integral membrane proteins and this included p 64, p 56 and p 26. *M. gallisepticum* p 56 was detected by immunoblot in 18 of 19 strains suggested that it could serve as antigen for serological tests. The membrane protein p 26 was evidenced in13 of 19 strains. Hyper immune sera to p 64 reacted with a 64 kDa protein in 19 *M. gallisepticum* strains but did not react with seven other avian *Mycoplasma* species (Avakian *et al.*, 1991).

Avakian and Kleven (1990b) demonstrated the presence of major and minor immunogenic antigens of *M. gallisepticum* membranes by western blot.

Barbour and Newman (1990) compared *M. gallisepticum* subunit and whole organism vaccines adjuvanated with multilamellar positively charged liposomes or oil-emulsion by western blotting. The chicken sera were used in western immunoblotting against whole *M. gallisepticum* polypeptides. Vaccination with the subunit (*M. gallisepticum* adhesin) bacterin containing positively charged liposomes resulted in antibody response specific to adhesion band (75 kDa) three weeks post the first and second vaccination. However, cross reactions of the same antibodies occurred to *M. gallisepticum* proteins of 85 kDa (three weeks after the first vaccination) and 56 kDa (three weeks after the second vaccination). Vaccination with whole *M. gallisepticum* proteins containing positively charged liposomes resulted in significant immunopotentiation of antibodies against low molecular weight polypeptides of *M. gallisepticum* (< 48 kDa).

Jan *et al*. (1996) identified *M. gallisepticum* membrane proteins p 52, p 67 and p 77 as distinct antigens by western blotting and crossed immunoelectrophoresis.

2.9 Serological tests

Serological procedures are useful for flock monitoring in *M. gallisepticum* control programmes and to aid in diagnosis when infection is suspected. The most commonly used tests are serum plate agglutination (SPA), HI and ELISA though several others have been described such as radioimmunoassay, micro IF and IP assay.

2.9.1 Serum plate agglutination

Probably SPA is the most commonly used test using commercial stained antigen. Non specific reactors, however, may occur in some flocks infected with *M. synoviae*, or those recently been vaccinated with oil-emulsion vaccines and/ or tissue-culture vaccines originated against various organisms (Roberts, 1970; Cullen and Timms, 1972; Glisson *et al*., 1984; Yoder, 1989). Certain non specific SPA reactions may be reduced by producing SPA antigen in medium containing liposomes instead of serum (Ahmad *et al*., 1988) or diluting the test serum (Ross *et al*., 1990). SPA test is quick, relatively inexpensive, and sensitive (Ley and Yoder, 1997).

The Serum Plate Agglutination test is not suitable for detecting egg yolk antibodies or maternally-derived antibodies in young poultry because such antibodies are mainly IgG and the test detects mainly IgM antibodies (Bradbury, 2001).

2.9.2 Haemagglutination inhibition (HI)

Mycoplasma gallisepticum and *M. synoviae* are capable of haemagglutinating avian red blood cells and specific antibodies in sera cause inhibition. The HI test requires a satisfactory haemagglutinating antigen, washed fresh chicken RBC's and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the *Mycoplasma* cells in PBS.

A study conducted by Adler and Da Massa (1967) found that formalin treated *M. gallisepticum* antigens haemagglutinated fresh and formalinized chicken erythrocytes and they retained this haemagglutinating ability for about seven months.

Krogsgaard-Jensen (1972) investigated the influence of different factors affecting the sensitivity of the indirect haemagglutination test and found that the

greatest sensitivity was obtained with fresh erythrocytes than with formalinized erythrocytes and the sensitivity could be increased by performing the sensitization at a lower pH.

Chhabra and Goel (1981) studied the cellular, humoral, and local immune responses of chickens to *M. gallisepticum* infection at weekly intervals for 10 weeks. A cellular response was indicated by significant LMI, demonstrated as early as one week post infection, but the maximum LMI of 36 per cent was observed at seven weeks post infection. The antibody response, as determined by the HI test, was found to be provoked very early in infection and the mean HI titer peaked seven weeks post infection. A highly positive correlation ($p<0.05$) was found between LMI percentages and mean HI titers. In the infected chickens a significant elevation of mean serum immunoglobulin IgG level was noted from the first week post infection onwards and this correlated well with the rise of HI antibodies.

Lin and Kleven (1984) evaluated the sensitivity and specificity of the microagglutination (MA), SPA and HI tests in chickens infected with *M. gallisepticum*, *M. synoviae*, or *Pasteurella multocida* or inoculated with bacterins prepared from *Staphylococcus aureus* or *Erysipelothrix rhusiopathiae*. Of the three tests evaluated, the HI test had the highest specificity, but it was the least sensitive. Both the MA and SPA tests were more sensitive than the HI test but lower in specificity. MA was less sensitive in detecting antibodies against heterologous *M. gallisepticum* strains when compared to the SPA test.

Talkington *et al*. (1984) evaluated an indirect ELISA with membrane derived sodium dodecyl sulphate–solubilized preparation without been and been passed through an immunoadsorbent column containing rabbit anti-medium antibodies as antigens and found that ELISA was more sensitive with the former mode of antigen preparation.

Studies conducted by Piela *et al*. (1984) revealed that egg yolk prepared by chloroform extraction and low speed centrifugation would prove an alternative to serum for antibody determination by ELISA for *M. gallisepticum.*

Haemagglutination inhibition test was found to be useful to confirm reactors detected by SPA, though it was time consuming and the reagents were not commercially available (Kleven *et al*., 1988; Dingfelder *et al*., 1991).

Kleven (1994) and Mendonça *et al*. (2001) opined that HI interpretation should be standardized and positivity for *M. gallisepticum* and *M. synoviae* should consider a cut-off point of 1:40, as previously suggested for *M. gallisepticum* since HI titres of 1:40 were fairly common in most of the tested birds in observational studies (Nascimento *et al*., 1999).

Ewing *et al*. (1996) compared HI assay and a new affinity-purified ELISA for detection of antibody to *M. gallisepticum* for use as confirmatory tests for the National Poultry Improvement Plan program. Results from the study showed that there were no differences between ELISA and HI as confirmatory tests in populations with a low and high prevalence of *M. gallisepticum* infection. However, ELISA was superior to HI in a population with moderate levels of *M. gallisepticum* infection.

Kleven (1998) suggested that flocks infected with some strains of *M. gallisepticum* strains might not give positive results with the HI test.

Abou El Makarem (2003) collected a total of 150 serum samples from chickens showing respiratory signs, and 150 blood samples were collected from experimentally infected chicken, for the detection of *M. gallisepticum* antibodies in serum by different serological tests. Results suggested that SPA could be used as an easy and simple screening test at one day to two weeks of age, while HI test and ELISA tests could be used for confirmation. ELISA could be used in birds more than two weeks of age.

In Haemagglutination Inhibition test for the flock screening of *Mycoplasma* antibodies, serum giving non-specific reactions to SPA usually does not give a positive reaction in HI using HA antigen and for confirming positive reactions sera should be taken two to three weeks after the infection (OIE 2008).

Haemagglutination Inhibition titers equal or higher than 1:80 are regarded as positive and titers between 1:20-1:40 are suspicious, whereas negative titers are below 1:20 (OIE, 2008).

2.9.3 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay was found to be a sensitive and specific test for the detection of antibodies against *M. gallisepticum* in flock screening tests (Kleven, 1998; Avakian and Kleven, 1990b).

Selected immunogenic proteins of *M. gallisepticum* strain R and *M. synoviae* purified from SDS-PAGE gels were evaluated as potential antigens for an ELISA test by Avakian and Kleven (1990a). They could observe that the *Mycoplasma gallisepticum* antigen p 64 detected antibodies three days after the Serum Plate Agglutination test and seven days before the Haemagglutination Inhibition test.

According to Stipkovits and Kempf (1996) ELISA was the most promising technique to substitute serum agglutination reaction (SAR) or even HI, but according to a previous field study by Nascimento *et al*. (1993), ELISA and SAR evidenced negative results whereas HI and PCR were positive. On the contrary, SPF chickens exposed to live vaccines (MG-F, MGTS-11 and 6/85) and a low virulent *M. gallisepticum* strain (*M. gallisepticum* -70) were positive in ELISA as early as 21 days post exposure. Positivity for *M. gallisepticum* in exposed chickens was detected by SAR and HI only at 35 and 42 days post-exposure, respectively, whereas positivity for the other *M. gallisepticum* strains (TS11 6/85 and *M. gallisepticum* -70) in SAR and HI could be detected only after challenge with *M. gallisepticum*-R, at 63 days post exposure (Nascimento *et al*., 1993).

May and Branton (1997) developed an antigen-based ELISA for the rapid identification of *M. gallisepticum* and *M. synoviae* and compared it with the conventional FAT. They observed that ELISA was able to identify the strain of *Mycoplasma* although it did not identify as many isolates as the fluorescent antibody procedure.

2.9.4 Latex agglutination test (LAT)

Latex agglutination test is a simple macro agglutination test which combines sensitivity with low cost and ease of application in the field conditions, without the need for any trained specialist or equipment.

Morton (1966) adsorbed human, avian, swine, and goat cells to latex particles and then these were agglutinated by immune sera. He found that in the sera from rabbits undergoing immunization with *Mycoplasma* antigens, the presence of anti-*Mycoplasma* antibodies was detected much sooner in the *Mycoplasma*-latex agglutination reaction test than in the agar-gel diffusion reaction and the growth inhibition tests. He also found that latex particles showed excellent uniformity of behaviour and stability during storage and testing.

The standardization, application and usefulness of latex agglutination test to detect *M. pneumonia* antibody were investigated and compared with tetrazolium reduction inhibition and complement fixation and found that latex agglutination procedure was comparable with complement fixation and metabolic inhibition tests for evaluating vaccine efficacy (Kende, 1969).

Rurangirwa *et al.* (1987) sensitized latex beads with a polysaccharide isolated from a F38 culture supernatant and used in a slide agglutination test to detect serum antibodies in goats with contagious caprine pleuropneumonia. The results were compared with that of a complement fixation test. Besides being more sensitive than complement fixation, the latex agglutination test can be performed in the field using undiluted serum or whole blood and a result obtained within two minutes.

A protocol for the sensitization of latex beads with antigen using carbonate-bicarbonate buffer was described by Ramadass *et al*. (1999). He indicated that the method was found to be very sensitive and the sensitized beads could be stored at 4°C for about four months and at room temperature for two months, without loss of antigen activity.

Ramadass *et al*. (2007) developed a LAT using *M. gallisepticum* PG 31 strain and compared it with an ELISA. They found that LAT and ELISA were of equal sensitivity.

2.9.5 Other diagnostic tests

Dot immunobinding assay was also used for the detection of antibodies against *M. gallisepticum* and *M. synoviae* by Avakian and Kleven (1990b) and Cummins *et al*. (1990). They observed that immunobinding assay was at least 20 times more sensitive in detecting antibodies to *M. synoviae* and 75 times more sensitive in detecting antibodies for *M. gallisepticum* than HI test.

Radi *et al*. (2000) developed an avidin-biotin immunoperoxidase test using polyclonal antibodies to facilitate the rapid identification of *M. gallisepticum* in respiratory tissue of turkeys. They suggested the use of multiple monoclonal antibodies directed against several different epitopes specific to the cell membrane of *M. gallisepticum* to improve the specificity of the test.

A single radial haemolysis test was used for the detection of antibodies to *M. gallisepticum* in sera of chickens by Khokhar and Prasad (1983) and they opined that the test was at least as sensitive as HI test.

Flow cytometry was utilized as a tool for detection of epitope diversity of F strain of *Mycoplasma gallisepticum* by Brown *et al*. (1997).

2.10 Tests for cell mediated immune response

Studies on cell-mediated immunity (CMI) in *Mycoplasma* infections in several species have indicated that CMI was involved in the development of immunity to mycoplasmal respiratory tract diseases (Fernald *et al*., 1972; Roberts, 1973; Taylor *et al*., 1974; Biberfeld *et al*., 1974; Kristensen *et al*., 1981; Gaunson *et al*., 2000).

2.10.1 Leukocyte migration inhibition test (LMIT)

The principle behind leukocyte migration inhibition is that the sensitized lymphocytes from an immune animal when stimulated by specific antigen, release factors that are capable of inhibiting the migration of macrophages and blood leukocytes. The test doesn't require any expensive equipment and the advantage of this technique is the plates can be fixed, stained and evaluated at any time and/or save as a permanent record.

Timms (1974) suggested that LMIT could be used to evaluate the cell mediated immunity against infectious diseases.

Studies conducted by Naot *et al*. (1977) indicated that slight mitogenic activity was associated with various *Mycoplasma* species, which was responsible for the stimulation of control lymphocytes that are not stimulated with mitogen during leukocyte migration inhibition test wheras, the studies by Nicolet *et al*. (1980) showed that the mitogenic effect could be attributed due to contaminating serum components in *Mycoplasma* preparations.

Leukocyte migration inhibition test was used as an *in vitro* measure of cell-mediated immune response (Carson *et al*., 1977; Nyindo *et al*., 1980; Azadegan *et al*., 1981).

Direct migration inhibition assay under agarose was proved to be a convenient, rapid and easily reproducible method for leukocyte migration inhibition assay (Clausen, 1971; Bendixen, 1977; Azadegan *et al*., 1981).

Chhabra and Goel (1981) used LMIT for the assessment of cellular immune response in *M. gallisepticum* infection and found that significant leukocyte migration

inhibition was demonstrated as early as one week post infection (PI), but the maximum LMI of 36.4 per cent was observed at seven weeks PI.

The importance of cell-mediated immune response in establishing a protective immunity against *M. gallisepticum* infection was documented by Tiwary and Goel (1986) in a study conducted on thymectomized birds, which showed an impaired resistance to *M. gallisepticum* infection,

A method for the separation of leukocytes from peripheral blood of chicken using a one-step Percoll density gradient was described by Mills and Wilcox (1993). They found that lymphocytes were found predominantly in fraction A and erythrocyte contamination was negligible.

The comparative efficacy of cell mediated immune response among different *Mycoplasma mycoides* subsp. *capri* immunogens was assessed by Rajneesh and Srivastava (1993) by using LMIT and delayed type hypersensitivity (DTH) and found that highest response in LMIT was observed at sixth week post vaccination.

The leukocyte migration inhibition test for the evaluation of cell mediated immunity was developed by Al-Shahery *et al*. (2008) for use in chicken vaccinated with Newcastle disease and others infected with local virulent strain of Newcastle disease virus. Results indicated that LMIT was reproducible and easy to be performed. Serum antibody titres were determined to study the correlation between HI titres and LMI level using a HI-macro assay. Results of the study indicated that cellular immunity was also important for the evaluation of immune response in addition to humoral immunity.

2.10.2 Blastogenic proliferation assay

A tetrazolium salt had been used by Mosmann (1983) to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detected living, but not dead cells and the signal generated was dependent on the degree of activation of the cells. This method could therefore be used to measure cytotoxicity, proliferation or activation. The results could be read on a multiwell scanning spectrophotometer (ELISA reader) and showed a high degree of precision. No washing steps were used in the assay. The main advantages of the colorimetric assay were its rapidity and precision, and the lack of any radioisotope. He used this assay to measure proliferative lymphokines, mitogen stimulations and complement-mediated lysis.

Bounous *et al*. (1992) compared the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay with the conventional tritiated thymidine deoxyriboside (3H-TdR) incorporation for assay of lymphocyte blastogenesis using mononuclear cells isolated from the spleens of specific-pathogen-free chickens. The study was undertaken in an effort to simplify methods for assessing avian lymphocyte proliferation, specifically for evaluating response to mitogens or for indirect measurement of T-cell growth factors. The results from stimulated cells in both assay methods were significantly different from results from the control cells, and the MTT assay results regressed in a significant linear manner on counts from 3H-TdR incorporation. On this basis, the MTT assay was a valid test for evaluation of lymphocyte proliferation of chicken splenocytes.

Assessment of cellular immune response by an *in-vitro* methyl- thiazolyl-tetrazolium (MTT) assay was found to be a simple and convenient method for assessing the cell activation rate and growth, obviating the need for radioactive material for the assay (Saravanan *et al*., 2003).

2.11 Estimation of Infective dose

The study conducted by Mc Martin and Adler (1961) indicated that the minimum CFU of *M. gallisepticum* required to produce air sac lesions was 10^2.

Nunoya *et al*. (1987) indicated that, for assessing the efficacy of *M. gallisepticum* vaccines by challenge studies, calculation of infective dose is essential. He found that scoring of lesions in trachea grossly and histopathological scoring of trachea were most suitable methods.

Adler and DaSilva (1970) studied the protective effect of an inactivated *Mycoplasma gallisepticum* bacterin against air sac infections with a challenge dose of 10^7 to 10^9 CFU.

Standard protocols for the histological preparation of tracheal specimens were described by Sheehan and Hrapchak (1980).

2.12 Immunization

Mycoplasma gallisepticum infection proved to be difficult to eradicate for many reasons. The in-house spread of *M. gallisepticum* is relatively rapid, thus necessitating the elimination of an entire flock rather than the individual reactor. A flock infected with *M. gallisepticum* is infected for life and will shed the organism for

entire life. Various techniques used to reduce egg transmission, such as heat treatment at 46°C for 12-14 hours or, more efficiently, by antibiotic treatment, either by *in-ovo* injection or, by dipping eggs in antimicrobial solutions (Nascimento and Nascimento, 1994; Stipkovits and Kempf, 1996) resulted in selection for more resistant *M. gallisepticum* survivors.

The failure to control *M. gallisepticum* infection on multiple production sites has led to the development of a number of vaccines, although vaccination is recommended only where field exposure is considered inevitable. Currently, inactivated and live attenuated vaccines are available to poultry farmers. Although inactivated vaccines (bacterins only for *M. gallisepticum*) were not well accepted in the past, they are often preferred today, mainly because there is no risk of infection and because they do not affect *M. gallisepticum* detection. Live and killed vaccines are marketed and regardless of the antigenic variation seen among *M. gallisepticum* strains, vaccination with a single strain seems to be effective (Bradbury, 2001).

2.12.1 Live vaccines

The live F strain *M. gallisepticum* vaccine was used extensively in chicken especially in multiple-age laying complexes and was successful in protecting against egg production drops (Branton and Deaton, 1985; Mohammed *et al*., 1987; Branton *et al*., 1988).

The F strain vaccine provided some protection to broilers from airsacculitis, following aerosol challenge with virulent R strain; however F strain vaccination did not prevent colonization by the challenge strain of *M. gallisepticum* (Lin and Kleven, 1982b; Levisohn and Dykstra, 1987).

Lin and Kleven (1982a) found that F strain could be transmitted through the egg and from bird to bird. Also vaccinal F strain was pathogenic in turkeys following experimental infection.

Ley *et al*. (1993) reported that F strain had been associated with *M. gallisepticum* outbreaks in meat and breeder turkeys under field conditions.

The live vaccines ts-11 and 6/85 have been reported to possess little or no virulence for chickens and turkeys (Evans and Hafez, 1992; Ley *et al*., 1997).

Whithear *et al*. (1990) and Abd-el-Motelib and Kleven (1993) found that ts-11 and 6/85 live vaccines offered less protective effect. The ts-11 vaccine organism persisted in the chicken for long periods, but the 6/85 strain vaccine did not persist and might be difficult to recover the organism after a few weeks. The strain 6/85 has been isolated from turkeys showing clinical disease in several instances (Kleven *et al*., 2004) and also from unvaccinated commercial layers housed near vaccinated birds (Throne Steinlage *et al*., 2003).

The strain ts-11 was transmissible from vaccinates when birds were in direct contact with each other and or shared a common feeder and waterer, but transmission was unlikely with indirect contact (Ley and Yoder, 1997).

The F strain displaces wild-type *M. gallisepticum* more efficiently than either ts-11 or 6/85, but ts-11 has been used to eradicate F strain *M. gallisepticum* from a commercial egg-production site (Turner and Kleven, 1998).

2.12.2 Inactivated vaccines

Yoder (1978) indicated that formalin at a final concentration of 0.1 per cent was very effective for the inactivation of *M. gallisepticum* for bacterin preparation.

Several investigators worked on killed oil adjuvanated *M. gallisepticum* vaccines (bacterins) to protect birds from infection and to lessen loss caused by *M. gallisepticum* and some found that such bacterins could protect layers from drop in egg production (Hildebrand *et al*., 1983., Yoder and Hopkins, 1985) and broilers from airsacculitis (Yoder *et al*., 1984; Karaca and Lin, 1987) while others did not give much protection in multiple–age site commercial layer flocks with endemic *M. gallisepticum* infection (Khan *et al*., 1986).

Vaccination with bacterins could reduce, but usually not eliminate, colonization by *M. gallisepticum* following challenge (Talkington and Kleven, 1983; Yoder and Hopkins, 1985; Yagihashi and Tajima, 1986).

Vaccination with an oil-emulsified *M. gallisepticum* bacterin available for commercial use in United States and similar products induced high levels of antibody response, prevented clinical signs and pathological lesions, and reduced the number of organisms colonizing the respiratory tract (Yoder, 1978; Panigraphy *et al*., 1981; Hildebrand *et al*., 1983; Yoder *et al*., 1984; Talkington and Kleven, 1985; Yagihashi *et al*., 1986).

The bacterin vaccines were also reported to reduce production losses and the rate of egg-transmission of *M. gallisepticum* (Glisson and Kleven, 1984; 1985; Yoder and Hopkins, 1985). However, little is known about the nature of immunity elicited by the bacterins.

Studies conducted by Yagihashi *et al*. (1986) on immunity induced with an aluminium hydroxide adsorbed *M. gallisepticum* bacterin in chicken suggested that vaccination with inactivated bacterin might be an effective means for controlling *M. gallisepticum* infection.

Barbour and Newman (1990) conducted a preliminary study on the efficacy of *Mycoplasma gallisepticum* vaccines containing different adjuvants in laying hens with a challenge dose of 1.3×10^5. The study indicated that vaccination with *M. gallisepticum* adjuvanted to multilamellar positively charged liposomes or oil emulsion prevented a significant drop in egg production following challenge.

2.13 Adjuvants

The adjuvants have been since long used along with inactivated vaccines for the augmentation of immune response in host against various diseases as it can enhance the immune response when administered with specific antigens than when the antigens are injected alone (Dalsgaard, *et al*., 1990; Edelman, 1980).

2.13.1 Aluminium hydroxide

Warren *et al*. (1968) described protection in chickens from formalin inactivated bacterins with aluminium hydroxide gel as adjuvant when protection against airsacculitis was used as indicative of protection.

Studies by Walls (1977) and Gupta *et al*. (1995) suggested that alum salts work by causing the formation of an antigen depot at the inoculation site from where antigen is released slowly. The trapping of soluble antigen in the alum gel may also increase the duration of antigen interaction with the immune system. Other mechanisms of action involve complement, eosinophil and macrophage activation and increased efficiency of antigen uptake by antigen presenting cells.

Harry (1978) evaluated the serological response of chickens vaccinated with several lots of inactivated oil emulsified *M. gallisepticum* vaccines prepared from R strain. The vaccines were inactivated with beta-propiolactone or formalin and

aluminium hydroxide was used as the adjuvant. There was little difference in HI response of young broiler and egg-laying-type chickens injected by either the intra footpad or subcutaneous routes with 0.5 ml doses of vaccine inactivated by either formalin or beta-propiolactone. Two doses eight weeks apart produced higher HI titres over a longer period than did a single dose.

Alum salts are relatively weak adjuvants and rarely induce cellular immune responses (Schirmbeck *et al*., 1995; Traquina *et al*., 1996; Brewer *et al*., 1996).

Comparative studies in humans and animals showed that aluminum was a weak adjuvant for antibody induction to recombinant protein vaccines and induced a Th2, rather than a Th1 response (Gupta, 1998).

The findings of Ulanova *et al*. (2001) suggested that aluminum hydroxide directly stimulates monocytes to produce pro-inflammatory cytokines activating T cells. Activated Th2 cells release IL-4, which in turn can induce an increase in the expression of MHC class II molecules on monocytes. The increase in the expression of antigen-presenting and co-stimulatory molecules leads to enhanced accessory functions of monocytes. These properties of aluminum hydroxide observed *in vitro* may explain its potent *in vivo* adjuvant effect to enhance antibody production.

Petrovsky and Aguilar (2004) described that aluminium hydroxide had little ability to stimulate Th1 immune responses, that was important for protection against many pathogens.

2.13.2 Saponin

A study was carried out by Mulira *et al*. (1988) to determine the efficacy of different adjuvants in enhancing antibody response to sonicated F-38 antigens. Goats were immunized against CCPP using antigens incorporated in Freund's incomplete adjuvant (FIA), saponin, aluminium hydroxide gel and Phosphate buffered saline (PBS) respectively. The goats were challenged four months after immunization to assess their immune status. Saponin and FIA were similar in their immune potentiation ability and were superior to aluminium hydroxide. As IFA has been considered unsuitable for use in food animals, saponin may prove valuable in vaccination of goats against CCPP caused by *Mycoplasma* strain F-38.

The ability of a saponin adjuvant, QS-21, to induce ovalbumin (OVA)-specific, class I MHC Ag-restricted cytotoxic T lymphocyte (CTL) was investigated

by Newman *et al*. (1992) in C57BL/6 mice, using different forms of soluble OVA and OVA adsorbed onto alum as immunogens. Ag-specific CTL responses were produced only if the QS-21 adjuvant was used. Similar responses were induced using alum-adsorbed OVA when mixed with the QS-21 adjuvant but not when used alone. The ability of the QS-21 adjuvant to induce class I MHC Ag-restricted CTL after immunization with soluble proteins is a characteristic unique to saponin adjuvants.

Kensil (1996) reviewed that naturally occurring triterpene glycosides (saponins) from *Quillaja saponaria* have considerable adjuvant activity. Adjuvant functions include stimulation of high levels of antibody to T-dependent and T-independent antigens, induction of mouse IgG1, IgG2b, and IgG2a isotypes, and induction of cytotoxic T lymphocyte responses.

Walduck and Opdebeeck (1996) reported that the use of saponin as a second adjuvant improves the immunogenic properties of aluminium hydroxide.

Saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity (Oda *et al*., 2000).

Iqbal (2007) reviewed that saponin was a promising adjuvant for vaccine production against animals and man, as it was able to stimulate humoral and cell mediated immune responses.

Cellular and humoral immune responses of dogs to a candidate vaccine, composed of *Leishmania braziliensis* promastigote protein plus saponin as adjuvant, have been investigated as a pre-requisite to understanding the mechanisms of immunogenicity against canine visceral leishmaniasis **(**CVL) by Giunchetti *et al*. (2007). The candidate vaccine elicited strong antigenicity related to the increases of anti-Leishmania IgG isotypes, together with higher levels of lymphocytes, particularly of circulating CD8 (+) T-lymphocytes and *Leishmania chagasi* antigen-specific CD8 (+) T-lymphocytes.

De la Fe *et al*. (2007) conducted a field trial of two dual vaccines against *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp *mycoides* in goats and found the use of combined aluminium hydroxide and saponin adjuvanated vaccines elicited better immune responses, than aluminium hydroxide alone.

2.14 Preservation of the isolate

Kelton (1964) reported that lyophilized cultures of *Mycoplasma* were preserved up to four years.

Harry (1964) could obtain viable mycoplasmas after fourteen years of storage in the freeze dried state at 4° C, whereas, the broth cultures were viable when sub cultured after three to four years of storage at -30° C.

Materials and Methods

MATERIALS AND METHODS

3.1 MATERIALS

Molecular grade chemicals and biologicals used in the study were obtained from Bangalore Genie, India unless and other wise mentioned. The analytical grade chemicals and media were purchased from Difco, France; Sigma, Germany; Hi Media, Mumbai and Sisco Research laboratory, Mumbai. Glass ware of Borosil and Riviera brands and Tarsons brand plastic ware were used in the study.

3.1.1. Samples for the Study

A total of 50 samples were obtained from sick/apparently healthy birds of different age groups from University Poultry Farm, Mannuthy, poultry farms in different parts of Kerala and birds brought for disease diagnosis to the Department of Veterinary Microbiology, birds necropsied in Centre of Excellence in Pathology, College of Veterinary and Animal Sciences, Mannuthy. Birds showing ailments such as nasal discharges, sinusitis, laboured breathing, conjunctivitis and/reported reduction in egg production were specifically included in the collection of samples for the study. Tracheal and conjuctival swab samples were collected from the birds, depending on the type of symptoms exhibited by the birds. In case of necropsied birds, lung samples and air sac materials were also collected. Reference strain (6/85) was obtained from Department of Microbiology, Veterinary College and Research Institute, Namakkal.

3.1.2 Detection of *M. gallisepticum* from clinical samples

3.1.2.1 Mycoplasma Genus-Specific Polymerase Chain Reaction (Genus-PCR)

3.1.2.1a Mycoplasma genus-specific primers GPO3 and MGSO (Marois et al., 2000)

5′GGG AGC AAA CAG GAT TAG ATA CCC T 3′: GPO3 (forward)

5′ TGC ACC ATC TGT CAC TCT GTT AAC CTC 3′: MGSO (reverse)

Primers were obtained as custom made 100 mM standard desalted oligonucleotides with OD-260 values of 17.2 and 18.1 respectively from Genetrix, Banglaore.

3.1.2.2 Mycoplasma gallisepticum Species Specific Polymerase Chain Reaction (Species-PCR)

3.1.2.2a Mycoplasma gallisepticum Species Specific primers MG IGSR F and MG IGSR R (Raviv et al., 2007)

5′ GTA GGG CCG GTG ATT GGA GTT A 3′: MG IGSR F (forward)

5′ CCG GTA GCA TTT CGC AGG TTT G 3′: MG IGSR R (reverse)

Primers were obtained as custom made 100 mM standard desalted oligonucleotides with OD-260 values of 17.2 and 18.1 respectively from Genetrix, Banglaore.

3.1.2.3 Deoxy ribonucleotide Triphospates *(dNTP)*

dNTP mix (10 mM) containing 2.5 mM each of dATP, dCTP, dGTP and dTTP.

3.1.2.4 Taq DNA Polymerase (Genetrix, Bangalore)

At a concentration of one unit per microlitre

3.1.2.5 PCR Reaction Buffer (10 ×)

Contains 500 mM KCl, 100 mM Trishydrochloride (pH 9) and 0.1 per cent gelatin in a total volume of 1ml.

3.1.2.6 Magnesium Chloride

At a strength of 25 mM

3.1.2.7 Extraction of DNA

3.1.2.7a Phosphate Buffered Saline (PBS) Stock (10 ×) pH 7.2

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate	11.32 g
Potassium dihydrogen phosphate	2 g

Distilled water up to	1000 ml

3.1.2.7b Working solution (1 ×)

PBS stock	10 ml
Distilled water up to	100 ml

3.1.2.8 Detection of amplified products

3.1.2.8a Submarine gel electrophoresis

i. Agarose

ii. EDTA Stock Solution (0.5 M) pH 8.0

Sodium EDTA. $2H_2O$	186.1 g
Distilled water up to	1000 ml

The solution was sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

iii. Tris Borate EDTA Buffer (TBE) pH 8.2

a. Stock solution (10 ×)

Tris base	108.0 g
Boric acid	5.0 g
EDTA (0.5 M, pH 8.0)	40 ml
Triple distilled water up to	1 litre

b. Working solution (1×)

TBE stock solution	10 ml
Triple distilled water to make	100ml

iv. Ethidium Bromide Stock Solution

Ethidium bromide	10 mg
Triple distilled water up to	1 ml

The solution was mixed well and stored in amber colored bottles at 4°C.

v. Gel Loading Buffer (6×)

a. Sucrose solution

Sucrose	40 g
Triple distilled water up to	100ml

b. Tracking dyes

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g

The gel loading buffer was prepared by carefully dissolving the tracking dyes in sucrose solution and kept at 4°C.

vi. DNA Molecular Size Marker

100 bp DNA Ladder consisting of 10-double stranded DNA segments of 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 bp.

3.1.3 Indirect Fluorescent Antibody Test

3.1.3a Hyper immune sera (3.2.7)

3.1.3b Fluorescent isothiocyanate conjugated anti-rabbit IgG (Bangalore Genei, India)

Used at the dilution of 1:200

3.1.4 Immunoperoxidase test

3.1.4.1 Horse raddish peroxidase conjugated anti-rabbit IgG (Bangalore Genei, India)

Used at the dilution of 1:2000

3.1.4.2 Substrate solution

3, 3'Diaminobenzidene (DAB)	9 mg
Tris (100 mM, p H 7.5)	7.5 ml
Nickel chloride (0.3 per cent)	1.5 ml
Distilled water	6 ml
Hydrogen peroxide (3 per cent)	150 µl

3.1.4.2a Tris (100 mM, p H 7.5)

Tris buffer	1.2114 g

Distilled water up to	100 ml

3.1.4.2b Nickel chloride (0.3 per cent)

Nickel chloride	30 mg
Distilled water up to	10 ml

3.1.5 Membrane protein extraction

3.1.5.1 Sodium chloride (0.25 M)

Sodium chloride	1.46 g
Distilled water up to	100 ml

3.1.5.2 Tris buffer (0.05 M, pH 8.5)

Tris buffer	0.6057 g
Distilled water up to	100 ml

3.1.5.3 RNAase (Bangalore Genei, India)

3.1.5.4 Magnesium chloride, 10 µM (SRL, India)

3.1.6 Preparation of RBC for HA/HI

3.1.6a Alsever's solution

Glucose	2.050 g
Sodium chloride	0.800 g
Sodium citrate	0.420 g
Citric acid	0.55 g
Distilled water up to	100 ml

3.1.6b PBS (***3.1.2.7b)***

3.1.7 Media for the Isolation of *M. gallisepticum*

3.1.7.1 PPLO broth base without crystal violet (Difco)

3.1.7.2 Swine serum

Blood was collected observing aseptic precautions from pigs slaughtered in the Centre of Excellence in Meat Science and Technology, College of Veterinary and Animal Sciences, Mannuthy. Serum was separated and sterilized by filtration using seitz filter and stored at –70°C in 15 ml aliquots till use.

3.1.7.3 Yeast extract

Two hundred and fifty gram of active dry baker's yeast (Difco) was suspended in one litre of distilled water, heated to boiling point, cooled and centrifuged for 20 min at 3000 × g. The supernatant was decanted and the pH of the supernatant was adjusted to 8.0 with 0.1 M NaOH. The solution was clarified by filtration through Whatman filter paper No.1 and then sterilized by filtration using seitz filter and stored –70°C in 10 ml aliquots.

3.1.7.3a Sodium hydroxide (0.1 M)

Sodium hydroxide	0.4 g
Distilled water up to	100 ml

3.1.7.4 Glucose

Ten per cent (w/v) solution of glucose was prepared by dissolving 10 g glucose in 100 ml distilled water. The pH was adjusted to 7.8 to 8 with 0.1 M NaOH and the solution was sterilized by filtration and stored at –70°C in five ml aliquots.

3.1.7.5 Thallium Acetate

Five gram of thallium acetate was dissolved in 100 ml of distilled water. The solution was sterilized by filtration and stored at –70°C in five ml aliquots.

3.1.7.6 Antibiotics

Benzyl penicillin (1000, 000 IU) was dissolved in 5 ml of distilled water. The solution was sterilized by filtration and stored at 4° C.

3.1.7.7 Phenol red

Phenol red (0.1 g) was ground in 0.1 M NaOH (2.8 ml) and made up to 100 ml with sterile distilled water. The solution was autoclaved at 121°C for 15 min at 15 lbs and stored at 4° C.

3.1.7.8 PPLO Broth

Pleuro pneumonia like organism broth base without crystal violet (1.47 g) was dissolved in 70 ml distilled water. The solution was autoclaved at 121°C for 15 min at 15 lbs and, after cooling, was fortified with the following enrichments.

Swine serum (heated at 56° C for 30 min)	15 ml

25% w/v Fresh yeast extract	10 ml
10% w/v Glucose	1 ml
5% w/v Thallium acetate	1 ml
Penicillin	1 lakh IU
0.1% w/v Phenol red solution	2 ml

3.1.7.9 PPLO Agar

One gram of PPLO agar was added to a solution of 1.47 g of PPLO broth base (without crystal violet). The mixture was autoclaved as before and kept in a water bath at 56°C. All the constituents of enrichments except phenol red were mixed separately and incubated at 56°C. The incubated enrichments were mixed carefully with the prepared agar and PPLO broth solution to avoid the production of air bubbles and dispensed into 55 mm diameter Petri dishes to a height of 5 to 6 mm. The prepared PPLO agar plates were kept for sterility check at 37°C for 48 h and the sterile plates were stored at 4°C to be used within 14 days.

3.1.8 Characterization of *M. gallisepticum* Isolates

3.1.8.1 Diene's staining technique

Diene's stain (stock solution)

Methylene blue	2.5 g
Azur II	1.25 g
Maltose	10 g
Sodium carbonate	0.25 g
Distilled water	100 ml

The solution was sterilized by filtration and stored at room temperature.

3.1.8.1a Working solution

Stock solution	1 ml
Normal saline up to	100 ml

3.1.8.2 Biochemical tests

3.1.8.2a Carbohydrate fermentation media

Heart infusion broth (Difco)	100 ml

Swine serum	10 ml
Potassium penicillin G	1000 units/ ml
Thallium acetate	0.1 per cent w/v

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

3.1.8.2b Arginine fermentation media

For fermentation of arginine, the medium was supplemented with one per cent L-arginine monohydrochloride and the pH was adjusted to 7.0.

3.1.8.3 Growth inhibition test (GIT)

Sterile filter paper discs were impregnated with 0.02 ml of undiluted hyper immune sera raised in rabbits against *M. gallisepticum* proteins (3.2.7). The discs were then dried in a Petri dish at 5°C over anhydrous calcium chloride and silica gel.

3.1.9 Protein Estimation: Lowry's Method

3.1.9.1 Solution A

Sodium hydroxide	2.8598 g
Sodium carbonate	14.3084 g
Distilled water up to	500 ml

3.1.9.2 Solution B

Copper sulphate	1.4232 g
Distilled water up to	100 ml

3.1.9.3 Solution C

Sodium tartarate	2.85299 g
Distilled water up to	100 ml

3.1.9.4 Lowry Solution (fresh; 0.7 ml/sample)

Solution A+ Solution B+ Solution C with a ratio (v/v) of 100:1:1

3.1.9.5 Folin Reagent (fresh; 0.1 ml/ sample)

Five milliliter of 2 N Folin and Ciocalteu's Phenol Reagent was mixed with five millilitre distilled water. The dilution ratio for the Folin and Ciocalteu's Phenol Reagent was 1:1, resulting in a 1 N Folin reagent.

3.1.9.6 Bovine Serum Albumin (BSA) Protein Standard Solution (fresh)

Bovine serum albumin (0.05 g) was weighed and dissolved in distilled water. The volume was made up to 500 ml. The final concentration of BSA was 100 mg/l.

Dilutions from the BSA stock (100 mg/l) for the standard curve

Volume of distilled water (ml)	Volume of stock BSA solution (ml)	Final concentration (mg/l)
10	0	0
8	2	20
6	4	40
4	6	60
2	8	80
0	10	100

3.1.10 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.1.10.1 Separating gel buffer (8 ×)

The following ingredients were dissolved in water.

Tris	36.6 g
SDS	0.8 g
Tetramethylethylenediamine dihydrochloride (TEMED)	0.232 ml

pH was adjusted with 1 N HCl to 8.4 to 8.6 and volume was made to 100 ml with distilled water.

3.1.10.2 Stacking gel buffer (8 ×)

Tris	12.11 g
SDS	3.2 g
TEMED	0.232 ml

pH was adjusted with 1 N HCl to 6.8 and volume was made to 100 ml.

3.1.10.3 Acrylamide Stock Solution (30 per cent)

Twenty nine gram of acrylamide and one gram of bis-acrylamide were dissolved in water by stirring and volume was made to 100 ml.

3.1.10.4 Sample buffer (5 ×)

Tris (1.8 g) was dissolved in 15 ml of water and pH was adjusted with 1 N HCl to 6.8. To this solution, SDS (five gram), bromophenol blue (0.0625 g) and glycerol (25 ml) were added and volume was made to 50 ml with water.

3.1.10.5 Ammonium persulphate solution (APS, 10% w/v)

Ammonium persulphate	10 g
Distilled water up to	100 ml

3.1.10.6 Electrode buffer

The following ingredients were dissolved in distilled water to make 500 ml

Tris	1.515 g
Glycine	7.2 g
SDS	0.50 g

Electrode buffer was prepared fresh.

3.1.10.7 Separating gel recipe (for 32.16 ml)

Separating gel buffer	4 ml
Acrylamide stock solution	5.34 ml
Water	22.66 ml
APS	0.16 ml

3.1.10.8 Stacking gel recipe (3.30 per cent acrylamide, for eight ml)

Stacking gel buffer	1 ml
Acrylamide stock solution	0.88 ml
Water	6.08 ml
APS	0.04 ml

3.1.10.9 Fixing solution

20 per cent (v/v) isopropanol and 10 per cent (v/v) acetic acid (for 1 to 2 h)

3.1.10.10 Staining solution

(0.175 per cent (w/v) Coomassie Brilliant Blue R-250 in 45 per cent (v/v) methanol and 10 per cent (v/v) acetic acid). Coomassie Brilliant Blue R-250 (0.875 g) was dissolved in 225 ml of methanol and 50 ml of acetic acid by stirring overnight. The volume was made to 500 ml with distilled water and the solution was filtered through Whatman No. 1 filter paper. The staining solution was stored at room temperature in an amber coloured bottle.

3.1.10.11 Destaining solution

20 per cent (v/v) methanol and 10 per cent (v/v) acetic acid (for 24 h with 3 to 4 changes), followed by 10 per cent (v/v) acetic acid (for 12 h).

3.1.10.12 Gel storage solution

7 per cent (v/v) acetic acid.

3.1.11 Western Blot

3.1.11.1 Transfer buffer

Tris base	18.2 g
Glycine	86.5 g
Methanol	1200 ml
Distilled water up to	6000 ml

3.1.11.2 Phosphate Buffered Saline with Tween-20 (PBST)

0.1 per cent Tween-20 in PBS

3.1.11.3 Blocking buffer

PBST	100 ml
Skim milk powder	5 g

3.1.11.4 Substrate solution

Tris (100 mM, pH 7.5)	7ml

Diaminobenzidine	9 mg
Nickel chloride (0.3 per cent)	1.5 ml
Distilled water	6 ml
Hydrogen peroxide (3 per cent)	150µl

3.1.12 Experimental Birds & Feed

Two hundred and seventy six chicks of five week age group (hatched out from parent stocks without any signs of respiratory illness) were purchased from University Poultry Farm, KAU, Mannuthy. Feed for the birds were purchased from University Feed Mill, KAU, Mannuthy.

3.1.13 Latex agglutination test

3.1.13a Latex beads (0.88 µm, Sigma)

3.1.13b Carbonate-bicarbonate buffer 0.06M, pH 9.6

Anhydrous sodium carbonate (Na_2CO_3)	1.15 g
Sodium bicarbonate ($NaHCO_3$)	4.13 g
Sodium azide	0.2 g
Distilled water up to	1000 ml

3.2 METHODS

3.2.1 Sample Collection

Sterile swabs made of cotton gauze were used for sampling. Just prior to sampling the cotton swabs were soaked with sterile PPLO broth and the excess broth was extruded by pressing the swab against the sides of the tube. Tracheal and conjuctival swab samples were collected from all the ailing birds. Twenty five tracheal swab samples were directly plated on to the PPLO agar plates also.

3.2.1.1 Tracheal swabs

For collection of samples from trachea, pre-soaked sterile cotton swab was carefully inserted after locating the oropharynx of a well restrained bird and the swab was pushed downwards through the tracheal tube gently, the region was swabbed and

the swab was immediately transferred back to the broth. Remaining thrust out portion of the swab beyond the tube was snipped off and the tube was screwed tightly.

3.2.1.2 Conjunctival swabs

Pre-soaked sterile cotton swabs were rubbed against conjunctiva and the swab was replaced back to the broth and the broth tube was closed tightly.

3.2.1.3 Air sac material

Air sac was collected directly into the broth by cutting it out using a pair of sterile scissors and transferred using sterile forceps and the tube was then tightly closed.

3.2.1.4 Lung

For the collection of lung samples, lung was scooped out using blunt scissors and the portions of lung showing lesions were cut and transferred to the broth and the cap was tightly closed.

Inoculated plates and broths were preserved over wet ice and transported to the laboratory. The agar plates and broth were then immediately transferred to a candle jar under humid conditions and incubated at 37°C under 5-10 per cent CO_2 tension.

3.2.2 Processing of Samples

Samples obtained at the Department of Veterinary Microbiology were processed immediately after collection. All the inoculated broths and agar plates were transferred without delay to a candle jar maintained at the aforesaid conditions. Broth tubes were shaken intermittently and caps were tightly closed before incubation to avoid changes in pH.

Fifty swab samples collected in PPLO broth were removed after four hour of incubation and one millilitre of the broth was transferred aseptically into sterile eppendorf tubes in a laminar airflow cabinet for the preparation of template DNA for *Mycoplasma* genus-specific PCR. The remaining broth media were further incubated till an appreciable color change of the broth to orange or yellow was evidenced or up to 21 days, whichever was earlier.

3.2.2a Extraction of DNA from clinical samples

The extraction of DNA was performed according to a previously described procedure (Liu *et al*., 2001). The procedure was as follows:

Clinical samples suspended in 1 ml of PPLO broth were spun at 13,000 × g at 4°C for 20 min. The supernatant was discarded and the pellet was re-suspended in PBS of pH 7.4 by agitating in a vortex mixer. The cell pellet was washed twice in PBS by centrifugation at 13,000 × g for 15 minutes at 4°C. Resuspended the pellet in a final volume of 20 µl of PBS, heated in a dry block at 110°C for 10 min and placed at -70°C for at least 15 min. Lysate was then centrifuged at 13,000 × g for 2 min to remove the debris. Supernatant containing DNA was transferred into a sterile eppendorf tube, numbered and stored at -70°C till use.

Twenty five agar plates directly streaked with tracheal swab samples were examined daily under microscope for the presence of *Mycoplasma* colonies. When growth was observed, subculture was made on to fresh broth by cutting a small block of agar from plate containing a single or homogenous colony as far as possible. The DNA was extracted from these broths as mentioned above.

3.2.2b Extraction of DNA from other bacterial strains

Pure colonies of *E. coli*, *S. aureus* and *P. multocida* were inoculated separately in to five millilitre of Brain heart infusion (BHI) broth and incubated at 37°C for 18 h. From this broth culture, 2 ml was transferred to an eppendorf tube and centrifuged at 3000 × g for 10 min. The supernatant was discarded and the pellet was washed twice with sterile PBS. The final pellet was re-suspended in 50 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The samples were thawed and centrifuged at 3000× g for 5 min and supernatant was stored at – 20° C for further use as template for PCR reactions.

3.2.3 Polymerase Chain Reaction

3.2.3.1 Reconstitution of primers

Lyophilized primers were reconstituted in sterile triple distilled water to a concentration of 200 picomoles per millilitre. The tubes were kept at room temperature with occasional shaking for one hour and then spun down to pellet the insoluble particles and the supernatant was distributed in 25 µl aliquots and stored at -70°C. At the time of use, aliquots were thawed and working solutions of the primers

prepared obtained by 10 fold dilutions of the stock to make the primer concentration to 20 picomoles per microlitre.

3.2.3.2 Mycoplasma Genus Specific PCR

The PCR reaction was carried out as per Marois *et al*. (2000) with slight modifications. A 25 µl reaction mixture was set up for the single PCR reaction consisting of

10 × PCR assay buffer	2.5 µl
$MgCl_2$ (0.5 mM)	0.5 µl
Forward primer (20 pM)	1 µl
Reverse primer (20 pM)	1 µl
dNTP's (100 µM)	1 µl
Taq DNA Polymerase	1 µl
Template DNA	5 µl
Triple distilled water	13 µl

One negative control with triple distilled water substituting the template DNA and one positive control incorporating template DNA from the reference strain (*Mycoplasma gallisepticum* 6/85) were incorporated. Template DNA prepared from *E .coli*, *S. aureus* and *P. multocida* were also included to check the specificity of primers selected in the study. The tubes were placed in a thermal cycler (Eppendorf Master Cycler). The reaction protocol was as follows

Initial denaturation		90° C for 1 min
40 cycles	Denaturation	95° C for 15 sec
	Annealing	58° C for 20 sec
	Elongation	75° C for 20 sec
One cycle of	Denaturation	95° C for 15 sec
	Annealing	58° C for 45 sec
	Elongation	75° C for 5 min

3.2.3.3 Mycoplasma Species Specific PCR

Samples that tested positive by genus specific PCR were further subjected for *M. gallisepticum* specific reactions. The PCR reaction was carried out according to Raviv *et al*. (2007), with some modifications. A 25 µl reaction mixture was set up for the single PCR reaction consisting of

10 × PCR assay buffer	2.5 µl
$MgCl_2$ (0.5 mM)	0.5 µl
Forward primer (20 pM)	1 µl
Reverse primer (20 pM)	1 µl
dNTP's (100 µM)	1 µl
Taq DNA Polymerase	1 µl
Template DNA	5 µl
Triple distilled water	13 µl

The reaction protocol was as follows

Initial denaturation		94° C for 3 min
30 cycles	Denaturation	94° C for 20 sec
	Annealing	55° C for 30 sec
	Elongation	72° C for 60 sec
	Final extension	72° C for 5 min

3.2.3.4 Identification of PCR product

Submarine agarose gel electrophoresis was performed using one per cent agarose in 1 ×TBE as the matrix. One gram of agarose was dissolved in 100 ml of TBE buffer by heating and then cooled to 50°C. To this, two microlitre of ethidium bromide was added. This was then poured on to a clean dry gel platform, the sides of which were sealed with adhesive tapes and a comb placed at proper position. The gel was then allowed to polymerize. Once the gel had set, comb was lifted gently and the adhesive tapes were removed and the gel tray was placed into the buffer tank. The

tank was filled with buffer till it covered the top of the gel. Five microlitre of the amplified product with one microlitre of 6 × gel loading buffer was loaded into the wells of the submerged gel. The negative and positive controls were also loaded in separate wells. The molecular weight marker used was100 bp DNA Ladder. Gel was run until the blue dye had migrated the full length of the gel.

The gel was visualized under UV trans-illuminator (Hoefer, USA) and the results were documented on gel documentation system.

3.2.4 Isolation and identification of *M. gallisepticum*

The swab samples positive for *M. gallisepticum* species specific PCR were streaked on to the solid media, after 24 h of incubation or a color change of the broth from pink to orange or yellow was evidenced, whichever was earlier. The inoculated agar plates were incubated at 37°C in a candle jar under 5-10 per cent CO_2 tension till visible colonies were evidenced or up to a maximum of 21 days.

The samples that were negative by PCR were serially diluted in fresh broth medium in an attempt to dilute out substances in the sample that might inhibit the growth of *Mycoplasma*. Such samples were kept up to 21 days before discarding.

The isolates obtained from species specific PCR positive samples were subjected to further characterization studies.

3.2.4.1 Microscopic examination

The colonies on PPLO agar plates were observed under 4 × and 10 × magnification of microscope.

3.2.4.2 Diene's staining

The colonies on agar plates were flooded with working solution of Diene's stain and kept for one minute. The excess stain was removed and the plates were kept at 37° C for one hour.

3.2.4.3 Biochemical Characterization

Biochemical characterization was done following the methods of Barber and Fabricant, (1971).

The fermentation media were inoculated with 48 h old broth cultures (0.1 ml). Inoculated media were held for 10 days at 37°C under 5-10 per cent CO_2 tension. A change in color from red to yellow was taken as evidence of carbohydrate fermentation due to acid formation.

For fermentation of arginine, inoculated media was held for five days at 37°C under 5-10 per cent CO_2 tension.

3.2.4.4 Indirect Fluorescent Antibody Test (IFAT)

The IFAT was done following the protocols of OIE, (2008). The protocol was as follows.

a) From colony bearing agar plates, blocks of about 1.0 × 0.5 cm were cut and placed on to labeled microscope slides with the colonies uppermost. Blocks were taken from the unknown isolate and from the known *M. gallisepticum* culture.
b) To make subsequent orientation possible, the lower right hand corner of the blocks were cut off.
c) A drop of suitably diluted *M. gallisepticum* anti-serum (3.2.13) was added to the surface of each block.
d) The slides were incubated for 30 min at room temperature in a humid atmosphere
e) Each block was then placed in a labeled tube containing PBS, pH 7.2 and washed for 10 min in a rotary mixer and finally the blocks were returned to original slides
f) Excess moisture from the sides of the blocks was removed by blotting. A drop of diluted anti-rabbit IgG conjugate (Genei) was added to each block and incubated and washed as before.
g) The blocks were returned to their original slides, and examined under the fluorescent microscope.

3.2.4.5 Indirect Immunoperoxidase Test (IPT)

The impression of *M. gallisepticum* colonies observed on the PPLO agar plates were made on nitrocellulose membrane and it was processed as in the case of Western blot (3.2.14.2). The principle for the test was similar to IFA test except that binding of specific antibody to colonies *in situ* was detected by adding an anti-rabbit IgG conjugated to the enzyme peroxidase. A positive reaction was developed by adding a specific substrate (3.1.4.2) which on oxidation produced colored colonies.

3.2.4.6 Growth Inhibition Test (GIT)

Agar plates inoculated with broth cultures of the organism were taken and anti-serum impregnated sterile filter paper discs were applied onto the surface of the agar plates. Filter paper disc impregnated with plain rabbit sera was also applied onto the agar surface to serve as a control. The surface area adjacent to each disc was examined microscopically for zones of inhibition, after incubation at 37°C under 5-10 per cent CO_2 tension for four days (Stanbridge and Hayflick, 1967).

3.2.4.7 Strain Differentiation by Sodium Dodecyl-Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

The protein profile of the samples that gave positive reaction with both genus and species specific PCR were deduced using a denaturing SDS-PAGE according to the method described by Laemmli, (1970), in a vertical electrophoresis apparatus (Hoefer, USA) with some modifications. Briefly, the following protocol was used.

3.2.4.7.1 Whole cell protein extraction

Whole cell proteins of *M. gallisepticum* were extracted according to the procedure described by Ramadass *et al*. (2007).

Log phase cultures of *M. gallisepticum* were harvested by centrifugation at 12,000 × g for 30 min and were washed three times with PBS. The *M. gallisepticum* cells were disrupted by sonication at constant pulse for 30 seconds with 30 seconds break. The sonication was repeated for 19 times.

3.2.4.7.2 Gel preparation

Discontinuous SDS-PAGE was performed according to Laemmli (1970). Glass plates with 1.5 mm spacers were assembled in gel casting mould. The comb was inserted between the plates. The notched plate was marked one centimeter below the comb teeth for stacking gel. The resolving gel solution of desired concentration was prepared and poured between the glass plates up to the mark made earlier. The gel surface was immediately layered with a small amount of water and the gel was allowed to polymerize for about half an hour. After the polymerization of resolving gel was complete, the water above the gel was decanted and the gel surface was rinsed twice with distilled water. The stacking gel solution was prepared and poured on the separating gel. Comb was inserted between the plates and the gel was allowed to

polymerize for an hour. After the polymerization of the stacking gel, the comb was removed carefully and the wells were rinsed with water. The plates with the gel were then carefully taken out of the mould and put in electrophoresis apparatus. Electrode buffer was poured in upper and lower tanks of the apparatus and it was connected to the power supply pack (Pharmacia, Model EPS 500/400).

3.2.4.7.3 Sample preparation and electrophoretic run

Protein samples were prepared by mixing protein solution with sample buffer (4:1) and two to five per cent mercaptoethanol. The processed protein samples were kept in water bath at 60 to 70°C for around five min. Around 50 to 100 µg of protein was loaded into the wells with a syringe.

Electrophoresis was carried out at room temperature in constant voltage mode at 50 volts till the dye front entered the resolving gel and thereafter electrophoresis was carried out at 100 volts. The power supply was disconnected after the dye front reached the bottom of the gel. The gel plates were seperated apart and the gel was carefully removed. The gel was put in fixing solution for one to two hour. After removing the gels from fixing solution the gel was stained with Coomassie Brilliant Blue R-250 for 10 to 12 h. It was then destained using the destaining solution (3.1.10.11) and the gels were then stored in seven per cent acetic acid.

3.2.5 Growth under Iron sufficient and deficient conditions

Mycoplasma gallisepticum cells were grown under iron sufficient and iron restricted conditions following the methods of Madsen *et al*. (2006).

Four 500 ml flasks, each containing 250 ml cultures were incubated at 37°C to early exponential phase, as determined by a change in colour of media and optical density. To two of the four flasks, 2, 2'–dipyridyl was added to a final concentration of one mg per ml for iron chelation. The remaining two flasks were left untreated and all of the flasks were incubated for 2 h at 37°C.

3.2.5.1 Whole Cell Protein Extraction

Log phase cultures of *M. gallisepticum* cells grown under iron sufficient and iron restricted conditions were harvested by centrifugation at 12,000 × g for 30 min at 4°C and were washed three times with PBS. The *M. gallisepticum* cells were

disrupted by sonication at constant pulse for 30 seconds with 30 seconds break. The sonication was repeated for 19 times.

3.2.5.2 Membrane Protein Extraction

Mycoplasma gallisepticum cells grown under iron sufficient and iron restricted conditions were harvested at the late exponential phase of growth by centrifugation at 12,000 × g for 15 min at 4°C, washed once and re-suspended in 0.25 M NaCl.

Membranes were isolated from cells, which were lysed by incubating them for 1.5 h at 37°C in 0.25 M NaCl containing 0.05 M Tris buffer, pH 8.5. The volume of NaCl –Tris buffer solution used was 20 per cent of that of the growth medium. The membranes were collected by centrifugation at 34,000 × g for 30 min and were re-suspended in a 0.25 M NaCl solution containing RNAse (10 μg/ml) and 10 μM $MgCl_2$ and incubated for 20 min at 37°C. The membrane preparation were harvested and re-suspended in 0.25 M NaCl solution (Shirvan *et al*., 1982).

3.2.6 Estimation of Protein Concentration

The whole cell and membrane protein concentration under iron sufficient and iron restricted conditions were estimated by the standard procedure given by Lowry *et al*. (1951).

3.2.7 Raising of Hyper Immune Sera in Rabbits against Whole Cell and Membrane Proteins Produced under Iron Sufficient and Restricted Conditions.

Eight Newzealand white male rabbits weighing about 1-1.5 kg were used for raising anti-sera. One millilitre of each set of protein (200 μg/ml) was mixed with an equal volume of Freund's complete adjuvant (FCA) to form an emulsion. Emulsification was checked by placing a drop of the mixture on the surface of water. Rabbits, (duplicate for each set of protein) injected subcutaneously with one millilitre of protein in FCA, were bled immediately before to obtain pre-immune serum.

Booster dose containing same concentration of protein emulsified with Freund's incomplete adjuvant (FIA) was given subcutaneously after two weeks. Rabbits were bled two weeks post booster to obtain the serum. About 10 ml rabbit blood was collected by puncturing heart with the help of a 20 ml syringe and hypodermic needle. Blood was kept undisturbed in a wide mouth test tube at room

temperature for one hour to allow clotting. Thereafter, it was kept overnight at 4°C. Then the serum was separated by centrifugation of the clotted blood at 1500 × g for 10 min. Separated sera were carefully aspirated by Pasteur pipettes. Sera were mixed with five ppm sodium azide and aliquoted into 0.5 ml aliquots and were stored at -70°C till further use.

3.2.8 Western Blot

3.2.8.1 Membrane transfer

The proteins were resolved on SDS-PAGE. The proteins on the gels were transferred electrophoretically onto nitrocellulose membrane using blotting apparatus as per protocol described below.

a)After electrophoresis, the gels were taken off from the plates and kept in transfer buffer to remove the excess SDS from the gels.

b) Eight to ten Whatman no.3 filter papers of a size larger than gels were stacked, one by one on the anode plate after soaking in the transfer buffer. Care was taken to avoid air bubbles in between the stacks.

c) Membranes kept pre wet in transfer buffer were placed over the paper stacks after marking the orientation of the membranes.

d) Then the gels were placed after marking the orientation and care was taken to avoid air bubbles.

e) Over the gels 8-10 filter papers (Whatman filter paper no. 3) exactly of gel size were placed after soaking them in transfer buffer.

The complete stack was saturated with ice-cold transfer buffer before the cathode plate was placed in position over the stack and a current of three to five mA/cm^2 was applied for one hour. After transfer, the gel was stained to check the efficiency of transfer and the membrane was subjected to immunological detection.

3.2.8.2 Development of blot

The membrane after transfer was incubated overnight in five per cent (w/v) skim milk in PBS-T (PBS containing 0.2 per cent Tween 20), pH 7.4, for blocking the non-specific binding sites. After blocking, the membrane was washed (five min each) in PBS-T and incubated with 1:200 diluted rabbit anti-*M. gallisepticum* whole cell protein and membrane protein serum at 37°C for 1 h.

Thereafter, the membrane was washed thrice with PBS-T as described above and incubated for 1 h at 37°C with 1:4000 diluted goat anti rabbit HRPO conjugate (Bangalore Genei, India). After washing, the protein antibody reaction was detected by incubating the membrane with substrate solution as mentioned in 3.1.4.2. The color reaction was terminated by washing the membrane with distilled water to prevent background colouration.

3.2.9 Determination of Infective Dose (ID)

Ten microlitres each of 48 h grown log phase cultures of *M. gallisepticum* in different dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were plated on PPLO agar in duplicate. After incubation at 37°C with 5 to 10 per cent CO_2 tension under humid atmosphere, plates were examined daily under low power objective of the microscope. Counts were performed when visible colonies were obtained, to find out the total number of *M. gallisepticum* cells present in one millilitre of the culture. The different dilutions were made from this original culture and five groups of chicks of eight weeks age were inoculated with five different dilutions, with each group containing six birds. An additional group of six birds served as the control group. Birds were sacrificed after two weeks and the air sac and tracheal lesions were scored as per Nunoya *et al.* (1987). The highest dilution of the culture showing significant lesion in the inoculated birds was taken as the infective dose.

3.2.10 Re-isolation of *M. gallisepticum* from birds with lesions

Samples were swabbed from the air sac and trachea of birds with lesions and inoculated into PPLO broth tubes and gently vortexed to release attached mycoplamas into the medium. The samples were filtered (to eliminate possible bacterial contaminants) and serially diluted (10-fold) four times and 20 µl of each sample was plated onto PPLO agar plates. The cultures were incubated at 37°C with 5-10 per cent CO_2 tension under a humid atmosphere and observed for colour change (acid shift) or *Mycoplasma* growth on plates.

3.2.11 Preparation of Vaccines

Whole cell and membrane proteins were produced under iron sufficient and iron restricted conditions, thereby producing four sets of proteins. The optimum concentrations of the proteins used per dose of each set of vaccines were 84.6 µg.

Formalin was added to a final concentration of 0.1 per cent with frequent agitation and kept at room temperature overnight (Yagihashi *et al*., 1986). Thereafter the vaccines were kept at 4°C till the next step.

3.2.11.1 Sterility checking

Sterility of the prepared vaccines was tested individually in PPLO agar for *M. gallisepticum*, blood agar and tryptic soy agar (TSA) for aerobic bacteria, modified thioglycollate medium for anaerobic bacteria and Sabouraud's dextrose agar (SDA) for detecting any fungal contaminant. The media except SDA were inoculated with 0.1 ml of the vaccine and incubated at five per cent CO_2 tension, at 37°C for a period of seven days. Sabouraud's Dextrose Agar was incubated at room temperature after inoculation with 0.1 ml of vaccine and observed for seven days for any growth.

3.2.11.2 Formulation with adjuvants

Saponin and aluminium hydroxide were used in different combinations to get the following eight different sets of vaccines.

Whole cell proteins + Saponin	WCS
Iron restricted whole cell proteins + Saponin	WC+S
Membrane proteins + Saponin	MPS
Iron restricted membrane proteins + Saponin	MP+S
Whole cell proteins + Saponin + $Al(OH)_3$	WCAS
Iron restricted whole cell proteins + Saponin + $Al(OH)_3$	WC+AS
Membrane proteins + Saponin + $Al(OH)_3$	MPAS
Iron restricted Membrane proteins + Saponin + $Al(OH)_3$	MP+AS

Aluminium hydroxide was incorporated in the vaccine at a concentration of 25 per cent v/v according to Yoder, (1978). Saponin was added to a final concentration of 100 μg/dose of vaccine. The vaccines were mixed in a stirrer for four hour. Thereafter, the vaccines were kept at 4°C till the next step.

3.2.11.3 Safety testing of vaccines

Safety of each of the eight vaccines prepared was assessed by injecting double field dose of the vaccines (one ml) subcutaneously to eight chicks of five weeks age group. The injected birds were observed for a period of seven days for any untoward reaction or clinical manifestations.

3.2.12 Vaccination Trials

3.2.12.1 Vaccination

A total of 240 five week old chicks were divided into 10 groups with 24 birds in each group. The birds of each group were vaccinated as detailed below.

Group	Vaccine characteristics	Dose and route of vaccination
Group I	WCS	0.5 ml s/c
Group II	WC+S	0.5 ml s/c
Group III	MPS	0.5 ml s/c
Group IV	MP+S	0.5 ml s/c
Group V	WCAS	0.5 ml s/c
Group VI	WC+AS	0.5 ml s/c
Group VII	MPAS	0.5 ml s/c
Group VIII	MP+AS	0.5 ml s/c
Group IX	Commercial vaccine (COMV)	0.5 ml s/c
Group X	Control – PBS	0.5 ml s/c

3.2.12.2 Collection of Serum Samples Pre and Post-Vaccination

Before vaccination serum was collected from birds in different groups including control. Post vaccinal sera were collected from vaccinated birds at weekly intervals up to three weeks, followed by a booster dose at day 28 and subsequent collection on 35^{th}, $49^{th\ and}$ 63^{rd} day post vaccination. Blood was collected by jugular veni puncture. The collected blood was allowed to clot and incubated at 37°C for one hour. Serum was separated following overnight incubation at 4°C and stored at -70°C until use.

3.2.13 Potency testing of vaccines

3.2.13.1 Assessment of Humoral Immune Response

3.2.13.1.1 Haemagglutination test (HA)

Haemagglutination and Haemagglutination inhibition tests were carried out according to the protocols of OIE, (2008), with slight modifications.

3.2.13.1.1.1 Preparation of RBC

a) Chicken blood was collected in equal amount of Alsever's solution and mixed well.
b) The mixture was centrifuged at 650 g for 15 min.
c) The supernatant was discarded and the pellet was suspended in PBS.
d) The suspension was centrifuged again and the supernatant was discarded.
e) The RBC pellet was re-suspended in PBS to give a final concentration of five per cent RBC, and this was diluted to 0.5 per cent with PBS for working in HA/HI.

3.2.13.1.1.2 Preparation of antigen

Fourty eight hour old broth cultures of *M. gallisepticum* were harvested by centrifugation at 13,000 × g for 30 min. The cells were washed twice and re-suspended in PBS.

3.2.13.1.1.3 Haemaggutination test proper

a. 25 µl of PBS was dispensed into each of the 12 wells in the first row of microtitre plate.
b. 25 µl of antigen suspension was taken into the first well with the help of micropipette and the contents of the well were mixed well.
c. 25 µl mixture was taken from the first well and dispensed into second well. This process was repeated till the last well and 25 µl was discarded from the last well.
d. 25 µl of 0.5 per cent RBC was taken and dispensed into each of the 12 wells in the first row.
e. In the last but one well of second row, 25 µl of PBS and 25 µl of 0.5 per cent RBC and in the last well of that row 50 µl of 0.5 per cent RBC alone were added.
f. The sides of the plate were gently tapped to mix the contents of the plate and the plates were covered.
g. The plates were allowed to stand for 45 min at room temperature before the results were read and recorded.

3.2.13.1.1.3a Interpretation of results

HA negative: the appearance of a sharp button of RBC at the bottom of the wells of microtitre plates indicated an absence of HA.

HA positive: appearance of a hazy film of RBC, no button or a very small button of RBC at the bottom of the microtitre plates indicated a positive result for HA.

HA titre was taken as the reciprocal of the highest dilution that produced complete HA. This dilution was considered to contain 1 HA unit of antigen.

3.2.13.1.2 Haemagglutination Inhibition Test (HI)

25 µl of PBS was dispensed into each of the 12 wells of first row and the last two wells of the second row of the microtitre plate.

a. 25 µl of serum was taken into the first well and the second last (positive control) well of the second row of microtitre plate.
b. Two-fold serial dilutions of the serum were done along the first row.
c. 25 µl of 4 HA dilution of antigen was added to each well including the control wells in the second row.
d. The sides of the micro well plates were gently tapped to mix the reagents and the plates were covered. The mixture was allowed to stand for 30 min at room temperature.
e. 25 µl of 0.5 per cent washed chicken RBC was added to each well including the control wells in the second row.
f. The sides of micro well plates were gently tapped to mix the reagents. The plates were covered with a lid and allowed to stand at room temperature for 45 min.
g. The control serum wells were first read and the settling patterns in the rest of the wells were read.
h. The highest dilution of serum where there was complete inhibition of haemagglutination was taken as the HI titre of that serum sample.

3.2.13.1.2a Interpretation of results

HI negative: appearance of a hazy film of RBC, no button or a very small button of RBC at the bottom of the microtitre plates indicated an absence of HI.

HI positive: the appearance of a sharp button of RBC at the bottom of the wells of microtitre plates indicated the presence of HI.

HI titre was taken as the reciprocal of the highest dilution that produced complete HI.

3.2.13.2 Assessment of Cell Mediated Immune Responses

3.2.13.2.1 Leukocyte migration inhibition test (LMIT)

3.2.13.2.1a Separation of leukocytes from peripheral blood

Blood was collected from all groups of vaccinated birds and control, six from each group, at third week post first vaccination, first and third week post booster vaccination, by juglar veni puncture using 22 gauge needle and 6 ml syringe containing 5 to 10 units of sterile anticoagulant sodium heparin per ml of blood. Blood samples were immediately diluted with an equal volume of cold Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 mM Hepes buffer and 10 mM sodium bicarbonate. Samples were kept at 4°C and processing commenced within 20 min of collection.

Percoll was diluted to an osmolality of 320 mOsm/kg, the mean measured osmolality of avian plasma, by adding 9 ml of 150 mM NaCl to 91 ml of percoll. Further 80 per cent (density 1.108 g/ml), 73 per cent (density 1.092 g/ml) and 60 per cent (density 1.056 g/ml) dilutions of percoll solution were made with PBS, pH 7.2.

Discontinuous percoll gradients were prepared in 12 ml polystyrene centrifuge tubes by sequentially layering two millilitre volumes of 80 per cent, 73 per cent and 60 per cent percoll solutions. Four millilitre of blood, previously diluted with an equal volume of cold RPMI 1640 medium, was applied to the top of gradients and the tubes centrifuged at 800 × g for 15 min. after centrifugation, fractions were removed sequentially from the top of the tube with Pasteur pipettes. The leukocyte concentration was determined using a haemocytometer. The cells were washed three times with sterile NS and re-suspended in NS to contain approximately 1.5×10^8 cells per ml (Mills and Wilcox, 1993).

3.2.13.2.1b Preparation of plates

Agarose (two per cent w/v in distilled water) was boiled and cooled to 45°C and equal quantity of double concentration of Hank's balanced salt solution (HBSS), pH 7.2, containing one per cent lactalbumin hydrolysate, 0.4 per cent yeast extract, 20 per cent foetal calf serum (FCS), streptomycin (200 μg/ml) and penicillin (200 IU per ml) was added. This agarose medium, kept at 45°C, was poured into Petri dishes

to a height of three millimeter thickness. The agar plates were incubated at 37°C for one hour prior to use.

3.2.13.2.1c Leukocyte migration inhibition test proper

Leukocyte Migration Inhibiton Test was conducted following the procedure described by Bendixen, (1977).

Leukocyte suspension prepared from the peripheral blood was divided into two equal parts and to one portion 0.1 ml of antigen was added and to the other portion 0.1 ml of sterile NS was added. Contents of each tube were thoroughly mixed and incubated for one hour at 37°C with occasional shaking to avoid cell clumping. The contents of each tube were filled in six wells of three millimeter diameter, cut eight millimeter apart in the agarose gel. The charged plates were incubated at 37°C in a humid chamber for 20 h. At the end of incubation period, the cells were fixed to the glass surface by flooding the plates with methanol acetic acid fixative for 15 min (seven parts methanol + one part acetic acid + two parts distilled water). The agar gels in the plates were then partially dried to facilitate their peeling off from the plates.

Migration area of leukocytes was measured by taking average diameter of the opaque zone around the wells. The migration index was calculated as the average area of migration of cells treated with antigen divided by the average area of migration of cells treated with NS. A LMIT index less than 0.8 was considered as a positive reaction.

3.2.13.2.2 Blastogenic calorimetry

Spleen was harvested on fifth week post booster vaccination from six birds of each of the vaccinated groups and the control group and then washed in PBS. Using a 20 G needle, one millilitre of sterile NS was injected into each spleen and the spleenic cell suspensions were aspirated. The isolated splenic cells were washed by centrifugation in RPMI 1640 solution. The cell pellet was suspended in RPMI such that each millilitre of the suspension contained 5×10^7 leukocytes. The test proper was conducted in a 96 well flat bottom microtitre plate (Nunc, USA). Roswell Park Memorial Institute medium 1640 (200 μl) containing 10 per cent FCS, streptomycin 1000 μg/ml and penicillin 1000 IU/ml was taken in each well of the plate. In duplicate wells of the microtitre plate, 10 μl cell suspension of each spleen was taken. To one of the cell suspension, 10 μg of prepared antigen (3.2.5.1) was added, which served as the stimulated culture. To the other well, an equal volume of RPMI 1640 was added

and the well served as the unstimulated culture. To each of the wells, five µg of mitogen (phytohaemagglutinin, PHA) was added and the contents were mixed well. The microtitre plates were incubated in a humid atmosphere of five per cent CO_2 at 37°C for 90 h. After incubation, 20 µl of methyl- thiazolyl-tetrazolium (MTT) (10 mg/ml) was added to each of the wells and the plate was further incubated under the stated conditions for a period of three hour. The plates were centrifuged at 1650 g for 10 min after completion of incubation. The supernatant was removed and 50 µl of DMSO was added to each well and the plates were gently shaken for 15 min for color development. The absorbance was measured at 570 nm with a reference wavelength of 630nm using an ELISA reader (Mosmann, 1983).

The response was calculated as the stimulation index (SI), given as,

$$\text{Response (SI)} = \frac{\text{Mean absorbance of stimulated culture}}{\text{Mean absorbance of unstimulated culture}}$$

3.2.14 Challenge studies

Two challenge studies were conducted on birds of all vaccinated groups and the control group, eight from each group on day 38 and 52 post vaccination with the ID of 1.1×10^5.

3.2.14.1 Scoring of tissue lesions in challenged birds

3.2.14.1a Air Sac Lesions

The air sacs of chickens were grossly examined and lesions in cranial thoracic sacs, caudal thoracic and abdominal sacs were graded by the following scoring system according to Nunoya *et al*. (1987).

0 = no significant changes

1 = cloudy appearance or 1-3 yellowish foci

2 = cloudy thickening and / >4 yellowish foci

3 = diffuse yellowish thickening with caseous exudates

3.2.14.1b Tracheal Lesions

The middle part and lower part just above the syrinx of the trachea, each approximately five millilitre long, were collected from each bird and fixed in 10 per cent formalin. The sections were embedded in paraffin and cut to four µm size. The

cut sections were stained with hematoxylin and eosin (H&E) according to standard histological protocols (Sheehan and Hrapchak, 1980).

Cross-sections of the trachea were microscopically examined and mucosal lesions were evaluated by the following scoring system.

0 = no significant changes

0.5 = very small aggregates (1 to 2 foci) or very slight, diffuse infiltration of lymphocytes

1 = small aggregates (>4 discrete foci) of lymphocytes or slight thickening of the mucosa due to diffuse lymphocytic infiltration

2 = moderate thickening of the mucosa due to heterophil and lymphocyte infiltration and edema accompanied by epithelial degeneration with or without luminal exudation

3 = extensive thickening due to heterophil and lymphocyte infiltration and edema with squamous metaplasia or degeneration of epithelia with luminal exudation.

3.2.14.2 Re-isolation of M. gallisepticum from challenged birds

Re-isolation of *M. gallisepticum* was carried out from the birds with lesions as per the procedure described in 3.2.10.

3.2.15 Latex Agglutination Test (LAT)

3.2.15.1 Sensitization of latex beads with whole cell proteins (Ramadass *et al*., 1999)

Latex beads (Sigma, 0.88 μm) were used. Latex bead suspension (10 per cent) was washed twice by centrifugation at 6700 × g for three minute each time in carbonate-bicarbonate buffer (3.1.13b). Finally the latex beads were made into a two per cent suspension with carbonate-bicarbonate buffer, which was later mixed with an equal volume of *M. gallisepticum* iron restricted whole cell protein antigen (25 μg/ml) diluted in the same buffer. The mixture was incubated at 37°C for six hour with constant shaking at 250 g. The sensitized beads were centrifuged at 6700 × g for three minute and the pellet was re-suspended as a two per cent suspension in PBS containing five mg/ml of BSA. The latex beads were left at 37°C in a water bath overnight. Finally, the beads were centrifuged as before and the pellet was re-

suspended in PBS containing 0.5 mg/ml of BSA and 0.1 per cent sodium azide as a 0.5 per cent suspension. Sensitized latex beads were stored at 4°C until use. The stability of latex beads was determined by storage at 4°C and at ambient temperature.

3.2.15.2 Latex agglutination test proper

The LAT was performed on a glass slide by mixing equal volumes of (20 µl) of serum sample and sensitized latex beads. The slide was rocked gently for two to five minutes. PBS and normal chicken sera were used as negative controls and rabbit anti-*Mycoplasma gallisepticum* hyper-immune serum (3.2.7) was used as a positive control. A total of 30 sera samples were subjected to LAT.

3.2.15.3 Grading of Latex Agglutination Test

Results were read on a +1 to +4 scales depending on the extent of agglutination and time taken for the development of agglutination. The serum samples were considered to be negative if no agglutination was observed within five minutes.

++++: heavy flocculent agglutination formed immediately (clear back ground)

+++ : heavy flocculent agglutination taking 1-2 min to form (clear back ground)

++ : light flocculent agglutination against mostly clear back ground occurring in 2-3 min

\+ : light flocculent agglutination against cloudy homogenous background after extended incubation

– : no agglutination, cloudy homogenous background

3.2.15.4 Evaluation of LAT in comparison with HI

Sensitivity = (a/a+c) × 100 where ‘a’ – no. of sera positive by HI & LAT and‘c’ – no. of sera positive by HI but negative by LAT

Specificity = (d/b+d) × 100 where‘d’ – no. of sera negative by HI & LAT and ‘b’ – no. of sera negative by HI but positive by LAT

Percentage agreement of LAT with HI was found out by means of kappa statistics denoted by ‘κ’

κ=a+d-P/1-P and P = (a+b)(a+c)+(c+d)(b+d) where ‘P’ is the probability.

3.2.16 Preservation of the isolates

Forty eight hour broth cultures of *M. gallisepticum* were mixed with an equal quantity of sterile 10 per cent skim milk powder (sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure) and dispensed as 5 ml aliquots into freeze drying vials of 40 ml capacity. The cultures were frozen in a deep freezer (-20°C), and transferred to the freeze drier while frozen. Vacuum reading was 80 μ and the vials were left for 12 h. Vials were vacuum-sealed and stored at -70°C.

Results

RESULTS

4.1 Samples for the study

A total of 50 samples including tracheal and conjuctival swabs, air sacs and lung materials, were collected from ailing birds with respiratory problems. These samples were collected from birds maintained at poultry farms in different parts of Kerala, University Poultry Farm (UPF), Mannuthy and also from birds brought to Dept. of Veterinary Microbiology for disease diagnosis and birds necropsied at Centre of Excellence in Pathology, College of Veterinary and Animal Sciences, Mannuthy. The swabs were wetted with pluero pneumonia like organism broth before collecting the samples and then transported to the laboratory in the same broth. In addition, twenty five tracheal swabs were directly streaked on to PPLO agar plates. All the samples were transported under cold conditions to the laboratory for further processing.

4.2 Processing and detection of avian *Mycoplasma* in clinical samples

The samples were thoroughly agitated in the broth and kept at 37° C under 5-10 per cent CO_2 tension in screw capped tubes. The agar plates were also kept under similar conditions in a humid atmosphere. One millilitre of broth culture from each sample was taken after a period of four hours and processed for the extraction of genomic DNA. Remaining broth cultures were incubated as mentioned above. The agar plates were observed daily under microscope for the presence of any colonies. The genomic DNA of *E. coli*, *S. aureus* and *P. multocida* were also extracted for checking the specificity of the selected primers. After extraction of the genetic material, PCR was carried out using *Mycoplasma* genus specific primers.

4.2.1 Mycoplasma genus specific polymerase chain reaction

The primers GPO3 and MGSO were used in this study for the amplification of DNA from *Mycoplasma*.

The amplified products were visualized by sub-marine agarose gel electrophoresis. A positive PCR result for *Mycoplasma* was indicated by the presence

of a 270 bp fragment in electrophoresed gel under UV transillumination, whereas, no amplification indicated a negative result. In negative control, amplification was not detected and in positive control (reference strain 6/85) 270 bp band was observed. The DNA prepared from *E. coli*, *S. aureus* and *P. multocida* did not evidence any amplification with these primers, indicating the specificity of the selected primers (Fig. 1).

Out of the total 50 samples (collected in broth and agar) subjected to genus-specific PCR, 12 were found positive. These included eight samples collected both in PPLO broth and streaked on to agar. Four more samples from agar plates also gave a positive result. Thus, 12 samples were positive for *Mycoplasma* genus specific PCR. Among these samples, ten were collected from trachea and two from conjunctiva. These samples were collected from the birds in UPF, Mannuthy, showing respiratory ailments and conjunctivitis.

4.2.2 Species differentiation of avian Mycoplasma from clinical samples

Samples those were positive in genus specific PCR were then subjected to a species specific PCR using another set of selected primers**.**

4.2.2a Mycoplasma species specific PCR – samples collected in PPLO broth

The primers MG IGSR F and MG IGSR R could not amplify the DNA isolated from *E. coli*, *S. aureus* and *P. multocida*.

Agar gel electrophoresis of the amplified PCR products were carried out along with a positive control and negative control in 0.5 X TBE buffer. A positive PCR was indicated by the presence of a band in the 660 bp region in the test and positive control samples. Out of the eight genus-specific PCR positive samples subjected to species specific PCR, only one sample was positive, collected from trachea.

4.2.2b Mycoplasma species specific PCR – samples cultured on PPLO agar

The individual colonies observed after three days on the twenty five agar plates streaked with samples were picked up and put in PPLO broth and kept at 37°C under 5-10 per cent CO_2 tension. The broth tubes were daily examined for colour change from pink to orange or yellow (Fig. 2). Samples from those tubes with colour

change were used for extraction of DNA. The species specific PCR was carried out in above mentioned conditions.

Among the twenty five samples swabbed onto agar, 12 were positive in genus specific PCR and these samples were further subjected to species specific PCR. Out of these samples, three were positive in species specific PCR (Fig. 3).

4.3 Isolation of *M. gallisepticum* from PCR positive samples

Out of the four samples which gave a positive result for species specific PCR, *Mycoplasma* could be isolated from two. One sample which came negative by species specific PCR was positive for genus specific PCR. One conjuctival sample also gave a negative result in species specific PCR after subculture. Thus two isolates were obtained. They were sub cultured and maintained for further characterization. No isolates could be obtained from the lung and air sac materials.

4.4 Characterization of *Mycoplasma* isolates

4.4.1 Microscopic examination

Pluero pneumonia like organism agar plates streaked with *M. gallisepticum* cultures presented colonies after three to four days only. The colonies were observed under 4 × and 10 × objectives of the microscope. They showed a typical "fried" egg appearance, with a dark raised centre and a depressed light coloured periphery (Fig 4).

4.4.2 Diene's staininig

The colonies obtained on PPLO agar plates were stained with Diene's stain and those with a dark blue centre and a light blue periphery were observed under 4 × and 10 × magnifications (Fig 5).

4.4.3 Carbohydrate fermentation

The 48 h broth cultures of *M. gallisepticum* (0.1 ml) were subjected to carbohydrate fermentation tests in PPLO broth incorporated with specific carbohydrates and the following results were obtained (Fig 6, Table 1).

Positive fermentation test was indicated by the presence of yellow colour in the broth medium after 10 days incubation at 37°C under 5-10 per cent CO_2 tension. *Mycoplasma gallisepticum* isolate fermented glucose and fructose. But the fermentation of galactose, mannose, cellobiose and xylose were negative.

4.4.4 Arginine fermentation

Mycoplasma gallisepticum broth cultures (0.1ml) were subjected to arginine fermentation test in PPLO broth incorporated with arginine and incubated for five days at 37°C under 5-10 per cent CO_2 tension. Both the isolates gave a negative result for arginine fermentation, as indicated by the red colour of the medium (Fig 6, Table 1).

4.4.5 Indirect Fluorescent Antibody Test (IFAT)

The PPLO agar blocks containing colonies of *M. gallisepticum* were cut and further processed for IFAT. Positive result was indicated by the presence of a greenish fluorescence under the fluorescent microscope (Fig 7).

4.4.6 Indirect Immunoperoxidase Test (IPT)

Impression of the *M. gallisepticum* colonies on the PPLO agar plates were made on nitrocellulose membrane and were processed for IP test. A positive IP test was given by colonies, by presenting brown spots on nitrocellulose membrane (Fig 8).

4.4.7 Growth inhibition test (GIT)

Agar plates were inoculated with 48 h broth culture of *M. gallisepticum* and sterile filter paper discs impregnated with *M. gallisepticum* specific anti-sera were applied onto the surface of the plates. Other filter paper discs impregnated with normal rabbit sera were also applied onto surface of agar plates to serve as controls. The plates were incubated at 37°C under 5-10 per cent CO_2 tension in a humid chamber and were examined daily under microscope. After three days, zones of inhibition could be detected around the filter paper discs impregnated with specific anti-sera. Meanwhile, no inhibition zones were detected around the normal rabbit sera impregnated discs (Fig 9).

4.4.8 Strain differentiation by SDS-PAGE

Whole cell proteins of the two *M. gallisepticum* isolates that gave positive reaction with species specific PCR were subjected to SDS-PAGE. The whole cell extract profile showed protein bands ranging in size from 24 kDa to 200 kDa. The protein profile was similar for the isolates indicating their homogeneity (Fig 10).

4.4.9 Profile of the whole cell and membrane proteins of M. gallisepticum grown under iron sufficient and iron restricted conditions

The whole cell and membrane proteins were obtained from the exponential phase cultures of *M. gallisepticum* grown under iron sufficient and restricted conditions. These proteins were subjected to a denaturing SDS-PAGE (Fig 11).

The profile of whole cell and membrane proteins deduced on SDS-PAGE showed a series of bands with molecular weights ranging from 24 kDa to 200 kDa. The whole cell protein profile under iron sufficient and restricted conditions showed the presence of two prominent bands and eight faint bands that were not detected in the iron sufficient and restricted membrane protein profile. One major band was in the region of 75 kDa and other in the region of 35 kDa.

The iron restricted whole cell and membrane proteins when subjected to SDS-PAGE revealed a profile similar as in the case of iron sufficient conditions, except for the presence of one additional band observed in the region of 52 kDa.

4.5 Determination of whole cell and membrane protein concentration

The concentration of *M. gallisepticum* whole cell and membrane proteins was estimated by Lowry's method. The test samples were diluted to ten times for estimation. The optical density and concentration obtained with BSA standards were plotted on a graph. From this, the concentrations of the test samples were deduced. The concentration of the BSA standard and the corresponding optical density obtained are given in the Fig.12 and Table 2.

The concentration of the test samples were estimated from their OD values plotted on the graph. The following concentrations were obtained for the test samples.

1. Whole cell proteins = 860 µg/ml
2. Iron restricted whole cell proteins = 910 µg/ml
3. Membrane proteins = 440 µg/ml
4. Iron restricted membrane proteins = 490 µg/ml

The concentration of iron restricted whole cell extracts and membrane proteins were higher than that of the same proteins produced under iron sufficient conditions.

4.6 Western blot

The whole cell and membrane proteins produced under iron sufficient and iron restricted conditions were subjected to immunoblot studies using hyper immune sera raised against respective proteins. The whole cell and membrane proteins produced

under both the conditions were found to be antigenic in western blot (Fig 13). In the immunoblot profile of membrane proteins produced under iron sufficient conditions, the predominant bands detected were in the region of 24, 26, 35, 56, 64, 67, 77, 82 and 105 kDa. The membrane proteins under iron restricted conditions showed the similar profile except for the presence of an extra band in the region of 52 kDa. Of the two prominent bands detected in SDS-PAGE of iron sufficient and restricted whole cell proteins, the one at 75 kDa was found to be highly antigenic, while the other at 35 kDa produced only faint band in the immunoblot. The eight faint bands detected in the whole cell protein profile under iron sufficient and restricted conditions were not detected in the immunoblot.

4.7 Calculation of infective dose (ID)

To ascertain the infective dose of *M. gallisepticum* isolates for challenge studies, the log phase cultures of *M. gallisepticum* were subjected to different dilutions and then plated onto PPLO agar plates in duplicate. The plates were kept at 37°C under 5-10 per cent CO_2 tension in a humid chamber and were examined daily under the microscope for the presence of *M. gallisepticum* colonies. The resultant colonies obtained after four days in readable dilution were counted under microscope and colony count present per millilitre of the original culture was found to be 7.1 x 10^8. From this, different dilutions were made. The colony counts obtained with different dilutions are given in Table 3.

Thirty six chicks of eight weeks age group were divided into six groups (I-VI), each containing six birds. Group VI was kept as control which was inoculated with plain PPLO broth. Remaining groups were inoculated with different dilutions of *M. gallisepticum* culture. After a period of two weeks, the birds were sacrificed and the air sac lesions were scored grossly. Tracheal lesions were scored after histopathological examination. The mean lesion scores in the air sacs and trachea, obtained with different dilutions of *M. gallisepticum* inoculum and plain PPLO broth, are given in Table 4 and 5.

The mean lesions were analyzed statistically using non-parametric tests and the mean ranks obtained in different groups were compared in order to find out the statistical significance. The highest dilution of *M. gallicepticum* cells that showed significant lesion scores in air sac and trachea was obtained with the infective dose of

1.1 x 10^5 CFU/ml. This was taken as the infective dose for challenge studies. The air sac and tracheal lesions observed in challenged birds is depicted in Figures 14 and 15.

4.8 Preparation of Vaccine

4.8.1 Sterility checking

The sterility of iron sufficient and iron restricted whole cell extract and membrane protein vaccines were checked by plating the vaccines on different media like PPLO agar, blood agar, tryptic soy agar, modified thioglycollate medium and SDA. This was done to check the presence of any live cells of *M. gallisepticum* and aerobic or anaerobic bacteria. No detectable growth was observed on any media after incubation for a period of seven days at 37°C. No growth was detected even after seven days in the inoculated SDA agar plates kept at room temperature.

4.8.2 Formulation with adjuvants

Aluminium hydroxide and saponin were the adjuvants used in the vaccines. Aluminium hydroxide gel was used at the concentration of 25 per cent v/ v and saponin was used at a final concentration of 100 μg per dose of the vaccine. Following inactivation and sterility checking, vaccines were mixed with these adjuvants in different combinations to obtain eight different sets of vaccines. Table 6 depicts the different types of vaccines prepared in the present study. They were injected into eight groups of chicken, each containing twenty four birds of five weeks age. Birds in group I to VIII were injected with 0.5 ml each of the respective vaccine subcutaneously. Birds in group IX were injected with a commercial vaccine. Group X served as control which received PBS instead of vaccine.

4.8.3 Safety testing

Before injecting the different types of vaccines to experimental birds, the toxicity of the vaccines was checked by injecting double the field dose of the each vaccine (one ml) subcutaneously to eight birds of five weeks age group and no untoward reactions or clinical manifestation detected during the observation period of seven days.

4.9 Vaccination trials

Each group, containing chicken of five weeks age, was injected with 0.5 ml of different types of experimental vaccine subcutaneously. Group IX was injected with a commercial *M. gallisepticum* bacterin vaccine and the control group (group X) birds were injected with PBS. Serum samples were collected from all groups of chicken on

0^{th} day before vaccination. Following the first dose of vaccine, serum samples were collected at 7, 14 and 21 days post vaccination. After this, a booster dose was given on day 28. Following booster vaccination, serum samples were collected at 35^{th}, 49^{th} and 63^{rd} day post vaccination.

4.10 Potency testing of the vaccine

4.10.1 Humoral immune response

Humoral immune responses of the vaccines were assessed by the estimation of haemagglutination inhibition titres obtained with the sera collected from chicken inoculated with different sets of vaccines.

4.10.1a Haemagglutination

Haemagglutination test was carried out to find out the titre of haemagglutinating antigen required to carry out haemagglutination inhibition test. The highest dilution of *M. gallisepticum* whole cell antigen that showed complete haemagglutination was 1:32. The reciprocal of the highest dilution was taken as the haemagglutination titre and this was found to be 32.

4.10.1b Preparation of 4 HA units of antigen for HI test in microwell plates

The dilution factor required to prepare 4 HA units was obtained by dividing the titre (32) with four. The volume of 4 HA unit dilution of antigen required for conducting the test was found out by multiplying 2.5 ml (diluted antigen required per plate) with number of plates required for conducting the test at a time. Then dividing this volume of antigen with dilution factor (eight) gave the antigen suspension required. This stock antigen suspension was diluted to the required volume of 4 HA unit antigen with PBS as diluent.

4.10.1c HI titre of pre-immunization sera

Some of the pre-immunization sera randomly collected from different group of chicken have shown HI titres ranging from 1 to 2 $\log_2$.

4.10.1d HI titre of vaccinated birds

The HI test was carried out on serum samples collected at different time intervals from chicken inoculated with different sets of experimental vaccines. Antibody response was detected in the post vaccinal sera as early as seven days in the vaccinated groups and the titres increased during subsequent days, though the pattern differed on different days. Haemagglutination inhibition test showing the

representative titres of each group on 35th, 49th and 63rd day post vaccination (first, third and fifth week post booster vaccination) are shown in Figures 16a, 16b and 16c.

4.10.1e Statistical analysis

Analysis of variance (one way) of mean logarithm of HI titres of sera collected on different days from chicken vaccinated with different experimental vaccines was done.

The data were analyzed statistically using one way analysis of variance (ANOVA). The antibody production started from seventh day onwards in all the vaccinated groups. The antibody response increased on day 14 in all the vaccinated groups, without any significant difference between groups. The mean logarithmic HI titre remained more or less the same on day 21 in all the vaccinated groups.

Since the cut off value needed for *M. gallisepticum* vaccine to afford protection against natural infection is fixed at 1: 80, a booster dose of different vaccines was given to all the vaccinated groups. Following the booster dose, there was significant increase in HI titre in all the vaccinated groups and groups V, VI, VII, VIII and IX showed significantly high titres than other groups. Groups I, II, III and IV showed almost similar titres without any significant difference.

At third week post booster vaccination, groups V, VI, VII, VIII and IX maintained the same titres without significant difference. But the titres in groups I, II, III and IV showed a decline in their mean logarithmic HI values. At fourth week post vaccination, the same titre was maintained in groups V, VI, VII, VIII and IX without significant difference but the titres in groups I, II, III and IV showed a further decline. The four experimental vaccine groups, V, VI, VII and VIII produced similar response when compared to the commercial vaccine group (group IX) without significant difference. There was significant difference in the mean titres between control and different vaccinated groups during all time periods.

The mean logarithmic HI titre post vaccination on different time intervals in different vaccinated groups is graphically represented in Figure 17 and Table 7.

4.10.2 Cell mediated immune response

4.10.2a Leukocyte migration inhibition test

The peripheral blood leukocytes of the vaccinated birds and the control group, six from each group, were harvested at three different time periods (3rd week post vaccination, 1st week post booster vaccination, 3rd week post booster vaccination) to

conduct leukocyte migration inhibition test in order to assess the cellular immune response evoked by the different sets of vaccines, in comparison to control group. The migration index less than 0.8 was considered as an indication of good CMI response.

The data of LMI test was analyzed statistically with T- test. The migration index was greater than 0.8 in all groups of birds at 3rd week post first vaccination, without significant difference. Following 1st week booster vaccination, there was significant increase in cellular immune response in all groups of vaccinated birds as indicated by the mean migration index value less than 0.8, without significant difference except in commercial vaccine group and control group which differed significantly. At 3rd week post booster vaccination, there was slight decrease in the leukocyte migration index in all vaccinated groups but not significant. The commercial vaccine group did not show any CMI response, as indicated by a migration index higher than 0.8. During all time periods, the migration index of control group differed significantly from vaccinated groups. The mean LMI index obtained for the vaccinated groups is depicted in Figure 18 and Table 8.

4.10.2b Blastogenic calorimetry

The spleen was collected from chicken, inoculated with different types of vaccines and from the control group on 5th week post booster vaccination. Six samples were collected from each group. The splenic cell suspensions were made in RPMI medium and the cells were subjected to blastogenic proliferation assay using the mitogen phytohaemagglutinin. The optical density obtained in different groups of chicken was read in an ELISA reader at 570 nm.

The data were analyzed statistically and found that all vaccinated groups except the commercial vaccine group showed higher SI values, without differing significantly from each other (Table 9). The commercial vaccine group showed a lesser SI value that differed significantly from other vaccinated groups. The mean stimulation index of the control group was significantly different from that of all vaccinated groups.

4.11 Challenge studies

Two challenge studies were conducted two weeks apart on 38th and 52nd day post vaccination in chicken inoculated with different sets of vaccines and in the

control group. The air sac lesions of the chicken were scored grossly and the tracheal lesions were scored after histopathological examination.

The data were analyzed statistically using non-parametric tests and the mean ranks obtained with different groups were compared. In the first challenge study, the air sac and tracheal lesions of different groups except the control did not differ significantly from each other (Table 10 and 11). The mean lesion scores of the control group were significantly higher than the vaccinated groups.

In the second challenge study, the mean lesion scores of the vaccinated groups did not differ significantly from each other (Table 12 and 13). The control group showed a lesion score significantly higher than the vaccinated groups.

4.12 Re-isolation of *M. gallisepticum* from birds with lesion scores

From the birds sacrificed during the first challenge study, samples were collected from trachea and air sacs, thoroughly dispensed in PPLO broth and incubated at 37° C under 5-10 per cent CO_2 tension. *Mycoplasma gallisepticum* could be isolated from all the control birds with lesions, which were confirmed by PCR, biochemical and serological tests. But during the second challenge study, the isolation rate of *M. gallisepticum* from the control birds with lesions was approximately 70 per cent. *Escherichia coli* was isolated from rest 30 per cent of the cases and confirmed by different biochemical tests. In both challenge studies *M. gallisepticum* could not be isolated from all the vaccinated groups.

4.13 Latex agglutination test

Latex beads were sensitized with *M. gallisepticum* whole cell proteins produced under iron restricted conditions. These beads were then used for carrying out the agglutination test with serum samples from chicken (Fig. 19, Table 14)). A total of 30 serum samples were tested using LAT and the results were compared with HI test (Table 15).

The sensitivity and specificity of the test were found to be 95.24 per cent and 93.33 per cent respectively. A kappa value of greater than 0.81 was obtained indicating that LAT was in agreement with HI.

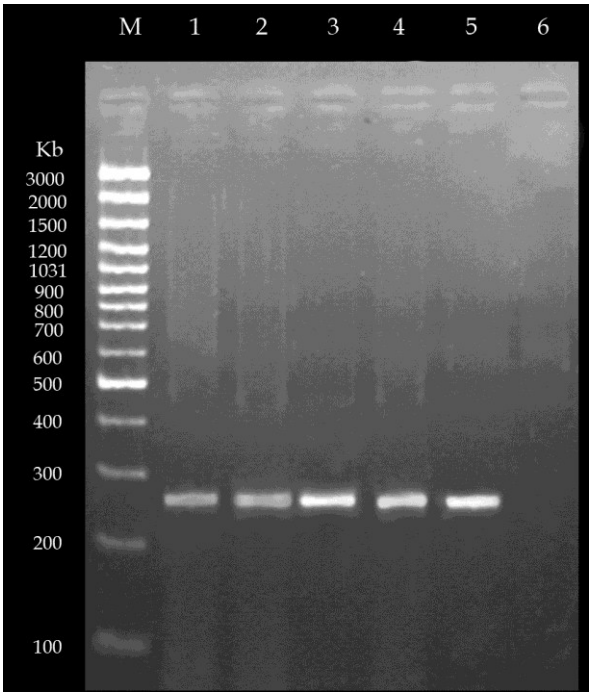


Fig. 1. Agarose gel electrophoresis of *Mycoplasma* genus specific PCR products

Lane M: Molecular weight marker
Lane 1, 2, 3, 4: *Mycoplasma* isolate
Lane 5: Reference strain 6/85
Lane 6: Negative control

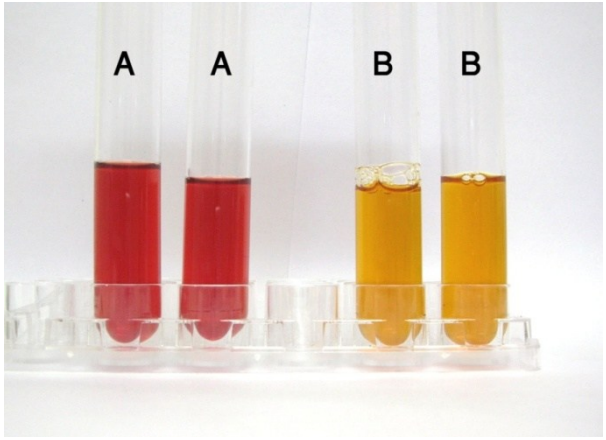


Fig. 2. Broth media before and after growth of *Mycoplamsa* culture

A: *Mycoplasma* broth before growth

B: *Mycoplasma* broth after growth

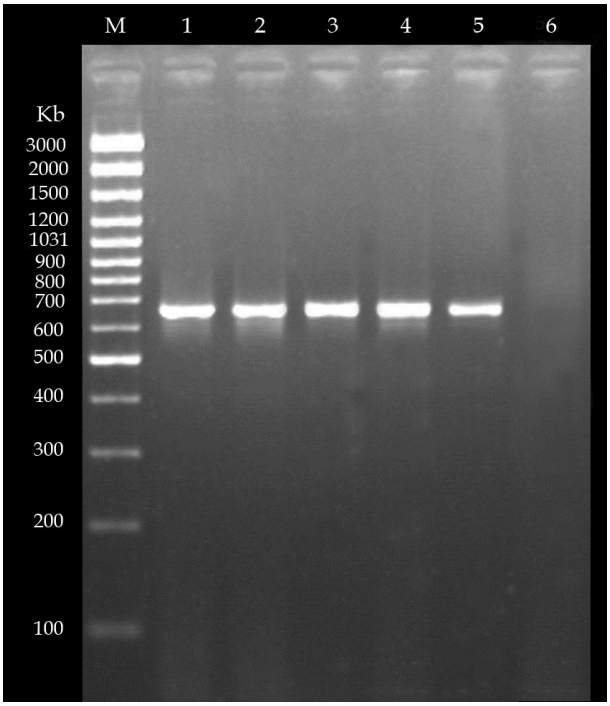


Fig. 3. Agarose gel electrophoresis of *Mycoplasma gallisepticum* species specific PCR products

Lane M: Molecular weight marker

Lane 1, 2, 3, 4: *M. gallisepticum* isolate

Lane 5: Reference strain 6/85

Lane 6: Negative control

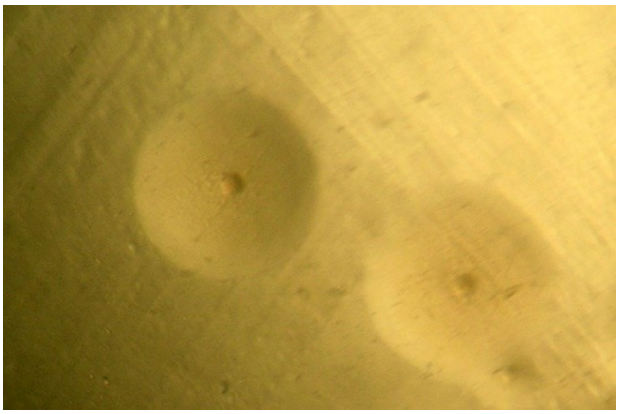


Fig. 4. Colonies of *M. gallisepticum* showing fried egg appearance (10 × magnification)

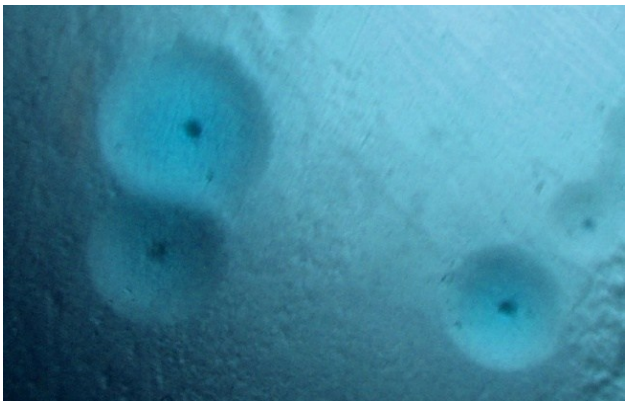


Fig. 5. Colonies of *M. gallisepticum* stained by Diene's method of staining (10 × magnification)

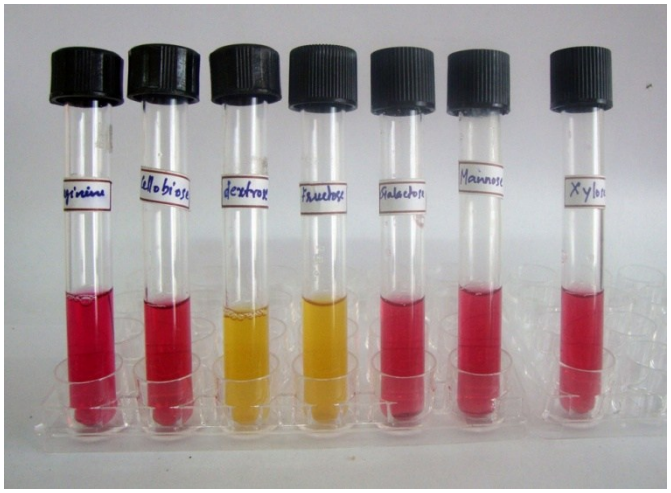


Fig. 6. Biochemical characterization of *M. gallisepticum* isolate (tubes from left to right: Arginine, cellobiose, dextrose, fructose, galactose, mannose, xylose)

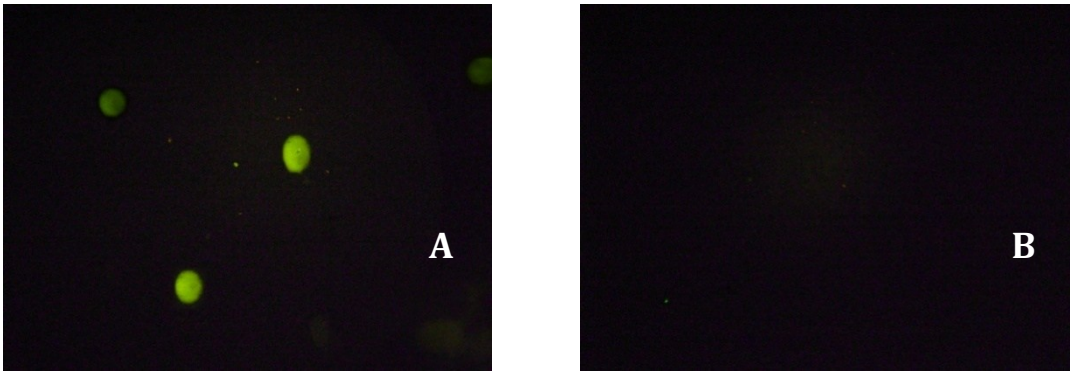


Fig. 7. Immunofluoroscence assay of *M. gallisepticum* isolate (A: positive reaction, B: negative control)

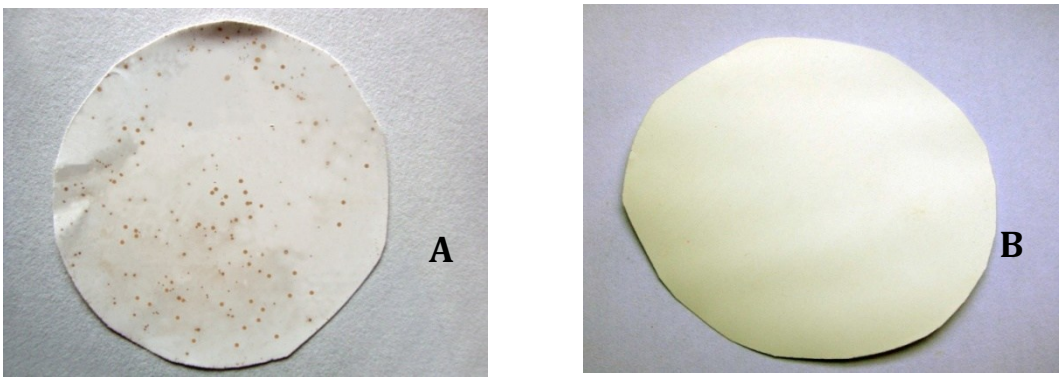


Fig. 8. Immunoperoxidase assay of *M. gallisepticum* isolate (A: positive reaction, B: negative control)

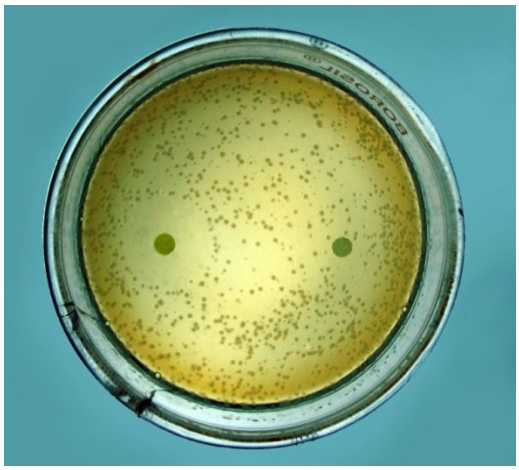
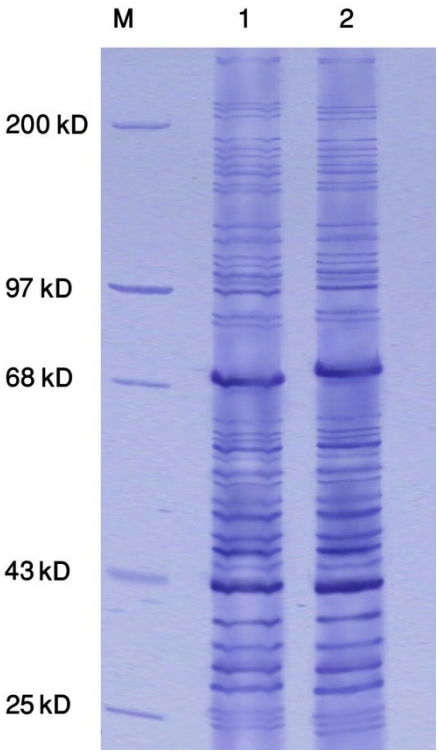


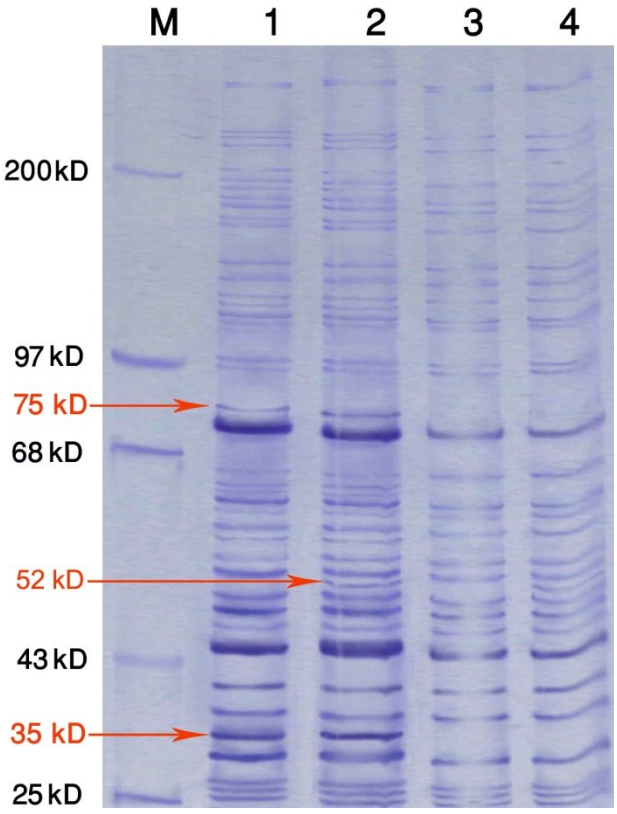
Fig. 9. Growth inhibition observed with *M. gallisepticum* isolate showing zone of inhibition (Disc on the left side had been impregnated with *M. gallisepticum* antisera and disc on the right side had been impregnated with pre immune sera)



Lane M: Molecular weight marker

Lane 1, 2: M. gallisepticum isolates

Fig. 10. Polyacrylamide gel electrophoresis of the whole cell proteins of *M. gallisepticum* isolates



Lane M: Molecular weight marker

Lane 1: Whole cell proteins

Lane 2: Iron restricted whole cell proteins

Lane 3: Membrane proteins

Lane 4: Iron restricted membrane proteins

Fig. 11. Polyacrylamide gel electrophoresis of the whole cell and membrane proteins produced under iron sufficient and iron restricted conditions

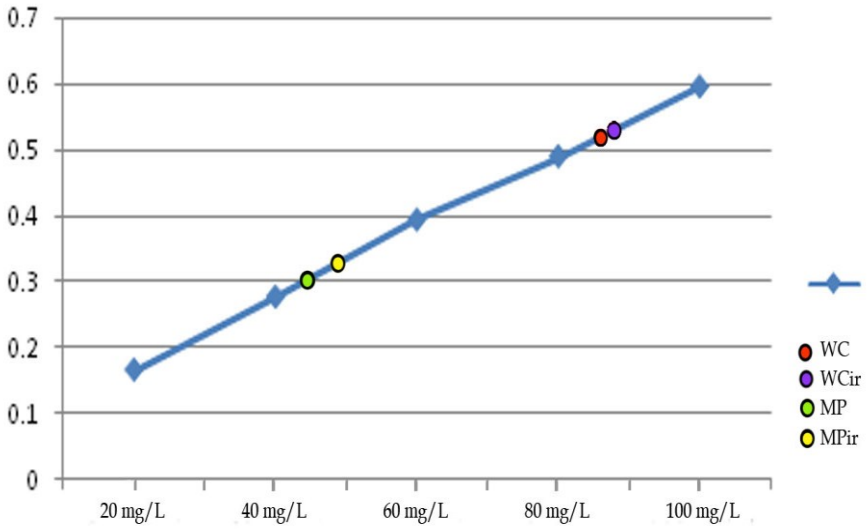


Fig. 12. Graphical representation of optical density and concentration of standards as estimated by Lowry's method of protein estimation

WC : Whole cell proteins

WCir : Iron restricted whole cell proteins

MP : Membrane proteins

MPir : Iron restricted membrane proteins

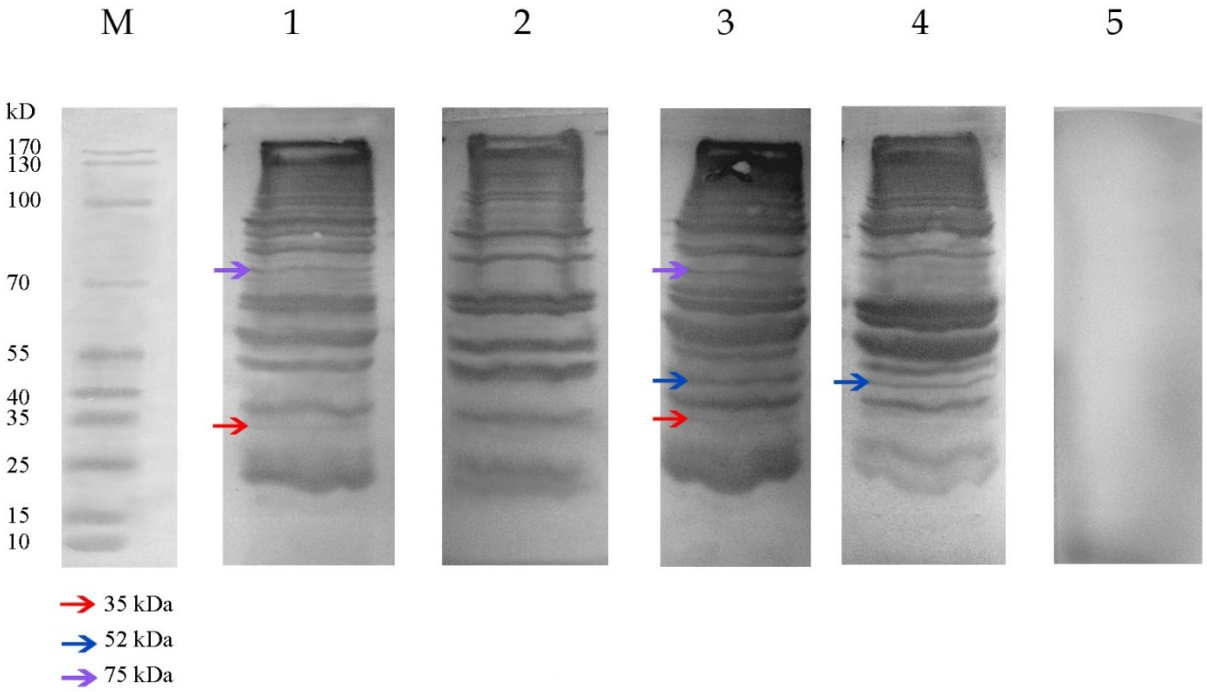
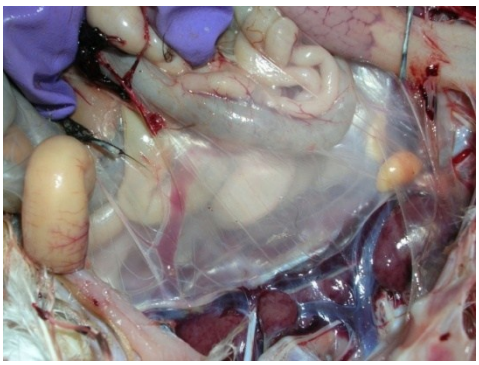
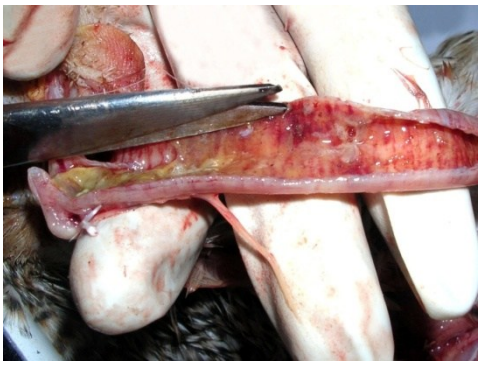


Fig. 13. Immunoblot profile of whole cell and membrane proteins of *M. gallisepticum* produced under iron sufficient and iron restricted conditions

Lane M: Molecular weight marker
Lane 1: Whole cell proteins
Lane 2: Membrane proteins
Lane 3: Iron restricted whole cell proteins
Lane 4: Iron restricted membrane proteins
Lane 5: Pre immune rabbit sera (Negative control)

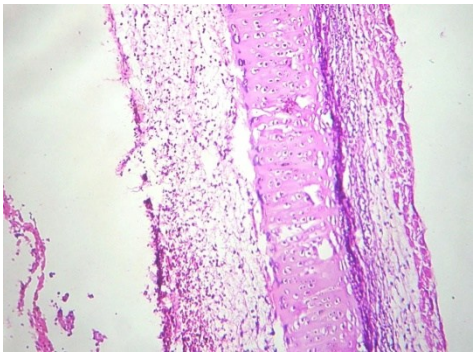


Cloudy thickened abdominal air sacs



Haemorrhages and mucous plugs in trachea

Fig. 14. Air sac and tracheal lesions observed in birds subjected to challenge studies with *M. gallisepticum* culture



Thickened mucosa, degenerated epithelial cells and infiltration of inflammatory cell in the trachea

Fig. 15. Histopathological tracheal lesions observed in birds subjected to challenge studies with *M. gallisepticum* culture

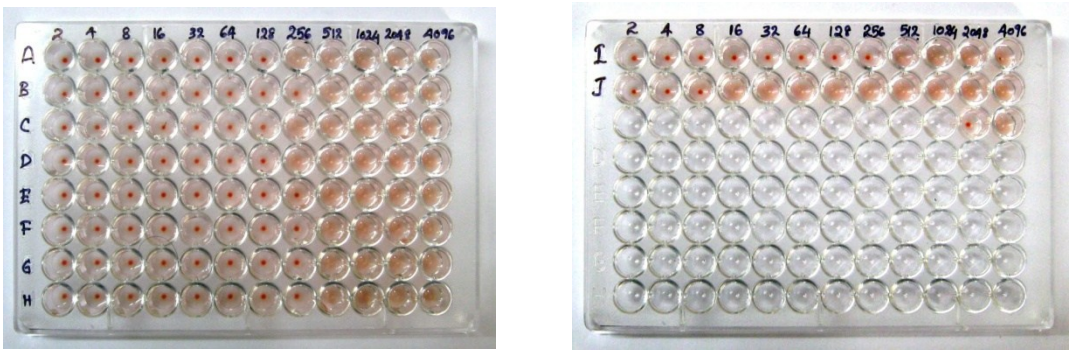


Fig. 16a

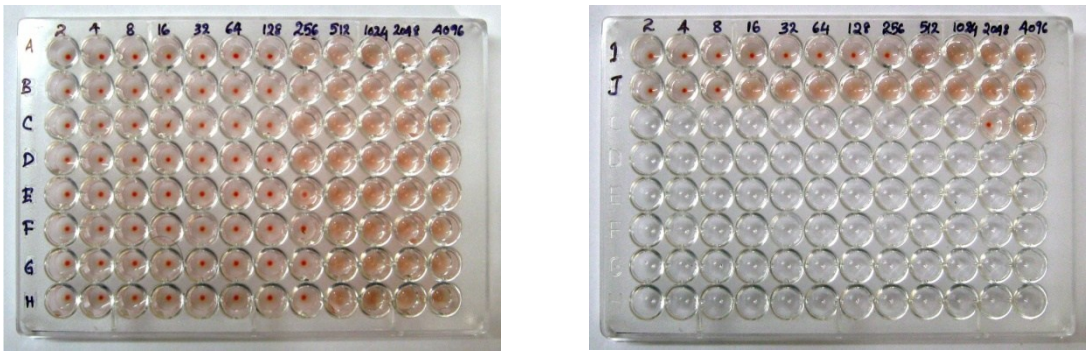


Fig. 16b

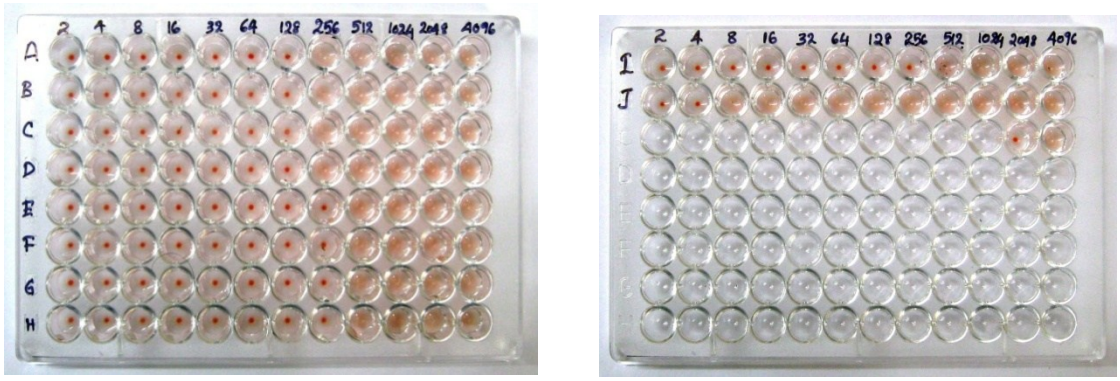


Fig. 16c

Fig. 16. Haemagglutination inhibition test showing the representative titres of each group after

a. first week of booster vaccination
b. third week post booster vaccination
c. fifth week post booster vaccination

A: Group I, B: Group II, C: Group III, D: Group IV, E: Group V, F: Group VI, G: Group VII, H: Group VIII, I: Group IX, J: GroupX

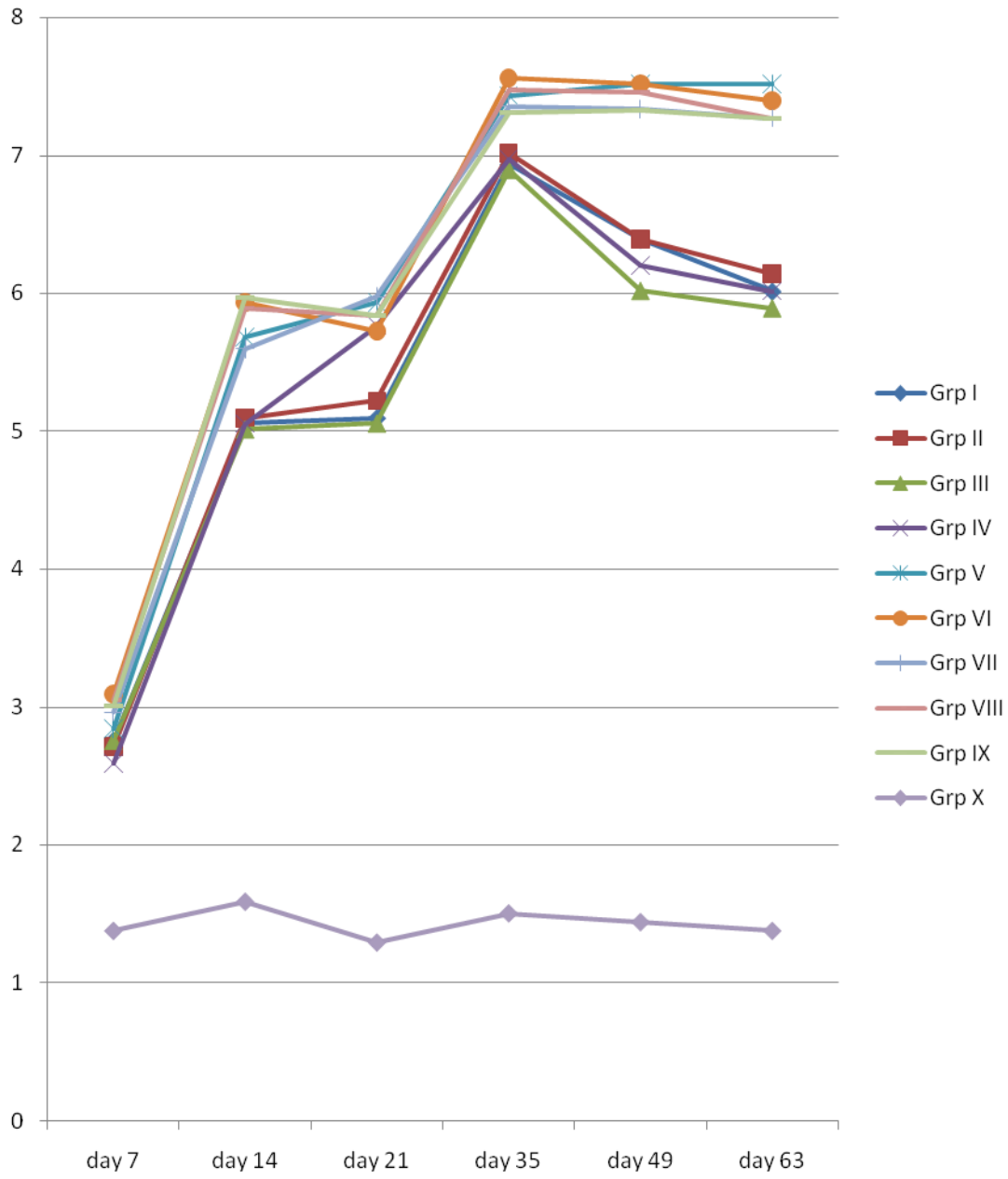


Fig. 17. Comparative mean HI titre of different vaccinated groups during different time periods

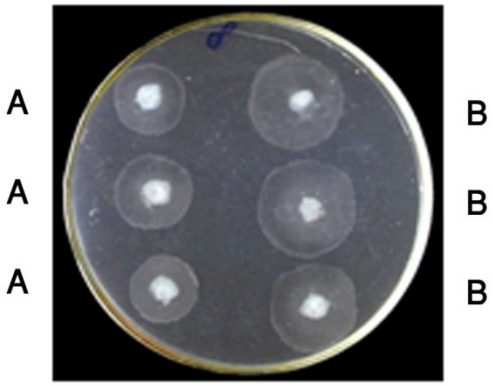


Fig. 18. Leukocyte migration inhibition test of the vaccinated and unvaccinated birds

Fig. 19. Latex agglutination test for detection of *M. gallisepticum* antibodies

A: Negative test

B: Positive test

Table 1. Carbohydrate fermentation and Arginine hydrolysis test results of isolated *M. gallisepticum* cultures

Substrate	***Result***
Glucose	+
Fructose	+
Galactose	_
Mannose	_
Cellobiose	_
Arginine hydrolysis	_

Table 2. Optical density values and the corresponding concentration obtained for standards in the Lowry's method of protein estimation.

Sl. no	***Samples***	***Concentration mg/litre***	***Optical density***
1	Standard 1	0	0.000
2	Standard 2	20	0.165
3	Standard 3	40	0.276
4	Standard 4	60	0.393
5	Standard5	80	0.489
6	Standard 6	100	0.595

Table 3. Colony counts obtained of PPLO agar plates with different dilutions of *M. gallisepticum* culture

Dilutions	***CFU/ml***
10^{-1}	4.5×10^{8}
10^{-2}	3.1×10^{7}
10^{-3}	2.2×10^{6}
10^{-4}	1.1×10^{5}
10^{-5}	1.5×10^{4}

Table 4. Mean air sac lesion scores obtained in birds inoculated with different dilutions of *M. gallisepticum* culture for estimation of infective dose

Group	***No. of birds inoculated***	***Inoculum (CFU/ml)***	***Gross lesions of air sac (Mean ± SE)***	***Mean Rank***
I	6	4.5×10^{8}	$8.6667^{b} \pm 0.8433$	26.17^{b}
II	6	3.1×10^{7}	$8.50000^{b} \pm 0.6191$	24.08^{b}
III	6	2.2×10^{6}	$8.5000^{b} \pm 0.4282$	24.42^{b}
IV	6	1.1×10^{5}	$8.5000^{b} \pm 0.3416$	23.25^{b}
V	6	1.5×10^{4}	$8.1667^{a} \pm 0.6009$	9.58^{a}
VI	6	Plain PPLO broth	$0.3333^{a} \pm 0.2108$	3.5^{a}

Means bearing different superscript in a column differ significantly ($P<0.05$)

Table 5. Mean histopathological tracheal lesion scores obtained in birds inoculated with different dilutions of *M. gallisepticum* culture for estimation of infective dose

Group	***No. of birds inoculated***	***Inoculum (CFU/ml)***	***Histopathological lesions of trachea Mean ± SE***	***Mean Rank***
I	6	4.5×10^8	$1.0000^{b} \pm 0.2236$	22.45^{b}
II	6	3.1×10^7	$1.0833^{b} \pm 0.2007$	24.42^{b}
III	6	2.2×10^6	$1.0000^{b} \pm 0.2236$	22.25^{b}
IV	6	1.1×10^5	$1.0000^{b} \pm 0.2236$	22.25^{b}
V	6	1.5×10^4	$0.5000^{a} \pm 0.1291$	12.75^{a}
VI	6	Plain PPLO broth	$0.1667^{a} \pm 0.1667$	7.08^{a}

Means bearing different superscript in a column differ significantly (P<0.05)

Table 6. Different types of *M. gallisepticum* vaccines formulated for the study

Group	***Vaccine characteristics***
I	Whole cell proteins + Saponin (WCS)
II	Iron restricted whole cell proteins + Saponin (WC+S)
III	Membrane proteins + Saponin (MPS)
IV	Iron restricted membrane proteins + Saponin (MP+S)
V	Whole cell proteins + Saponin + $Al(OH)_3$ (WCAS)
VI	Iron restricted whole cell proteins + Saponin + $Al(OH)_3$ (WC+AS)
VII	Membrane proteins + Saponin + $Al(OH)_3$ (MPAS)
VIII	Iron restricted Membrane proteins + Saponin + $Al(OH)_3$ (MP+AS)
IX	Commercial vaccine (COMV)
X	Control – PBS

Table 7. Logarithmic HI titre values of vaccinated and unvaccinated groups of chicken (Mean ± SE)

Days post vaccination	***Grp I***	***Grp II***	***Grp III***	***Grp IV***	***Grp V***	***GrpVI***	***GrpVII***	***GrpVIII***	***Grp IX***	***GrpX***
7th	2.757^{bc} ± 0.138	2.714^{bc} ± 0.128	2.757^{bc} ± 0.11	2.59^{b} ± 0.119	2.84^{bc} ± 0.116	3.091^{c} ± 0.147	2.966^{c} ± 0.095	3.049^{c} ± 0.113	3.01^{c} ± 0.105	1.378^{a} ± 0.101
14th	5.054^{b} ± 0.154	5.096^{b} ± 0.147	5.012^{b} ± 0.085	5.054^{b} ± 0.073	5.68^{cd} ± 0.099	5.931^{d} ± 0.103	5.597^{c} ± 0.119	5.889^{cd} ± 0.11	5.973^{d} ± 0.073	1.587^{a} ± 0.103
21st	5.096^{b} ± 0.134	5.221^{b} ± 0.085	5.054^{b} ± 0.095	5.764^{b} ± 0.091	5.931^{c} ± 0.103	5.722^{c} ± 0.095	5.973^{c} ± 0.113	5.842^{c} ± 0.115	5.842 ± 0.115	1.295^{a} ± 0.095
Ist wk post booster	6.934^{b} ± 0.058	7.017^{b} ± 0.085	6.892^{b} ± 0.069	6.975^{b} ± 0.073	7.435^{c} ± 0.103	7.56^{c} ± 0.104	7.351^{c} ± 0.099	7.477^{c} ± 0.104	7.309^{c} ± 0.095	1.504^{a} ± 0.121
3rdwk post booster	6.391^{b} ± 0.125	6.391^{b} ± 0.125	6.015^{b} ± 0.183	6.203^{b} ± 0.164	7.518^{c} ± 0.129	7.518^{c} ± 0.129	7.33^{c} ± 0.12	7.456^{c} ± 0.128	7.33^{c} ± 0.12	1.441^{a} ± 0.158
4thwk post booster	6.015^{b} ± 0.189	6.14^{b} ± 0.125	5.889^{b} ± 0.227	6.015^{b} ± 0.268	7.518^{c} ± 0.189	7.393^{c} ± 0.183	7.268^{c} ± 0.164	7.268^{c} ± 0.164	7.268^{c} ± 0.164	1.378^{a} ± 0.183

Means bearing different superscript in a column differ significantly (P<0.05)

Table 8. Leukocyte migration inhibition index of vaccinated and unvaccinated groups of chicken (Mean ± SE)

	3rd week post first vaccination	***1st week post booster vaccination***	***3rd week post booster vaccination***
I	$0.995^{a} \pm 0.039$	$0.758^{a} \pm 0.014$	$0.748^{a} \pm 0.012$
II	$1.015^{a} \pm 0.049$	$0.75^{a} \pm 0.0112$	$0.743^{a} \pm 0.02$
III	$0.977^{a} \pm 0.024$	$0.767^{a} \pm 0.012$	$0.747^{a} \pm 0.017$
IV	$0.963^{a} \pm 0.017$	$0.763^{a} \pm 0.011$	$0.752^{a} \pm 0.016$
V	$0.982^{a} \pm 0.02$	$0.762^{a} \pm 0.011$	$0.73^{a} \pm 0.086$
VI	$0.984^{a} \pm 0.022$	$0.752^{a} \pm 0.06$	$0.733^{a} \pm 0.088$
VII	$0.972^{a} \pm 0.017$	$0.752^{a} \pm 0.016$	$0.732^{a} \pm 0.087$
VIII	$0.95^{a} \pm 0.024$	$0.762^{a} \pm 0.011$	$0.742^{a} \pm 0.015$
IX	$0.957^{a} \pm 0.032$	$0.863^{b} \pm 0.013$	$0.87^{b} \pm 0.012$
X	$1.047^{a} \pm 0.038$	$1.083^{c} \pm 0.044$	$1.072^{c} \pm 0.038$

Means bearing different superscript in a column differ significantly (P<0.05)

Table 9. Mean blastogenic stimulation index obtained in vaccinated and unvaccinated groups of chicken

Group	***5th week post booster vaccination (Mean ± SE)***
I	$1.759^{cd} \pm 0.025$
II	$1.764^{d} \pm 0.012$
III	$1.688^{c} \pm 0.027$
IV	$1.692^{c} \pm 0.023$
V	$1.739^{cd} \pm 0.015$
VI	$1.752^{cd} \pm 0.018$
VII	$1.789^{d} \pm 0.099$
VIII	$1.881^{e} \pm 0.045$
IX	$1.267^{b} \pm 0.09$
X	$0.9853^{a} \pm 0.03$

Means bearing different superscript in a column differ significantly (P<0.05)

Table 10. Mean air sac lesion scores obtained in birds inoculated with *M. gallisepticum* culture in the first challenge study

Group	***Gross lesions of air sac (Mean ± SE)***	***Mean Rank***
I	0.3750^{a}± 0.375	38.31 [a]
II	0.1250 [a] ± 0.125	37.94 [a]
III	0.2500 [a] ± 0.164	42.38 [a]
IV	0.1250 [a] ± 0.125	37.94 [a]
V	0.0000 [a] ± 0.0	33.50 [a]
VI	0.1250 [a] ± 0.125	37.94 [a]
VII	0.0000 [a] ± 0.0	33.50 [a]
VIII	0.0000 [a] ± 0.0	33.50 [a]
IX	0.0000 [a] ± 0.0	33.50 [a]
X	8.1250^{b}± 0.515	76.50 [b]

Means bearing different superscript in a column differ significantly (P<0.05)

Table 11. Mean histopathological tracheal lesion scores obtained in birds inoculated with *M. gallisepticum* culture in the first challenge study

Group	***Histopathological lesions of trachea (Mean ± SD)***	***Mean Rank***
I	0.1250 [a] ±0.125	39.88 [a]
II	0.1250 [a] ±0.125	39.88 [a]
III	0.0000 [a] ±0.0	35.00 [a]
IV	0.1250 [a] ±0.1250	39.88 [a]
V	0.0000 [a] ±0.0	35.00 [a]
VI	0.0000 [a] ±0.0	35.00 [a]
VII	0.0000 [a] ±0.0	35.00 [a]
VIII	0.0000 [a] ±0.0	35.00 [a]
IX	0.0000 [a] ±0.0	35.00 [a]
X	1.3750 [b] ±0.246	75.38 [b]

Means bearing different superscript in a column differ significantly (P<0.05)

Table 12. Mean air sac lesion scores obtained in birds inoculated with *M. gallisepticum* culture in the second challenge study

Group	***Gross lesions of air sac (Mean ± SD)***	***Mean Rank***
I	2.0 [a] ± 0845	49.94 [a]
II	0.875 [a] ± 0.56	35.88 [a]
III	1.25 [a] ± 0.726	40.44 [a]
IV	1.375 [a] ± 0.905	37.25 [a]
V	0.125 [a] ± 0.125	29.94[a]
VI	0.25 [a] ± 0.164	33.88 [a]
VII	0.25 [a] ± 0.164	33.88 [a]
VIII	0.25 [a] ± 0.164	33.88 [a]
IX	0.25 [a] ± 0.164	33.88 [a]
X	8.5 [b] ± 0.627	76.06[b]

Means bearing different superscript in a column differ significantly (P<0.05)

Table 13. Mean histopathological tracheal lesion scores obtained in birds inoculated with *M. gallisepticum* culture in the second challenge study

Group	***Histopathological lesions of trachea (Mean ± SD)***	***Mean Rank***
I	0.188 [a] ± 0.091	44.44 [a]
II	0.188 [a] ± 0.013	41.00 [a]
III	0.313 [a] ± 0.016	46.19 [a]
IV	0.125 [a] ± 0.082	40.13 [a]
V	0.0 [a] ± 0.0	31.50 [a]
VI	0.0 [a]± 0	31.50 [a]
VII	0.0 [a] ± 0.0	31.50 [a]
VIII	0.0 [a] ± 0.0	31.50 [a]
IX	0.0 [a] ± 0.0	31.50 [a]
X	1.50 [b] ± 0.019	75.75 [b]

Means bearing different superscript in a column differ significantly (P<0.05)

Table 14. Grading of Latex agglutination test for detection of *M. gallisepticum* antibodies in sera collected from chicken

No of birds	***4 +***	***3 +***	***2 +***	***1 +***	***Negative***
30	1	3	2	4	20

Table 15. Compatative evaluation of Latex agglutination test with Haemagglutination inhibition in the detection of *M. gallisepticum* antibodies in sera collected from chicken

LAT	***HI***		
	Positive	***Negative***	***Total***
Positive	8 (a)	1 (b)	9
Negative	2 (c)	19 (d)	21
Total	10 (a + c)	20(b + d)	30

Discussion

DISCUSSION

Avian mycoplasmosis is one of the economically significant diseases affecting poultry (Jordan, 1979). The pathogenic avian *Mycoplasma* species affecting poultry are *M. gallisepticum*, *M. synoviae*, *M. iowae* and *M. meleagradis*. Among this, *M. gallisepticum* is the most pathogenic and occur world-wide, causing chronic respiratory disease in chickens and infectious sinusitis in turkeys. The organism causes decreased egg production, hatchability, growth rate and feed conversion rate; increased mortality and condemnation rate of carcasses as well as indirect losses due to increased sensitivity of infected birds to management failures and associating agents, such as Infectious Bronchitis, Laryngeotracheitis, or Newcastle disease viruses and *E. coli* (Carpenter *et al*., 1981).

Successful control of the disease, including eradication of *M. gallisepticum*, depends very much on reliable diagnosis of infection. This can be done by isolation and identification of the organism and or by serological assays. Isolation techniques are laborious, time consuming and expensive. Problems posed due to the overgrowth of faster-growing saprophytic *Mycoplasma* species or no growths in subculture are other difficulties underlying the isolation of pathogenic mycoplasmas. Systemic antibody response is widely used as a diagnostic and epidemiologic tool in the poultry industry to identify or determine the prevalence of infectious agents. Serological tests such as RSA, HI and ELISA are widely used for flock monitoring of *Mycoplasma*. Other detection methods like GIT, MIT, IFAT, IPT and agar gel precipitin test are also used.

Among the various diagnostic methods, PCR that detect nucleic acid from pathogenic mycoplasmas is an effective tool for the laboratory detection since it is rapid, sensitive and specific (Nascimento *et al*., 1991; Lauerman *et al*., 1993; Zhao and Yamamoto, 1993; Geary *et al*., 1994; Silveira *et al*., 1996).

Mycoplasma gallisepticum infection proved to be difficult to eradicate for the following reasons. The in-house spread of *M. gallisepticum* is relatively rapid, thus necessitating the elimination of an entire flock rather than the individual reactor. This is practically not feasible because it can amount to huge financial loss. Layers are commonly kept on multiple age farms. A flock infected with *M. gallisepticum* is

infected for life and will shed the organism for life; therefore older flocks on a multiple age farm serve as a source of infection for younger flocks. The spread of *M. gallisepticum* cannot be prevented on a multiple age farm area once the organism is introduced, without slaughtering all the infected hens. The range of air-borne spread and the survival of *M. gallisepticum* outside are longer than originally believed. Perhaps the techniques applied to reduce egg transmission, such as heat treatment at46°C for 12-14 hours or, more efficiently, by antibiotic treatment, either by *in-ovo* injection or, by dipping eggs in antimicrobial solutions (Nascimento and Nascimento, 1994; Stipkovits and Kempf, 1996) resulted in selection for more resistant *M. gallisepticum* survivors.

Strategies to reduce the adverse impact of *M. gallisepticum* infection in commercial poultry include surveillance, control and eradication programme; use of antimicrobials such as tetracyclines, macrolides, quinolones and tiamulin and vaccination. Both live and killed vaccines are available for the control of *M. gallisepticum* infection. However, neither type of vaccine has earned universal acceptance. Ideally *M. gallisepticum* vaccine should be safe and effective in preventing infection as well as disease, for all types of poultry and in multiple-age flocks, should be capable of displacing wild-type *M. gallisepticum* and allowing a return to *M. gallisepticum* free status when vaccination is stopped.

The three currently available live *M. gallisepticum* vaccines are F, 6/85 and ts-11 strains. The F strain vaccine had been used extensively in chickens especially in multiple-age laying complexes and was successful in protecting egg production drops. It provided some protection to broilers from airsacculitis, following aerosol challenge with virulent R strain. But F strain vaccination did not prevent colonization by the challenge strain of *M. gallisepticum*. Strain F can be transmitted through the egg and from bird to bird. It was found that vaccinal F strain was pathogenic in turkeys, following experimental infection and had been associated with *M. gallisepticum* outbreaks in meat and breeder turkeys under field conditions.

The live vaccines 6/85 and ts-11 have been reported to possess little or no virulence for chickens and turkeys. The ts-11 vaccine persists in the chicken for long periods, but the 6/85-strain vaccine does not persist and may be difficult to recover after a few weeks. Transmission of strain 6/85 from vaccinates to commingled pullets or sentinel birds in adjacent pens was not observed. The strain ts-11 is transmissible

from vaccinates when birds are in direct contact with each other and/or share a common feeder and waterer, but transmission is unlikely with indirect contact.

One major concern about live *M. gallisepticum* vaccines is safety. There have been numerous instances of clinical respiratory disease caused by "escaped" F strain vaccine in turkeys; this strain should probably not be used if there is potential danger of spread to turkeys, even though it is the most efficacious strain in chickens. There have been several instances of isolation of 6/85-like *M. gallisepticum* strains from turkeys showing clinical disease (Kleven *et al*., 2004). In some cases there was a history of vaccination of nearby chickens or turkeys. There has been detection of 6/85-like isolates of *M. gallisepticum* in unvaccinated commercial layer housed near 6/85-vaccinated birds (Throne Steinlage *et al*., 2003). The ts-11 strain also had been detected in unvaccinated chicken flocks. In both instances there was a history of possible use of contaminated vaccination equipment and in one of the instances, subsequent spread to neighboring broiler breeders. Strain ts-11 can spread from vaccinated spike males to unvaccinated breeder females. These experiences suggest that, even though the live vaccines are very effective, they do have the potential for spread, and their safety should be very carefully examined before a decision is made to vaccinate. In India only the inactivated vaccines are in use.

Good effective bacterins can reduce and control the clinical signs associated with the *M. gallisepticum* infection as well as inhibit vertical transmission of the disease. The post-vaccinal serological response is directly correlated with overall immunity in vaccinated birds. It is claimed that bacterins can improve hen housed egg prodcution (HHP-15 eggs), livability and feed efficiency. Bacterins also aid in eradication of *M. gallisepticum* from vertical transmission, within three consecutive production cycles. So nowadays the uses of inactivated vaccines are recommended. But the use of inactivated bacterin vaccines so far developed is limited due to higher costs associated with vaccination protocols and lesser effectiveness.

The adjuvants have been since long used for the augmentation of immune response in host against various diseases of bacterial, mycoplasmal and viral origin. It is a well known fact that adjuvants can enhance the immune response when administered with specific antigens than when the antigens are injected alone (Dalsgaard *et al*., 1990). The use of adjuvants in inactivated vaccines could enhance the immunogenicity of weaker antigens, reduce the antigen amount needed for a successful immunization, reduce the frequency of booster immunization needed and

improve the immune response in elderly and immunocompromised vaccinates (Edelman, 1980).

Even though the aluminium hydroxide is widely used in *M. gallisepticum* bacterin vaccines as an adjuvant for the production of a good antibody response, it has little capacity to stimulate cellular immune response (Petrovsky and Aguilar, 2004). Studies conducted by several researchers indicated that CMI also plays a major role in the development of immunity to mycoplasmal respiratory tract diseases (Roberts, 1973; Biberfeld *et al*., 1974). Thus the incorporation of an adjuvant in bacterin vaccine which stimulates cell mediated immune response could enhance the protection against *Mycoplasma* infections. Saponin is an adjuvant which has the unique ability to stimulate cell mediated arm of the immune system as well as to enhance antibody production. Additionally a low dose is needed for adjuvant activity (Iqbal, 2007). Hence a combination of aluminium hydroxide-saponin in bacterin vaccines could be able to stimulate and prolong both the humoral and cell mediated immune responses against *Mycoplasma* infections.

Iron is involved in basic cellular functions and hence, an essential micronutrient for all living systems. Iron deprivation by sequestering the available iron in iron binding proteins is a prominent feature of the innate immune responses of the host to microbial infections. Bacteria have evolved several strategies for iron acquisition from the host system, which involve various transcriptional regulators in genes involved in iron uptake. During iron depletion, bacteria up regulate and down regulate many bacterial proteins and might also produce new immunogenic proteins. The antibodies produced against these proteins might offer better protection to host against natural infections (Salyers and Whitt, 2002).

Taking into consideration all these factors, our study was designed to develop and evaluate whole cell and membrane protein vaccines of *M. gallisepticum* grown under iron sufficient and restricted conditions, incorporating adjuvants like saponin and aluminium hydroxide individually and in combination and their comparison with commercial vaccine.

5.1. Samples for the study

A total of 50 samples including tracheal and conjuctival swabs, air sacs and lung materials, were collected from the birds showing respiratory ailments from farms in different parts of Kerala, University poultry farm, Mannuthy, birds brought to Dept. of Veterinary Microbiology and birds necropsied at Centre of Excellence in

Pathology, College of Veterinary and Animal Sciences, Mannuthy. The swabs were pre wetted in *Mycoplasma* broth before collecting the samples in the same. In addition to collection in broth, twenty five tracheal swabs were plated directly onto PPLO agar also. The samples were transported under cold conditions to the laboratory for further processing. Wet swabs yielded significantly greater number of mycoplasmas than dry swabs and this was in accordance with Zain and Bradbury (1995).

5.2 Detection of avian *Mycoplasma* in clinical sample

5.2.1 Mycoplasma genus-specific PCR

A total of 12 samples were found positive when 50 samples collected in PPLO broth and agar were subjected to *Mycoplasma* genus-specific PCR. An amplified product of 270 bp was obtained in the case of positive samples and positive control (reference strain 6/85) by using the primers MGSO and GPO3. There was no amplification of the DNA from other bacterial strains like *E.coli*, *S. aureus*, and *P. multocida* with the primers used, which indicated this specificity. Marois *et al*. (2000) and Dipu (2006) used these primers for the amplification of DNA from *Mycoplasma* and confirmed these as genus specific.

5.2.2 Mycoplasma species-specific PCR

Out of the 12 genus positive PCR samples obtained in the study when subjected to species specific PCR, only four were found positive. It might have happened due to the over-growth of non-pathogenic saprophytic species of avian *Mycoplasma* in broth, over whelming the growth of *M. gallsepticum* (Liu *et al*., 2001).The overgrowth by faster growing *Mycoplasma* sp. or other organisms is a frequently encountered problem experienced with culture of *Mycoplasma* (Kempf, 1998).The primers used for the amplification were constructed from the intergenic spacer region located between the 16s and 23s rRNA and were confirmed as *M. gallisepticum* specific **(**Raviv *et al*., 2007). Specificity of the primers was indicated by the absence of amplification of DNA from other bacterial species tested. The positive samples and the reference culture gave an amplified product of 660 bp by using the primers MG IGSR F and MG IGSR R.

5.3 Media for the isolation of avian *Mycoplasma*

For the isolation of all the pathogenic mycoplamas the primary requirement is to have a good medium base. Most media formulations for the isolation of mycoplasmas have as base components those first recommended by Edward (1947),

which included a meat-infusion (usually beef heart), tryptone and/or peptone, yeast extract, glucose and 10 to 20 per cent horse or swine serum. The significance of bacterial inhibitors penicillin and thallium in *Mycoplasma* media had been well documented (Frey *et al*., 1968; Branton *et al*., 1984).

The medium used in the present study contained buffalo heart infusion as the base (Lecce and Sperling, 1954) and this medium was found ideal for the isolation and propagation of avian mycoplasmas. Similar results have been reported by George (2003). It appears that buffalo heart infusion broth supplemented some of the unspecified nutrients required for the growth of avian mycoplasmas. Manohar (2001) compared eight different media and found that buffalo heart infusion broth could give better growth of avian mycoplasmas than the various other media compared. All the inoculated media were incubated at 37°C under 5 - 10 per cent CO_2 tension. Increased humidity and CO_2 tension between 5-10 per cent have been reported to enhance growth of *Mycoplasma* (OIE, 2008).

5.4 Isolation of *Mycoplasma gallisepticum*

Out of the total 50 samples collected, 25 tracheal swab samples were directly streaked onto PPLO agar plates also. Among the samples inoculated in broth, only eight were found positive for genus specific PCR whereas, in case of those plated onto PPLO agar, 12 were positive. Out of these 12, four samples were negative for PCR, when collected in broth. Thus, a total of 12 samples (including the four samples that were additionally positive when collected onto PPLO agar plates) were positive for genus specific PCR. This was in agreement with the finding that for the isolation of avian *Mycoplasma*, solid medium was found to be more effective than liquid medium (Ronglian *et al*., 1996).

Out of the 12 *Mycoplasma* genus specific PCR positive samples obtained, only four were found to be positive in *M. gallisepticum* specific PCR. These samples were collected from birds maintained at UPF, Mannuthy. From these four species specific PCR positive samples, only three isolates could be obtained. This might be due to the fact that the viability of the organism in the sample did not influence the result obtained with PCR, whereas it was the most critical factor as far cultural isolation was concerned. The potential for detecting non-viable mycoplasmas explains the discrepancies between PCR and culture results, especially following antibiotic therapy (Kempf *et al*., 1994; Moalic *et al*., 1997). Ley *et al*. (1993) suggested DNA based tests such as PCR were capable of targeting organism without reliance on the viability of the organism.

Conjunctival swabs, air sac and lung materials did not yield any isolate. Air sac materials, lung materials and conjuctival swabs collected from advanced stages of infection showed heavy contamination with other bacteria so that the isolation of mycoplasmas was unsuccessful.

All the *M. gallisepticum* isolates showed growth only after three or four days. This might be due to the fact that pathogenic mycoplasmas are usually more fastidious than less pathogenic ones (Yoder, 1991).

The growth of mycoplasmas was indicated by a change in colour of the broth from pink to yellow. This was because of the change in colour of indicator phenol red to yellow, when dextrose in the media was fermented by *Mycoplasma* (Yoder, 1991).

A few broth cultures were contaminated during the study and they were passed through 0.2 micro meter Millipore filter in an attempt to selectively retain other bacteria in the filter and the filtrate was used as the inoculum. Filtrate was streaked onto the solid media after 24 h of incubation or till a colour change of the broth from pink to orange or yellow was evidenced, whichever was earlier. Such broth which evidenced no indication of growth was serially diluted in fresh broth medium in an attempt to dilute out specific antibody or antibiotics or inhibitory substances in tissues that might inhibit the growth of *Mycoplasma*. The presence of arginine hydrolyzing (alkali producing) *Mycoplasma* species if present might mask the acid colour change produced (OIE, 2008). In the present study no growth could be obtained on the agar plate upon plating of serially diluted broth.

Among the three *M. gallisepticum* isolates obtained only two could be cultured and maintained. Kempf *et al*. (1997) reported that difficulty in sub culturing was one major problem encountered during the cultivation of *Mycoplasma*.

5.5Characterization of avian mycoplasmas

Serological screening of new isolates against all other recognized species is too cumbersome. However, preliminary grouping of new isolates by simple biochemical reactions reduces the necessity for comparisons by more complex procedures and aids in detecting mixed cultures. Biochemical and serological procedures should be used together, as the independent use of either might not reveal mixed cultures (Edward and Freundt, 1969).

5.5.1 Colony characters

The colonies obtained with *Mycoplasma* isolates in general showed fried-egg appearance with a dark raised centre and a light coloured depressed periphery. The fried egg appearance of the *Mycoplasma* colonies had been well documented (Frey *et*

al., 1968; Jain *et al*., 1971; Power and Jordan, 1976). Apart from this, coalesced colonies were also obtained. This is in agreement with the finding of Yoder and Hofstad (1964), who observed that closely adjacent colonies readily coalesced. There was appreciably no morphological difference between the colonies of *M. gallisepticum* and other *Mycoplasma* colonies obtained. The species designation of mycoplasmas could not be determined by their colony characters alone even though variation in colonies of different species of avian mycoplasmas had been noted (Yoder and Hofstad, 1964).

5.5.2 Diene's staining

Diene's stain will be taken up by *Mycoplasma* and bacterial L-forms. But only colonies of *Mycoplasma* retain the stain, while others get decolourised in 15-30 min. This was in agreement with Dienes and Weinberger (1951). In the present study, *M. gallisepticum* isolates produced typical fried egg appearance on the *Mycoplasma* agar surface, with a dark blue raised centre and a light blue depressed periphery as confirmed by the Diene's method of staining (Madoff, 1960). Diene's reagent, confirmed the isolates as true mycoplasmas rather than as bacterial L-forms (Timms, 1967).

5.5.3 Biochemical tests

Mycoplasma gallisepticum isolates identified by PCR in the present study gave a positive result for fermentation of glucose and fructose and a negative test for arginine hydrolysis. The biochemical test results such as fermentation of glucose and fructose and non-fermentation of galactose, mannose, xylose, cellobiose and the absence of hydrolysis of arginine confirmed the isolate as *M. gallisepticum* as reported earlier by Fabricant (1969) and Aluotto *et al*. (1970).

Poveda (1998) stated that identification of mycoplasmas was greatly reliant on serological tests based on the recognition of structural membrane proteins by specific antiserum, because they had limited biosynthetic capacity due to their small genome size. He suggested that a preliminary biochemical characterization could reduce the battery of sera required for serological identification.

5.5.4 Growth Inhibition

There was inhibiting growth of *Mycoplasma* colonies around the filter paper discs incorporated with *M. galliseptiucm* anti-sera. Growth inhibition test using known specific antiserum against *M. gallisepticum* confirmed the isolates since the

species specific antiserum inhibited the growth of the specific species. This finding is in accordance with the observation of Clyde (1964) and Woode and Mc Martin (1973). The antigen on the *Mycoplasma* associated with inhibition of the growth by antiserum appears to be essentially physiologically active receptors (Woode and Mc Martin, 1973). Studies conducted by Domermuth and Gourlay (1967), Stanbridge and Hayflick (1967), Davies and Read (1968) and Ogra and Bohl (1970) indicated that GI test was simple and had a high degree of specificity.

5.5.5 Indirect fluorescent antibody test

Colonies of *M. gallisepticum* on agar blocks gave a greenish fluorescence on addition of secondary antibody conjugated with fluorescene isothiocyanate dye. Del Guidice *et al*. (1967) and Polak-Vogelzang et *al*. (1979) found that immunofluorescene and immunoperoxidase techniques were the most suitable methods for the identification of *Mycoplasma* species in mixed cultures on primary isolation plates. Direct immunofluorescence employing colonies on the surface of agar plates, or colony imprints, have been found very effective for culture identification (Talkington and Kleven, 1983; 1984; Morse *et al*., 1986). Corstvet and Sadler (1964) and Talkington and Kleven (1983) indicated direct staining of *Mycoplasma* colonies on agar surface or colony imprints with fluorescent antibody as the most commonly used method to determine species of avian *Mycoplasma* isolates. Although, biochemical characterization could help in preliminary classification of *Mycoplasma* isolates, final identification must be done by serological tests and most satisfactory of these are Indirect Fluorescent Antibody Test and Immunoperoxidase Test (OIE, 2008).

5.5.6 Immunoperoxidase test

The replica of the *Mycoplasma* colonies made on nitrocellulose membrane gave brown spots on addition of *M. gallisepticum* specific anti-sera raised in rabbit followed by addition of secondary antibody conjugated with horse raddish peroxidase enzyme and specific substrate solution. Immunobinding based on indirect immunoperoxidase test of *Mycoplasma* cells blotted on a nitrocellulose paper was reported to be useful for rapid identification of mycoplasmas in broth medium and clinical specimens (Kotani and McGarrity, 1989). The indirect immunoperoxidase test was used for rapid identification of mycoplasmas by Imada *et al*. (1987) and colonies

of type strains of 22 *Mycoplasma* species, 3 *Acholeplasma* species, and 3 *Ureaplasma diversum* serogroups were identified by this test with high sensitivity and specificity.

This test was proved to be a simple and useful technique for rapid identification of many *Mycoplasma* species grown on agar medium. Studies conducted by Polak-Vogelzang *et al*. (1979) and Quinn *et al*. (1981) showed that IP test was not only as simple as the fluorescent antibody test and as specific as the GI test, but also more sensitive than these tests.

5.5.7 Strain differentiation by PAGE

Forty eight hour grown log phase cultures of *M. gallisepticum* were harvested by centrifugation. The cells were washed and re-suspended in PBS and the whole cell proteins of *M. gallisepticum* were produced by sonication. Shirvan *et al*. (1982) used ultrasonication for the extraction of whole cell proteins of *M. gallisepticum* and was found to be an effective method for the detection of cytoplasmic proteins. Whole cell proteins of the two *Mycoplasma* isolates confirmed by *M. gallisepticum* PCR were analyzed in SDS-PAGE. The whole cell protein profile in the present study showed protein bands ranging in size from 24 kDa to 200 kDa. The protein profile of the two isolates was almost similar.

Polyacrylamide gel electrophoresis of whole-cell proteins was used for the first time for differentiation of *Mycoplasma* species by Razin and Rottem (1967). The present study was in accordance with Khan *et al*. (1986) who examined various strains of *M. gallisepticum* by SDS-PAGE and detected minor but distinct and reproducible differences in protein banding patterns between strains which included the vaccine F strain from various sources, an atypical (variant) strain, and the standard (A 5969, S6) strains. He observed that protein banding patterns in the lower part of the gel (approximately below the 65 kDa level) could be used to identify the species and variations in the banding pattern, particularly around the 68 kDa level, could be used for strain detection. In the present study these observations were similar for both the isolates indicating their homogeneity.

The principal reasons for examining whole-cell proteins were to achieve sufficient separation on the basis of molecular mass to allow individual proteins to be identified. This technique is suitable for most applications and allows direct comparisons to be made between different samples run on the same gel (Duffy *et al.*, 1998). Ferraz *et al*. (2000) subjected the *M. gallisepticum* strains (wild type S6 and a

vaccine strain F-K810) to polyacrylamide gel electrophoresis and differentiated both strains on SDS-PAGE analysis by a peptide band p75, which was specific for *M. gallisepticum* F-K810 strain.

5.6 Membrane protein preparation

Membrane proteins were extracted from late exponential phase cells of *M. gallisetpicum* by a mild alkaline-induced lysis of non-energized cells. Razin and Rottem (1967) disrupted cell membranes of *Mycoplasma* by osmotic lysis and the membrane proteins were separated electrophoretically in polyacrylamide gels. They found that electrophoretic patterns of membrane proteins were highly specific for the different *Mycoplasma* strains examined and the use of this method could prove the identity or dissimilarity of *Mycoplasma* strains. The membrane preparations produced by alkaline lysis method were found to be free of cytoplasmic contaminants through electron microscopy, chemical, density gradient and enzymatic analysis (Shirvan *et al*., 1982).

5.7 Profile of the whole cell extract and membrane proteins of *M. gallisepticum* grown under different conditions

In general, the whole cell extracts and membrane proteins of *M. gallisepticum* grown under iron restricted conditions showed an increase in concentration of proteins as estimated by Lowry's method, when compared to the whole cell and membrane proteins extracted from *M. gallisepticum* grown under iron sufficient conditions. This might be due to the up regulation of various genes and the subsequent expression of proteins when the bacterium is subjected to iron deprivation. This is in accordance with Madsen *et al*. (2006) who conducted a transcriptional profiling of *M. hyopnuemoniae* during iron depletion using microassay and identified that 27 genes were either up or down regulated in response to low-iron growth conditions.

In the profile of whole cell and membrane proteins on SDS-PAGE, bands were detected ranging in size from 24 kDa to 200 kDa. The whole cell proteins produced under iron sufficient and restricted conditions showed the presence of two major bands and eight minor bands that were not observed in iron sufficient and restricted membrane protein profile. Among the two major bands, one was in the region of 75 kDa and other in the region of 35 kDa. These extra bands observed in whole cell extracts might be the cytoplasmic proteins.

The whole cell proteins produced under iron sufficient and restricted conditions showed similar profile except for the extra band detected in the region of 52 kDa in iron restricted conditions. The membrane proteins produced under iron restricted conditions also showed the presence of one additional band in the region of 52 kDa, which indicated that the protein might be of membrane origin. This is in agreement with Harry and Potter (1989) who conducted a study on the outer membrane protein profile of *Actinobacillus pleuropneumoniae* grown under iron restricted conditions by polyacrylamide gel electrophoresis and immunoblotting. They found that a virulent serotype synthesized a novel protein.

5.8 Raising of hyper immune sera

Hyper-immune serum was raised against iron sufficient and iron restricted whole cell extracts and membrane proteins in rabbits. Eight rabbits were used in the study, two for each set of proteins.

5.9 Western blot

The antigenicity of iron sufficient and iron restricted whole cell extracts and membrane proteins was assessed by conducting immunoblot studies using rabbit anti-*M. gallisepticum* sera. The whole cell and membrane proteins produced under both conditions were found to be antigenic in western blot. In immunoblot, bands were detected ranging in size from 24 kDa to105 kDa. The whole cell proteins produced under iron sufficient and restricted conditions produced the same profile in immunoblot except for the presence of one extra band detected in the region of 52 kDa in iron restricted conditions. This was absent in the profile of whole cell proteins produced under iron sufficient conditions. In the profile of membrane proteins produced under iron restricted conditions also, the extra band in the region of 52 kDa was detected. Among the major cytoplasmic protein bands, one in 75 kDa region was highly antigenic as indicated by the density of the band, but the band in 35 kDa region was a faint one indicating low antigenicity. Other cytoplasmic proteins were meagerly detected in immunoblot. The antigenic membrane proteins detected were in the region of 24, 26, 35, 56, 64, 67, 77, 82 and 105 kDa, which was in accordance with the following studies where similar immunogenic protein bands were detected.

Avakian and Kleven (1990b) studied the humoral immune response in White Leghorn chicken experimentally infected with *M. gallisepticum* by immunoblotting

and identified six immunogenic species specific proteins of *M. gallisepticum* with relative molecular masses of 82, 64, 56, 35, 26 and 24 kDa.

Avakian *et al*. (1991) compared *M. gallisepticum* strains for identification of immunogenic integral membrane proteins (which included three immunogenic species specific proteins, p 64, p 56 and p 26) by immunoblotting and found that p 64 was detected in 18 of the 19 strains compared, p 26 in 13 strains and p 64 in all the strains. Jan *et al*. (1996) purified *M. gallisepticum* membrane proteins p 52, p 67 and p 77 by a high performance liquid chromatography and showed these three proteins as distinct antigens by western blotting and crossed immunoelectrophoresis.

5.10 Estimation of infective dose

Different dilutions from 10^{-1} to 10^{-5} of forty eight hour grown broth culture of *M. gallisepticum* were made and plated on PPLO agar plates in duplicate. The plates were incubated at 37°C under 5-10 per cent CO_2 tension in a humid atmosphere and the plates were observed daily under microscope for the presence of colonies. The visible growths of colonies were detected after three days and the counts were performed at the readable dilution. From these, the total counts present per ml of the original culture were calculated. This was found to be 7.1×10^8. From this, above mentioned dilutions were made and inoculated into five different groups of birds, each group containing six birds. A sixth group maintained as control was inoculated with plain PPLO broth.

The birds were sacrificed after a period of two weeks and the lesions in air sac were scored grossly. Tracheal lesions were scored after histopathological examination. The highest dilution of culture that showed significant lesion scores was taken as the infective dose for challenge studies. Nunoya *et al*. (1987) described that calculation of infective dose was required to establish screening methods for studying the pathogenicity of various strains of *Mycoplasma* in order to evaluate the effects of various immunization procedures. They found that scoring of lesions in air sac and trachea seemed to be a desirable method.

In the present study the lesions in the air sacs of birds from groups I, II, III and IV inoculated with the dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively were significantly ($p < 0.05$) greater than those of birds inoculated with 10^{-5} dilution and the control. The highest dilution that showed significant lesion score was 1.1×10^5. The birds inoculated with plain PPLO broth showed no lesions. The infective doses used

in other studies were 10^2 by Mc Martin and Adler (1961), 10^7 to 10^{10} by Adler and Da Silva (1970) and 1.3 x 10^5 by Barbour and Newman (1990).

5.11 Vaccination

Due to the limitations of available live vaccines, alternative vaccines are being sought. Vaccines in their simplest form consist of one or more purified subunits (antigens) and are presented to the host in the presence of an adjuvant, a substance that enhances the immune response. More complex vaccine forms may include killed pathogens or bacterins presented in conjunction with an adjuvant. Research regarding the use and further development of *M. gallisepticum* subunit and bacterin vaccines had been reviewed and although these vaccine types were generally protective, the application of the vaccines thus far developed might be limited due to costs associated with vaccination protocols and overall effectiveness (Ley, 2003).

Whole cell and membrane proteins were produced from log phase cultures of *M. gallisepticum* grown under iron sufficient and iron restricted conditions. For producing iron restricted condition, 2, 2′ dipyridyl was added to the log phase cultures of *M. gallisepticum* as per the method described by Madsen *et al*. (2006). Whole cell proteins were obtained through the process of sonication and membrane proteins by an alkaline lysis method. These proteins were used for the preparation of vaccines. The optimum concentration of the proteins used in the study for vaccine preparation was 84.2 µg/dose of the vaccines. This was in accordance with Barbour and Newman (1990) who conducted a study on efficacy of *M. gallisepticum* bacterin vaccines containing these protein concentrations per dose of vaccine with different adjuvants (liposome or oil) in laying hens. Results showed that the vaccines provided significantly good antibody response and protection against challenge infection.

Following inactivation with formalin (0.1 per cent final concentration) different sets of vaccines were subjected to sterility checking. Formalin concentration of 0.1 per cent has been successfully used to inactivate *M. gallisepticum* by Yagihashi *et al*. (1986). This concentration could preserve the antigen leading to an improved immune response. Yoder (1978) used 0.5 per cent formalin and 0.1 per cent betapropiolactone for the inactivation of a *M. gallisepticum* bacterin vaccine and there was no significant difference in the HI response in birds with both types of inactivating agents.

The vaccine preparations were found to be sterile as no growth was observed in PPLO agar, blood agar, tryptic soy agar and modified thioglycollate medium, even after seven days of incubation at 37°C under 5-10 per cent CO_2 tension. Also no growth was detected in the inoculated SDA plates kept at room temperature and at 37°C even after seven days.

The inactivated sterile vaccines were then formulated with adjuvants *viz*., aluminium hydroxide and saponin alone and in combination. The studies conducted by Yoder (1978) found that chicken inoculated with the adjuvanated vaccines developed and maintained higher HI titres than did chicken injected with only the inactivated preparations. Warren *et al*. (1968) described protection in chicken injected with formalin inactivated aluminium hydroxide gel adjuvanated vaccine. Protection against airsacculitis was used as an indication of protection. Yagihashi *et al*. (1986) conducted studies on immunity induced with aluminium hydroxide adsorbed *M. gallisepticum* bacterin in chickens and suggested that vaccination with inactivated bacterin might be an effective means for controlling *M. gallisepticum* infections.

Petrovsky and Aguilar (2004) reported that aluminium hydroxide had the capacity to evoke good humoral immune response but little capacity to elicit cell mediated immune response. Studies by Fernald *et al*. (1972) and Roberts (1973) indicated that CMI response also played an important role in the control of mycoplasmal respiratory tract diseases. Iqbal (2007) reviewed that saponin was a promising adjuvant for vaccine production against animals and man, as it was able to stimulate both humoral and cell mediated immune responses. Hence, a combination of saponin and aluminium hydroxide might be able to stimulate both the humoral and cellular arms of the immune system. So in the present study, vaccines were formulated with the adjuvant saponin alone and saponin and aluminium hydroxide in combination.

Safety testing of the prepared vaccine is very important to determine whether the vaccine may produce any adverse local or systemic reactions. This test was done by injecting double the field dose of each set of vaccine to different groups of experimental birds and no ill effects were observed. Production of vaccine requires careful safety and potency testing before release for use in the field. Potency testing of

vaccine was carried out by assessing the humoral and cell mediated immune response evoked by the different sets of vaccines in different groups of experimental birds.

5.12 Potency testing of vaccines

5.12.1 Assessment of humoral immune response

5.12.1a Haemagglutination/haemagglutination inhibition

Analysis of variance (one way) of the mean logarithmic values of HI titres of sera collected on different time intervals from different vaccinated groups and the control groups was done.

The antibody response was observed from day seven post vaccination onwards for all the vaccinated groups without significant difference. The antibody titre of all groups increased up to 14 days post vaccination without significant difference. On day 21 post vaccination, there was no significant increase in titre in all the vaccinated groups.

The HI titre induced by all types of vaccine was below the cut off value 21 days post vaccination. In the present study since we have made serial double dilutions for conducting HI test, we could not detect an HI titre between 64 and 128. Since an HI titre of 1:80 or above is needed to afford protection against *M. gallisepticum infection* (OIE, 2008), a booster dose was given to all vaccinated groups on 28 days post vaccination and blood was collected on 35th day for serum seperation. There was significant increase in HI titre in all the groups. Groups V, VI, VII, VIII and IX showed a significantly higher titre than other groups. The HI titre of the blood collected on 49th day showed almost similar titre without significant difference. But for the groups I, II, III and IV, the HI titre showed a decline in their values. The blood collected on 63rd day showed almost similar titre for the groups V, VI, VII, VIII and IX without significant difference, but for groups I, II, III and IV, the HI titre showed a further decline in their values.

Comparing groups V, VI, VII and VIII, a slight increase in HI response was seen in groups VI and VIII, vaccinated respectively with iron restricted whole cell and membrane protein vaccines adjuvanated with saponin-aluminium hydroxide combination but it was not statistically significant. Increase in HI titre observed in these groups could be attributed to the up regulation of many proteins and production of new immunogenic proteins in response to iron restricted conditions which evoked

the production of good antibody response. Lack of statistical significance might be due to the lesser number of birds included in our study.

The higher HI titres obtained in groups V, VI, VII and VIII would presumably be attributable to the purified saponin present in these vaccines. Effectively, this agent has been satisfactorily used in vaccines protecting against other diseases caused by *Mycoplasma* (Rurangirwa *et al*., 1987), and its use as a second adjuvant improves the immunogenic properties of aluminium hdroxide, avoiding the use of oil components (Rurangirwa *et al*., 1987; Walduck and Opdebeeck, 1996). Moreover, the concentration of saponin used in the present study (100 µg/dose) was insufficient to produce the adverse reactions associated with the higher concentrations of this agent (Walduck and Opdebeeck, 1996).

De la Fe *et al*. (2007) conducted a field trial of two dual inactivated vaccines incorporated with adjuvants aluminium hydroxide and saponin alone and in combination against *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *mycoides* in goats. The results showed that the serological response was better in goats injected with vaccines incorporated with saponin plus aluminium hydroxide. Their findings indicated the effectiveness of the vaccines in preventing the clinical signs such as mastitis, abortion, pneumonia and polyarthritis in herds affected with contagious caprine agalactia.

Generally the antibody levels of all the vaccinated groups were significantly higher than the unvaccinated control group throughout the study period. The commercial vaccine showed a similar response pattern when compared to the V, VI, VII and VIII groups, without significant difference.

5.12.2 Cell mediated immune response

Thymectomized birds showed an impaired resistance to *M. gallisepticum* infection, suggesting the importance of cell-mediated immune response in establishing a protective immunity against *M. gallisepticum* (Tiwary and Goel, 1986). Results from the study conducted by Gaunson *et al*. (2000) indicated that cytotoxic T cells play an important role in evoking immune response to *M. gallisepticum* infection, particularly in the acute phase of the disease. Kristensen *et al*. (1981) documented the presence of humoral and cell-mediated immune responses in pigs vaccinated with *M. hyopnuemoniae.*

5.12.2a Leukocyte migration inhibition test

Leukocyte migration inhibition test was carried out using the peripheral blood samples collected from experimental birds (six from each group) inoculated with different sets of vaccine. The test was conducted during three time intervals at third week post first vaccination and at first and third week post booster vaccination. The leukocyte migration index of all sets of vaccines was above 0.8, when blood samples were collected at third week post vaccination. Leukocyte migration index reached its peak on first week post booster vaccination in all vaccinated groups except the commercial vaccine group and slight but not significant reduction was noticed on third week post booster vaccination. The LMI response in group inoculated with commercial vaccine was significantly higher than the cut-off value of 0.8 required for a protective CMI response. The LMI response in control group of birds was significantly different from the vaccinated groups during all these time intervals.

Rajneesh and Srivastava (1993) observed a maximum leukocyte migration index in goats inoculated with *Mycoplasma capri* sonicated (MS) cells with saponin adjuvant at third week, MS with Freund's complete adjuvant at fourth week, MS with vitamin E at sixth week and MS with potash alum at sixth week post vaccination. In the present study, the highest LMI was observed at third week post booster vaccination in birds inoculated with iron restricted whole cell and membrane protein vaccines adjuvanated with saponin-aluminium hydroxide combination. This might be due to the presence of saponin because of its capacity to stimulate cell mediated as well as humoral immune response. The difference in time required to achieve the peak response in both the studies might be attributed to the difference in species of *Mycoplasma* and host system, which would have contributed to different patterns of immune response. There was no saponin in the commercial vaccine used in the present study and hence might have failed to evoke a significant CMI response.

5.12.2b Blastogenic calorimetry

The splenocyte proliferation assay was conducted using splenic cells collected from experimental birds (six from each group) inoculated with different types of *M. gallisepticum* vaccine. Splenic cells were collected from the sacrificed birds at fifth week post booster vaccination. The mitogen phytohaemagglutinin induced significantly high proliferation of splenocytes from different groups of vaccinated birds at fifth week post booster vaccination.

The mean stimulation index values of all the vaccinated groups were significantly higher when compared to the control group. Highest stimulation index was noticed in birds inoculated with iron restricted whole cell and membrane protein vaccines adjuvanated with saponin-aluminium hydroxide combination. This might be due to the presence of saponin which is capable of stimulating the cellular arm of immune response.

Saravanan *et al*. (2003) found that *in-vitro* methyl- thiazolyl-tetrazolium (MTT) assay was found to be a simple and convenient method for assessing the cell activation rate and growth, obviating the need for radioactive material for the assay. Studies conducted by Kristensen *et al*. (1981) on the cell mediated immune response in swine after vaccination against *M. hyopnuemoniae* demonstrated a higher SI in lymphocytes from vaccinated animals than in lymphocytes from non-vaccinated animals. Slight stimulation of lymphocytes was seen in commercial vaccine inoculated group. This was in accordance with Naot *et al*. (1977) who reported the mitogenic activity of various *Mycoplasma* species, but an apparent mitogenic effect could be explained as being due to contaminating serum components in *Mycoplasma* preparations (Nicolet *et al*., 1980).

5.13 Challenge studies

Challenge studies were conducted in the vaccinated birds to assess the efficacy of the different types of vaccine against *M. gallisepticum* infections. Two challenge studies were conducted at two weeks interval.

First challenge study was conducted on 38th day post vaccination. Eight birds were taken from a group for each set of vaccine and a control group consisting of eight birds was also included in the study. The birds were sacrificed after two weeks and examined for any lesions in air sac and trachea. The air sac lesions were scored grossly and the tracheal lesions scored after histopathological examination of the tissues. The results of the study were analyzed statistically. All the vaccinated groups offered more or less similar protection without significant difference. The mean lesion score produced by control group was significantly higher than the vaccinated groups.

The second challenge study was done on day 52nd day post vaccination. The birds were sacrificed after two weeks as mentioned above and the lesions were scored and statistically analyzed. The mean lesion scores obtained in groups V, VI, VII, VIII

and IX was significantly less than that obtained in other vaccinated groups. The control groups have the highest mean lesion score which was significantly higher than other vaccinated groups.

5.14 Re-isolation of *M. gallisepticum* from challenged birds

The air sac and tracheal samples were collected from birds with lesions. *Mycoplasma gallisepticum* could be re-isolated on PPLO agar from all control birds with lesions in the first challenge study and were confirmed by PCR, biochemical and serological tests.

In the second challenge study, even though all the birds in control group was infected, in 30 per cent of cases, infection was not due to *M. gallisepticum*, since no *Mycoplasma* colonies could be detected in PPLO agar plates. Also, the isolates produced growth on Mac Conkey's agar. *M. gallisepticum* will not produce growth on Mc Conkey's agar (OIE, 2008). The isolated organism was confirmed as *E. coli* by morphological, cultural and biochemical tests. These birds might have become predisposed to *E. coli* infection by stressful environmental condition such as increased atmospheric humidity due to heavy rainfall at that time.

In the first challenge study, the similar protective effect given by all vaccinated groups without statistical significance might be due to the presence of an HI titre greater than 1:80 (cut-off titre) in all the vaccinated groups. Additionally, the presence of a CMI response was detected in the vaccinated groups except in commercial vaccine group first week post booster vaccination, which might also have contributed to the protective effect. In the second challenge study, there was no statistical difference in the protective effect evoked by different vaccinated groups, but the groups V, VI, VII and VIII showed least lesions. This might be due to the reduction in HI titre in other vaccinated groups on 49th and 63rd day post vaccination, even though, the CMI response remained more or less the same on all vaccinated groups without statistical difference.

In the present study, the iron restricted protein vaccines adjuvanated with saponin-aluminum hydroxide evoked a higher HI titre and CMI response than iron sufficient protein vaccines adjuvanated with saponin-aluminum hydroxide, even though it was not statistically significant. This was in agreement with Salyers and Whitt (2002) who reported that during iron depletion, bacteria up regulate and down regulate many bacterial proteins and may also produce new immunogenic proteins.

This might have contributed to the improved response produced by these vaccines. The lack of statistical significance could be attributed to the lesser number of birds included in our study.

5.15 Latex agglutination test

Latex agglutination test was carried out using latex beads sensitized with sonicated *M. gallisepticum* iron restricted whole cell proteins. Anti- *M. gallisepticum* whole cell protein serum raised in rabbits was kept as positive control and phosphate buffered saline as negative control. A total of 30 serum samples from chickens were used for the test. The results were graded and compared with HI test. The LAT got a 95.24 per cent sensitivity which indicated that the LAT failed to detect two HI positive samples as positive. The specificity was 93.33 per cent which means that one sample negative in HI came positive in LAT. This was in agreement with Ramadass *et al*. (2007), who developed a latex agglutination test using *M. gallisepticum* PG 31 strain and compared it with ELISA. He found that LAT and ELISA were of equal sensitivity.

In rapid serum agglutination test, small size of the *Mycoplasma* cells requires large number of cells to form visible aggregates or aggregates which will settle readily by gravity. Absorbing *Mycoplasma* cells to carrier particles like latex offers a means of increasing the mass of the cells and, at the same time, maintaining the specificity of the *Mycoplasma* surface antigens (Morton, 1966).

5.16 Preservation of the isolates

Log phase cultures of *M. gallisepticum* were lyophilized and stored at -70°C. Kelton (1964) reported that lyophilized cultures of *Mycoplasma* were preserved up to four years whereas Harry, (1964) could obtain viable mycoplasmas after fourteen years of storage in the freeze-dried state at 4°C.

In brief, saponin-aluminium hydroxide adjuvanated whole cell and membrane protein vaccines, especially those produced under iron restricted conditions, could be an effective tool to afford protection against *M. gallisepticum infection*, as it would stimulate both humoral and cell mediated immune responses in host. Commercial vaccine used in the study for comparison failed to evoke a protective CMI response. The LAT developed in the present study using locally predominant strain of *M. gallisepticum* would prove particularly useful in identifying infected birds as it

combines sensitivity with low cost and ease of application in the field, without the need for any trained specialist or equipment.

The future study should be aimed at the evaluation of saponin-aluminium hydroxide adjuvanated whole cell and membrane protein vaccines produced under iron restricted conditions employing more number of birds. Also, the study period should be extended, in order to see whether the humoral and CMI responses evoked by the vaccines will be maintained for a long time. In the case of Latex agglutination test, more number of sera samples should be screened to confirm the sensitivity and specificity of the test in comparison with HI test.

Summary

SUMMARY

Compared with rest of the livestock sector, the poultry industry in India is more scientific, well organized and is progressing towards modernization. The success story of Indian poultry industry is uniquely exceptional. From a backyard venture, it has made a quantum leap to emerge as a dynamic and sophisticated agro-based industry. But the problems facing the industry are many and diverse, the major problem being the lack of adequate facilities for the diagnosis and prevention of emerging diseases. Intensive breeding and managemental practices to enhance the production have greatly contributed to higher incidence of several infectious diseases.

Among the various ailments affecting poultry, mycoplasmosis is an economically decrepitating disease, causing significant reduction in growth and egg production. The infection is of larger duration and persists in the flock indefinitely, with intermittent shedding of the organism following a period of stress. Therefore, the elimination of the organism is extremely difficult and so vaccination is the only remedy to control mycoplasmosis.

Currently two types of vaccines are available against *M. gallisepticum* infection, live and inactivated vaccines. The currently available live attenuated *M. gallisepticum* vaccines have many drawbacks like transmissibility, chances of reversion and less protective effect. Among the inactivated vaccines, none has earned universal acceptance. Ideally *M. gallisepticum* vaccine should be safe and effective, preventing infections as well as disease, for all types of poultry and in multiple-age flocks, should also be capable of displacing wild-type *M. gallisepticum* and allowing a return to *M. gallisepticum* free status when vaccination is stopped.

Adjuvants have been used since long for the augmentation of immune response of inactivated vaccines in host against various diseases of mycoplasmal origin. They can increase the immunogenicity of poor antigen, improve the efficacy of vaccine, and reduce the amount of antigen or the number of immunizations. Inactivated vaccines using *M. gallisepticum* immunogens with improved adjuvants or antigen delivery system might be capable of achieving a return to *M. gallisepticum* free status.

There were no reports of the efficacy of currently used aluminium hydroxide adjuvanted vaccines on CMI response. Studies on CMI response in *Mycoplasma* infections in several species have indicated that it is involved in the development of immunity to mycoplasmal respiratory tract disease. So the present study was designed in such a way to develop a vaccine incorporating an adjuvant which is capable of stimulating CMI response also. Saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production with only a low dose needed for adjuvant activity.

Iron is an essential metal for almost all living systems as a cofactor or as prosthetic group for various enzymes involved in basic cellular functions. Iron deprivation by sequestering the available iron in iron binding proteins is a prominent feature of the host defense mechanism to microbial infections. Bacteria have alternate means of iron acquisition which includes up and down regulation of various existing proteins and the production of many new proteins which might be immunogenic.

Taking all these into consideration, the present study was undertaken to develop and evaluate the efficacy of vaccines against *M. gallisepticum* infections using whole cell and membrane proteins produced under iron sufficient and restricted conditions incorporating the adjuvants aluminium hydroxide and saponin either alone or in combination

A total of 50 samples, including tracheal and conjuctival swabs, lung and air sac materials, were collected from birds showing respiratory ailments. The samples were collected from University poultry farm, Mannuthy, poultry farms in different parts of Kerala, birds brought to Dept. of Veterinary Microbiology for disease diagnosis and also from birds necropsied in Centre of Excellence in Pathology, College of Veterinary and Animal Sciences, Mannuthy. In addition to collection in broth, 25 tracheal swab samples were streaked directly onto PPLO agar plates. The samples after collection were transported to the laboratory under cold conditions and processed immediately. The inoculated PPLO broths and agar plates were transferred without delay to a candle jar and incubated at 37°C under 5-10 per cent CO_2 tension and in addition, a humid chamber was provided for the inoculated agar plates. One milliliter of broth was taken after four hour incubation and processed for extraction of DNA. *Mycoplasma* genus specific PCR was performed using these DNA with specific primers. Among the 50 samples that collected in broth, eight were found positive for genus specific PCR. The twenty five tracheal swab samples that streaked directly onto

agar plates were examined daily for the presence of characteristic colonies, which were picked up and dispensed in PPLO broth for extraction of DNA.

Out of 25 tracheal swab samples, 12 were found positive for genus specific PCR, including four samples that were negative for genus specific PCR when DNA extracted from broth was used as template. Thus, a total of 12 samples were positive for genus specific PCR. The samples that came positive in genus specific PCR were incubated till a colour change to yellow was observed or after 24 h incubation, whichever was earlier. The samples was then subjected to species specific PCR. Among 12 genus specific PCR positive samples, four were positive for species specific PCR. The PCR negative samples were serially diluted and plated onto PPLO agar plates in an attempt to dilute out any inhibitory substance present. The plates and broths were kept up to 21 days before discarding. From four species specific PCR samples, two isolates could be obtained *viz*., MG UPF-1 and MG UPF-2. They were sub cultured and maintained for further characterization.

For morphological characterization, the colonies obtained on PPLO agar plates were observed under 4 × and 10 × magnifications of the microscope. Colonies with typical "fried egg" appearance was obtained with a dark raised centre and a depressed light coloured periphery. The colonies when subjected to staining with Diene's stain, showed a dark blue centre and a light periphery.

The isolates gave a positive test for fermentation of glucose and fructose but had negative response for galactose, mannose, cellobiose and xylose fermentation. The test for arginine hydrolysis was also negative.

After biochemical characterization the colonies were subjected to serological characterization. The growth inhibition test revealed the zones of inhibition around *M. gallisepticum* anti-serum impregnated filter paper disc applied onto the surface of agar plates containing colonies whereas, no inhibition zone was detected around normal rabbit sera impregnated disc.

Immunoperoxidase test was carried out on imprints of the colonies on nitrocellulose membrane. Presence of brownish spots representing individual colonies were obtained after addition of horse raddish peroxidase enzyme conjugated secondary antibody followed by substrate solution. In negative control no discolouration was observed.

The colonies on agar plates were subjected to indirect fluorescent antibody test. The presence of a greenish fluorescence was obtained after adding the secondary antibody conjugated with flouroscene isothiocyanate dye followed by diaminobenzidine substrate solution. No fluorescence was detected in negative control.

For differentiation of the isolates, whole cell proteins were extracted by sonication and subjected to SDS-PAGE. The whole cell protein profile in the present study showed protein bands ranging in size from 24 kDa to 200 kDa. The protein profile of the two isolates obtained on PAGE revealed same banding pattern, indicating their homogeneity.

Four sets of cultures of *M. gallisepticum* was grown to late exponential phase and for producing iron restricted conditions, 2, 2′ dipyridyl was added to two sets of culture. All the four sets were incubated for two more hours and cells were harvested by centrifugation and washed twice in PBS by centrifugation. Whole cell proteins were produced by the sonication of cells and membrane proteins by an alkaline lysis method. The concentration of whole cell and membrane proteins produced under iron sufficient and restricted conditions were estimated by Lowry's method. The concentration of different proteins obtained were 860 µg/ml, 910 µg/ml, 440 µg/ml and 490 µg/ml, respectively, for whole cell proteins, iron restricted whole cell proteins, membrane proteins and iron restricted membrane proteins respectively. In general, the concentration of iron restricted whole cell and membrane proteins were higher than that of the same proteins produced under iron sufficient conditions.

The profile of the iron sufficient and restricted whole cell and membrane proteins were analyzed in SDS-PAGE. In the profile of whole cell and membrane proteins on SDS-PAGE, bands were detected ranging in size from 24 kDa to 200 kDa. The profile of the whole cell proteins produced under iron sufficient and restricted conditions were same except for the presence of an additional band of 52 kDa seen only in iron restricted proteins. The profile of membrane proteins produced under both conditions was also the same except for the presence of additional band at 52 kDa region for iron restricted membrane proteins. Among the whole cell and membrane proteins, whole cell protein profile showed the presence of 10 additional bands, two prominent bands at 75 kDa and 35 kDa and eight faint bands, which were cytoplasmic proteins and were absent in membrane protein profile.

Hyper-immune serum was raised in eight Newzealand White rabbits, against these four different sets of proteins to assess their antigenicity in Western blot. The

whole cell proteins produced under iron sufficient and restricted conditions produced almost same profile in immunoblot except for the presence of one extra band detected in the region of 52 kDa which was absent in iron sufficient whole cell proteins. The profile of membrane proteins produced under iron sufficient and restricted conditions were also the same, except for the extra band at the region of 52 kDa detected in iron restricted proteins. The cytoplasmic protein band at 75 kDa region was highly antigenic indicated by the density of the band, but the band at 35 kDa region was a faint one. Other cytoplasmic proteins were meagerly detected in immunoblot. The antigenic membrane proteins detected were in the region of 24, 26, 35, 52, 56, 64, 67, 77, 82 and 105 kDa.

The optimum concentration of 84.2 µg per dose of iron sufficient and restricted whole cell and membrane proteins were inactivated with formalin at a final concentration of 0.1 per cent. The sterility of the vaccines were checked in different media like PPLO broth, TSA, blood agar, modified thioglycollate medium and SDA, and no growth was detected. The vaccines were then formulated with saponin and aluminium hydroxide alone and in combination at a final concentration of 100g/dose and 25% v/v respectively, thereby producing eight different sets of vaccines. The safety of the adjuvant formulated vaccines was checked in eight chicks of five weeks age group and no untoward reactions were observed.

The infective dose of *M. gallisepticum* cells was determined by inoculating different dilutions of 48 h grown cultures in different group's chicks of 5 weeks age group, with 6birds in each group. The birds were sacrificed after two weeks and the tracheal and air sac lesions of birds were scored. The highest dilution of *M. gallisepticum* cells showing significant lesions was taken as the challenge dose and this was found to be 1.1×10^5.

For vaccination studies, 240 chicks of five weeks age were divided into 10 groups and were inoculated with eight different sets of vaccines. One group was inoculated with commercial vaccine and other was kept as control.

Serum samples were collected from different groups of birds pre-vaccination and thereafter on 7, 14 and 21st day post vaccination. The data were analyzed statistically using ANOVA. Antibody response started as early as seven days post vaccination as determined by mean $\log_2$HI titre in all vaccinated groups. The HI titre

increased in day 14 in all groups without significant difference. The response in day 21 remained the same. Since the mean log_2 HI titre was below the cut-off value 1:80 as specified by OIE, a booster dose of different vaccine was given to different groups on day 28. Serum was collected post-booster dose on 35^{th}, 49^{th} and 63^{rd} day. The mean HI titre of groups vaccinated with iron sufficient and restricted saponin adjuvanted vaccines showed an increase on day 35^{th} followed by decline of the titre on subsequent collections. But the mean HI titres of groups vaccinated with iron sufficient and restricted saponin-aluminium hydroxide adjuvanted vaccines maintained their titres without decline and were significantly different from above mentioned groups. Among these groups, again iron restricted saponin-aluminium hydroxide adjuvanted vaccines showed a slight increase in their titres, but this was not statistically significant. Commercial vaccine group also showed a similar response. The control group showed significantly different titre from other vaccinated groups during the entire time period.

In order to assess the CMI response evoked by different sets of vaccines, LMIT and Blastogenic calorimetry were carried out.

For LMIT, the peripheral blood samples were collected during three different time periods (third week post first vaccination, first and third week post booster dose) from birds inoculated with different sets of vaccines. Six birds were included from each group. The data was analyzed using a T-test. The migration index of vaccinated groups did not show any significant difference on third week post first vaccination. During first week post booster collection, the index of vaccinated groups differed significantly from commercial vaccine group as indicated by a migration index less than 0.8. On third week post booster collection, migration index of all vaccinated groups remained more or less the same. Again migration index of commercial vaccine group was above 0.8. The mean leukocyte migration index of the control group was significantly different from other groups during all time intervals.

Blastogenic calorimetric study was conducted using spleenic cells harvested from birds of different vaccinated groups on fifth week post booster vaccination. Six birds were included from each group. The mean stimulation index values of all the vaccinated groups were significantly higher as compared to the control group. The index of commercial vaccine group was significantly less than that of other groups.

The highest stimulation index was seen in birds inoculated with iron restricted saponin-aluminium hydroxide adjuvanted vaccines.

To assess the protective response evoked by different vaccines, two challenge studies were conducted two weeks apart on the 38th and 52nd day post vaccination. In the first challenge study, all the vaccinated groups offered more or less similar protection as indicated by least lesion scores, without significant difference. In second challenge study, the mean lesion scores obtained in groups V, VI, VII, VIII and IX was significantly less than that obtained in other vaccinated groups. The control groups have the highest mean lesion score which was significantly higher than other vaccinated groups.

Mycoplasma gallisepticum was re-isolated from all challenged control birds with lesions during the first challenge study. In second challenge study the isolation rate was 70 per cent.

A latex agglutination test was developed using iron restricted *M. gallisepticum* whole cell proteins. The commercial latex beads were purchased and sensitized with *M. gallisepticum* proteins. Twenty five microlitres each of sensitized beads and test serum samples were mixed in a glass slide by gentle rocking movement. A total of 30 sera samples were subjected to the test and a positive reaction were indicated by the presence of agglutinated latex particles at periphery of slide in two to five minutes and a negative reaction was indicated by a uniform suspension. The results were compared with HI test and LAT had got a sensitivity of 95 per cent and specificity of 93 per cent. The test was statistically significant also as indicated by a kappa value greater than 0.81.

To conclude, with regard to humoral immune response the saponin cum aluminium hydroxide adjuvanted vaccines produced under iron restricted conditions produced a high protective titre than saponin-aluminium hydroxide adjuvanted vaccines produced under iron sufficient conditions, even though it was not statistically significant. The response obtained with commercial vaccine group was more or less similar. With regard to CMI response, the experimental vaccines produced a significant CMI response whereas commercial vaccines failed to evoke a protective CMI response. Latex agglutination test was found to be an effective tool for flock screening of birds for *M. gallisepticum* antibodies and the test is simple, rapid, and easy to conduct under field conditions.

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DEVELOPMENT AND EVALUATION OF WHOLE CELL AND MEMBRANE PROTEIN VACCINES AGAINST *Mycoplasma gallisepticum*

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ABSTRACT

A study was carried out to develop and evaluate the efficacy of vaccines against *M. gallisepticum* infections in *Gallus domesticus* using whole cell and membrane proteins produced under iron sufficient and restricted conditions, incorporating the adjuvants aluminium hydroxide and saponin either alone or in combination.

A total of 50 samples were collected in broth and in addition 25 tracheal swabs were streaked onto PPLO agar plates also, from birds showing respiratory ailments. *Mycoplasma* genus specific PCR revealed twelve positive samples and on subjecting these samples to species specific PCR, a total of four samples showed positive results. From four species specific PCR samples, two isolates could be obtained, named as MG UPF-1 and MG UPF-2, which were sub cultured and subjected to morphological, biochemical and serological characterization. The SDS-PAGE profile of whole cell proteins of the two isolates revealed similar profile, indicating their homogeneity.

Whole cell and membrane proteins were isolated from cultures grown under iron sufficient and iron deficient conditions. Both the whole cell and membrane proteins revealed bands ranging from 24 kDa to 200 kDa on SDS-PAGE. The whole cell proteins had two prominent bands at 75 kDa and 35 kDa and eight faint bands, which the membrane proteins lacked. The whole cell and membrane proteins produced under iron restricted conditions had an additional band of 52 kDa, which was not seen in the respective protein groups isolated under iron sufficient condition. Most of the proteins resolved on SDS-PAGE in the four sets of proteins were found to be antigenic as found out by western blot analysis.

The optimum concentration of the proteins used for vaccine preparation was 84.2 µg per dose of the vaccine. Formalin inactivated vaccines were formulated with saponin and aluminium hydroxide alone and in combination at a final concentration of 100 µg/dose and 25 per cent v/v respectively, thereby producing eight different sets of vaccines. The sterility and safety testing of the vaccines were carried before vaccination trials.

The infective dose of *M. gallisepticum* cells for challenge studies was found to be 1.1×10^5. For vaccination studies, 240 chicks of five weeks age were divided into 10 groups and were inoculated with eight different sets of vaccines. One group was inoculated with commercial vaccine and other was kept as control. Potency testing for humoral response was conducted with HI test and CMI response with LMIT and Blastogenic calorimetry. The mean HI titre of groups vaccinated with iron sufficient

and restricted saponin adjuvanated vaccines showed an increase in their titre at first week post booster vaccination followed by decline of the titre on subsequent collections on third and fifth week post booster vaccination. The mean HI titres of groups vaccinated with iron sufficient and restricted saponin-aluminium hydroxide adjuvanated vaccines maintained significantly high HI titres during the entire study period. Commercial vaccine group also showed a similar response. The control group showed significantly less titre from other vaccinated groups during the entire time period.

Leukocyte migration inhibition test and Blastogenic calorimetry were carried out to assess the CMI response evoked by different sets of vaccines. A significant CMI response was shown by all vaccinated groups except commercial vaccine and control group.

To assess the protective response evoked by different vaccines, two challenge studies were conducted two weeks apart. All the vaccinated birds were having significantly lower lesion score when compared to the control group. *Mycoplasma gallisepticum* was re-isolated from all the control birds with lesions during the first challenge study and from 70 per cent of the birds in the second challenge study.

A latex agglutination test was developed using iron restricted *M. gallisepticum* whole cell proteins. When compared with HI test, LAT had a sensitivity of 95 per cent and specificity of 93 per cent. The test was statistically significant as indicated by a kappa value greater than 0.81.

The saponin-aluminium hydroxide adjuvanated whole cell and membrane protein vaccines had elicited similar humoral immune response than vaccines adjuvanated with saponin alone. With regard to CMI response, the experimental vaccines produced a significant CMI response whereas, commercial vaccines failed to evoke a protective CMI response. In brief, saponin-aluminium hydroxide adjuvanated whole cell and membrane protein vaccines, especially those produced under iron restricted conditions, could be an effective tool to afford protection against *M. gallisepticum infection*, as it would stimulate both humoral and cell mediated immune responses in host

Latex agglutination test was found to be an effective tool for flock screening of birds for *M. gallisepticum* antibodies and the test is simple, rapid, and easy to conduct, with applicability under field conditions.