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MOLECULAR METHODS BASED DETECTION OF PATHOGENIC MYCOPLASMAS OF CHICKEN

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Thesis submitted in partial fulfilment of the requirement for the degree of

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2006

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DECLARATION

I hereby declare that this thesis entitled **"MOLECULAR METHODS BASED DETECTION OF PATHOGENIC MYCOPLASMAS OF CHICKEN"** 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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CERTIFICATE

Certified that the thesis entitled **"MOLECULAR METHODS BASED DETECTION OF PATHOGENIC MYCOPLASMAS OF CHICKEN"** is a record of research work done independently by **Dr. Dipu. M.K.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Introduction

1. INTRODUCTION

Poultry farming is recognized as one of the best in agriculture to provide protein source diet in South Asian countries due to explosion of human population, stagnation of pulse production, decreased soil fertility and change in climate. India ranks fourth in the world with 185 million birds (55 per cent of South Asia) and 2.2 million tons of egg production by the end of the year 2003. About four million people in India thrive on various poultry activities. In the chicken meat ranking table India has moved to the fifth place. Forty two per cent of the total birds in India are confined to the southern region. The increasing demand for poultry products has transformed poultry sector activity into a full-fledged industry from a mere household/backyard activity until recently.

In light of the growing importance of poultry sector to the economy it is critical to comprehend the various factors which may adversely affect the production performances of poultry including the quality and quantity of the feed, water intake, intensity and duration of light received, parasitic infestation, numerous managemental and environmental factors and diseases affecting the poultry. Of the various economically decrepitating diseases affecting the poultry, *Mycoplasma* infection has a special significance.

Bacteria referred to as mycoplasmas or "fungus forms" are phenotypically distinguished from other bacteria by their minute size (0.3-0.8 micrometer) and total lack of cell wall. The special appeal in the study of mycoplasmas stems from the fact that they are the smallest and simplest self-replicating prokaryotes. Mycoplasmas occur widely in nature, as parasites or commensals, not only of poultry but also of humans, other primates, mammals, reptiles, fishes and of many plants. They are mainly inhabitants of the host mucous membranes and possess the ability to adhere

to mucosal or epithelial surfaces lining the oral cavity and the respiratory and urogenital tracts of vertebrates. Transmission occurs directly through contact, egg transmission, genital route and secondary transmission through aerosols and fomites, food, water, insect vectors or carriers had been documented.

The pathogenic mycoplasmas of chicken include the *Mycoplasma gallisepticum*, the causative agent of Chronic Respiratory Disease (CRD); *Mycoplasma synoviae*, the causative agent of Infectious synovitis; *Mycoplasma iowae*, associated with reduced hatchability. *M. gallisepticum* infection accounts for decreased growth and decreased egg production. *Mycoplasma synoviae* causes sub clinical upper respiratory tract infection and may be involved in airsacculitis associated with Newcastle disease and infectious bronchitis.

When a chicken is infected with *M. gallisepticum*, the infection is of longer duration and the infection persists in the flock indefinitely and the chickens may shed the organism intermittently, especially following a period of stress. This characteristic makes the elimination of *M. gallisepticum* extremely difficult in multi-age breeder and laying complexes. If a laying flock is infected with *M. gallisepticum*, a successful elimination of the infection is possible only by the complete depopulation of the farm at the end of the laying cycle and providing down-time prior to the re-introduction of chickens. This has to be followed up with stringent control measures, vaccination programme and routine monitoring.

Thus, taking into account the immense economic significance and persistence of avian mycoplasmas, it is imperative to detect the infection in a flock at the earliest. Serology based detection of the disease is mostly of a retrospective kind and is not a method of choice that could be adopted in a control programme as it will not

aid in the early diagnosis of the disease. Antigen detection methods are superior when the aim is for an earlier, accurate and definitive diagnosis.

Demonstration of the organism or its nucleic acid is equally valuable in the diagnosis of a current infection. Direct demonstration of *Mycoplasma* in the clinical samples or culture by immunofluorescence has been widely adopted, for which specific antisera have to be maintained. *M. gallisepticum* strains vary widely in antigenic makeup and have the ability to alter the expression of major surface antigenic proteins. Although less well studied, strains of *M. synoviae*, *M. meleagridis*, and *M. iowae* appear to exhibit similar variability. This peculiar characteristic of *Mycoplasma* poses a serious hurdle in the diagnosis or if so in the differentiation of the organism.

Similar difficulties are encountered while adopting Enzyme Linked Immunosorbent Assay (ELISA) for diagnosis. It is often noted that flocks infected with *M. gallisepticum* strains may not give positive results with the Haemagglutination-inhibition tests because of strain variation (Kleven *et al.*, 1998). Same is the hurdle when monoclonal antibodies are used for detection of mycoplasmas.

As far as the isolation of mycoplasmas from clinical specimens are concerned, it is dependant upon a number of critical conditions including the presence of specific antibodies or antibiotics in the specimen, occurrence of inhibitory components in many cells and tissues, application of improved laboratory cultural technique and particularly the difficulty in the identification of the isolate which require a large battery of species-specific antisera and seed. It takes as long as 14 days to judge a sample to be negative on the basis of isolation trials. Moreover it is not unusual that the pathogenic and more exacting mycoplasmas getting

overgrown by the less fastidious ones. This is especially true in the case with *M. synoviae*. It is highly impractical to rely on the above methods for an earlier detection and differentiation of mycoplasmas.

On the contrary, *in vitro* methods of DNA amplification as Polymerase Chain Reaction (PCR) have large potential not only in the rapid detection but also in species differentiation of mycoplasmal infections of poultry at a single shot. The PCR assay is a very sensitive technique which can detect fewer particles of mycoplasmas, no matter whether viable or nonviable, than the conventional methods. The stable nature of the nucleic acid makes it possible to detect even the nonviable organisms by PCR.

The introduction of PCR has pushed aside the previously developed DNA probes, by providing faster and much more sensitive tests. The sensitive level of a PCR test can be as low as a single organism, enabling detection of mycoplasmas in poultry treated with antibiotics and in asymptomatic birds. A positive result is obtained with PCR much prior to that of serological response and is also effective in immunocompromised hosts. Polymerase chain reaction was shown to be most valuable in detection and identification of the non-culturable plant and insect-mycoplasma like organisms (MLOs).

Mycoplasmas are highly fragile and osmotically labile, which necessitate expensive transport media and immense care while transportation of the samples to the laboratory. This could be circumvented if PCR is adopted as the diagnostic tool, for the viability of the organism is not a critical factor affecting PCR. The PCR is a highly user friendly and simpler technique as compared to other diagnostic assays. Depending upon the primer sequences one select, a very high specificity equivalent to that of cultural procedure is often obtained with PCR. A comprehension on the

prevalent pathogenic species of avian mycoplasma in the region is an important basis for adopting effective treatment and adequate control measures. Thus application of molecular methods for the early detection of pathogenic mycoplasmas of chicken was considered worth studying. Moreover due to the wide strain variation among *M. gallisepticum*, an attempt to delineate the different strains of *M. gallisepticum* prevalent shall still foster the beneficial dimensions of the work.

On account of the above-discussed details, the present work has been undertaken with objectives laid down as

1. Detection of *Mycoplasma* DNA from the specimens by Polymerase Chain Reaction (PCR).

2. To differentiate the three significantly pathogenic mycoplasmas of chicken namely *M. gallisepticum*, *M. synoviae* and *M. iowae* from the other less pathogenic ones based on the result of polymerase chain reaction

3. Isolation of *Mycoplasma* from clinical samples testing positive by polymerase chain reaction and also from a few randomly selected negative samples.

4. To comprehend the different strains of *M. gallisepticum*, if any, among chicken of various age groups.

Review of Literature

2. REVIEW OF LITERATURE

Genus referred to as *Mycoplasma* belongs to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae and are minute cocco-bacillary bodies. *Mycoplasma* spp. was probably first encountered in chickens during the 1930s by Nelson.

Most important species of avian mycoplasma which is of greater concern is *Mycoplasma gallisepticum*. Other pathogenic mycoplasmas infecting poultry are *Mycoplasma synoviae, Mycoplasma iowae* in the chicken and turkey, *Mycoplasma meleagridis* in the turkey. *Mycoplasma gallisepticum* is particularly important in the chickens and turkeys as a cause of respiratory disease and decreased production (Jordan, 1979; Ley and Yoder, 1997). More recently it has been recognized as a cause of conjunctivitis in house finches in North America (Ley *et al.*, 1996; Luttrell *et al.*, 1996).

Of the various methods employed for the identification of mycoplasmas, molecular methods based tests are found to be highly specific and sensitive which enable rapid identification even in the absence of viable organisms. (Ley *et al.*, 1993).

2.1 HISTORY

Mycoplasma galllisepticum is one of the pathogens causing heavy economic loss to the poultry industry and is reported to have world wide distribution. Nelson (1936) isolated avian mycoplasma for the first time and described it as cocco-bacillary form bodies in coryza of chicken.

Markham and Wong (1952) demonstrated that the causative agents of Chronic Respiratory Disease (CRD) of chicken and Infectious Sinusitis of

turkeys were members of the pleuro-pneumonia group of bacteria, which opened an era of active research on avian mycoplasmosis.

Johnson and Domermuth (1956) reported the presence of Pleuro-Pneumonia Like Organisms (PPLO) in chicks and poults in small numbers by egg transmission, which led to clinical disease on subsequent exposure to viral infection or stress.

Edward and Kanarek (1960) suggested the name *Mycoplasma gallisepticum* to the cocco-bacillary form bodies reported by Nelson (1936) in coryza of chicken.

Mycoplasma gallisepticum was isolated from the brain tissue of naturally infected turkey by Zander (1961) and subcultured in the laboratory as his sixth stock culture and named it as S6 strain.

Jordan (1979) had reported the importance of *Mycoplasma gallisepticum* as a cause of respiratory disease and decreased egg production in chicken and turkeys. He also had formulated suitable culture media for the isolation of *Mycoplasma.*

2.2 DISEASE

More than one species of *Mycoplasma* infecting the same tissue in turkeys was reported by Jordan and Amin (1980).

Davidson *et al.* (1981) described unilateral or bilateral periocular swelling along with respiratory signs and sinusitis in infectious sinusitis of wild turkeys.

Reece *et al.* (1986) described conjunctivitis, sinusitis, mucoid tracheitis and airsacculitis in Infectious sinusitis caused by *Mycoplasma gallisepticum* in Japanese quails, chukar partridges and golden pheasants.

Mohammed *et al.* (1987) reported marked reduction in egg production in hens infected with *Mycoplasma gallisepticum*

Natural infection of *Mycoplasma gallisepticum* in pheasants, chukar partridges and peafowl characterized by foamy eyes, swollen infra orbital sinuses, respiratory distress and death was described by Cookson and Shivprasad (1994).

Occurrence of kerato-conjuctivitis in chickens associated with *Mycoplasma gallisepticum* was reported by Nunoya *et al.* (1995). He also found that the gross and microscopical lesions were similar to those in finch conjunctivitis caused by *Mycoplasma gallisepticum*.

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

Mycoplasma gallisepticum was described as the etiological agent of naturally acquired house finch conjunctivitis and 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).

Ley and Yoder (1997) found that although *Mycoplasma gallisepticum* has been associated with conjunctivitis in game birds and commercial layer chickens, it has not been considered as a natural pathogen of wild birds.

Mycoplasma gallisepticum strain isolated from house finches experimentally infected both turkeys and chickens (O'Connor *et al.*, 1997).

Naturally occurring mycoplasmal conjunctivitis was reported among wild-caught and initially sero-negative house finches maintained in captivity for

12 weeks (Luttrel *et al.*, 1998). Severe bilateral or unilateral ocular swelling, conjunctivitis and often death characterized the disease. They opined that infected birds without lesions act as asymptomatic carriers in the wild.

Mycoplasma gallinarum although regarded pathogenic, was found to induce fatty liver haemorrhagic syndrome in birds experimentally inoculated with them (Branton *et al.*, 2003).

2.3 INCIDENCE

Natural infections of *Mycoplasma gallisepticum* leading to conjunctivitis have been described in pheasants, chukar partridges, peafowl and quail (Wills, 1955; Wichman, 1957 and Reece *et al.*, 1986).

Organisms that have been encountered in concurrent infection with *Mycoplasma gallisepticum* were *Haemophilus gallinarum* (Kato, 1965), New castle disease virus (Timms, 1972) and *Escherichia coli* (Gross, 1961).

Mycoplasma gallisepticum strain, GM1125 was isolated and identified as a cause of bilateral conjunctivitis and sinusitis-respiratory disease outbreak in an integrated chukar partridges operation by McMartin *et al.* (1996). The morbidity reached 100 per cent while 60 per cent of losses occurred due to mortality and culling.

The prevalence of mycoplasmal conjunctivitis in house finches was studied by Dhondt *et al.* (1998) and they 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.

2.4 TRANSMISSION

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

Jordan (1979) observed that both egg and airborne transmission may be influenced by intercurrent infection and probably by other non-infectious factors, namely, trauma, excess of ammonia and social stress.

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

The studies conducted by Bencina *et al.* (1987) revealed that the chickens remained as *Mycoplasma gallisepticum* carriers many months after the acute phase of infection and they showed no signs of the disease.

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

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

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

Hartup and Kollias (1999) conducted a field study to investigate the occurrence of *Mycoplasma gallisepticum* in eggs and nestlings from nests of

house finches by polymerase chain reaction, isolation and serologic tests. The results of their study suggested that pseudovertical transmission of *Mycoplasma gallisepticum* between the breeding adults and their dependant offspring as the main mode of transmission of infection and there were no evidence supporting the transovarian transmission of *Mycoplasma gallisepticum* in these house finches.

2.5 DIAGNOSIS

2.5.1 Isolation

Avian mycoplasma was first isolated by Nelson (1936).

Delaplane and Stuart (1943) cultivated an agent in embryonated chicken eggs, which was associated with CRD of chicken, although they did not identify the agent as pleuro-pnuemonia like organisms.

Conversely, Van Herick and Eaton (1945) isolated a PPLO in artificial medium from embryonated chicken eggs, although they did not associate the organisms with respiratory disease of chicken.

Medium that contained beef heart infusion and yeast extract enriched with equine serum was used by Markham and Wong (1952) for the isolation of *Mycoplasma gallisepticum.*

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

Alder *et al.* (1954) suggested a medium composed of 10 per cent blood agar slope overlaid with 20 per cent horse serum. Among several media tested,

they found that PPLO broth was more satisfactory than agar for the isolation of *Mycoplasma gallisepticum.*

Lecce and Sperling (1954) proposed heart infusion agar with thallium acetate as selective medium for the growth of avian strains of PPLO.

Modified Grumble's medium was advised by Taylor and Fabricant (1957) for the isolation of avian mycoplasma.

Fabricant (1958) reported that chick embryo inoculation was superior compared to commercial media for the isolation and detection of avian mycoplasma.

The broth cultures of avian mycoplasma were viable when subcultured after three to four years of storage at –30°C, while lyophilized preparations contained viable *Mycoplasma* when subcultured after five to fourteen years of storage at 4°C (Harry, 1964).

Survival of *Mycoplasma* strains in the lyophilised state and at various storage temperatures was studied by Kelton (1964) and found that all strains survived the freeze-drying process and at least 3 or 4 years of storage could be obtained in the freeze-dried state.

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

French medium fourth formula supplemented with human plasma supported good growth of all the three pathogenic species of avian mycoplasma (Frey *et al.*, 1968).

Frey *et al.* (1968) found that avian meat infusion base medium was efficient for the isolation and propagation of *Mycoplasma gallisepticum* and for the isolation of *Mycoplasma meleagridis*, but did not support the growth of *Mycoplasma synoviae.*

Frey *et al.* (1968) compared various media for isolation of avian mycoplasmas and found that the modified Papageorgiou medium was superior to VF broth and avian meat infusion base medium.

Jain *et al.* (1971) used buffalo-heart infusion broth and agar for the isolation and maintenance of PPLO.

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 pH of the medium used by Shimizu *et al.* (1979) for the isolation of avian mycoplasma was 7.8.

Lin and Kleven (1983) demonstrated that the antigen yields of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* could be increased by readjusting the pH of the growth medium back to the original alkaline state when the pH had reached to the acidic side.

Branton *et al.* (1984) used Frey's medium with phenol red as the growth indicator for the isolation of avian mycoplasma.

Heavy contamination with bacteria was reported by Majid (1986) when specimen from fowl was directly inoculated on to *Mycoplasma* agar plates for *Mycoplasma gallisepticum* isolation.

Branton *et al.* (1991) studied the effect of hydration of swab material with Frey's broth media and found that wetting of swab prior to swabbing did not improve the recovery of *Mycoplasma gallisepticum* from commercial layers.

Antibiotics effective in treating the *Mycoplasma gallisepticum* infection may interfere with immune response and also makes it difficult to isolate, giving the false impression that the flock is free from the infection (Kempf, 1991).

Zain and Bradbury (1995 and 1996) reported that cotton swabs could retain a large per cent of the total numbers of colony forming units of *Mycoplasma gallisepticum* even after thorough agitation. The experiment revealed that wet swabs yielded significantly greater number of mycoplasmas than dry swabs.

For the isolation of avian mycoplasma solid medium was found to be more effective than liquid medium (Ronglian *et al.*, 1996).

Zain and Bradbury (1996) reported that survival of *Mycoplasma gallisepticum* was best on wet swabs stored at 4^0C than on dry swabs.

Transportation medium for avian mycoplasma with peptone, glycerine and penicillin was used by Kempf *et al.* (1997).

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

Branton *et al.* (1999) conducted an experiment to determine the effects of age at inoculation and induced moult on the reisolation of *Mycoplasma gallisepticum* from commercial leghorn hens that had been eye-drop inoculated with F strain *Mycoplasma gallisepticum* at either 10 or 66 weeks of age. He

could observe a significant decrease in the *Mycoplasma gallisepticum* isolations from post moult swabs compared with the pre moult swabs.

Increased humidity and CO_2 tension between five to ten per cent have been reported to enhance the growth of mycoplasma (OIE, 2000).

Manohar (2001) compared six media for isolation of *Mycoplasma 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.

George (2003) found Buffalo heart infusion broth ideal for the isolation and propogation of avian mycoplasmas.

2.5.2 Characterization of Mycoplasma

Somerson and Morton (1953) reported that the tetrazolium compounds were reduced by all six strains of PPLO of human origin. This reduction was visible only when incubated anaerobically in the presence of tetrazolium compounds, indicating the presence of flavoproteins in PPLO.

Several serological and pathological variants of PPLO of avian origin were isolated, of which some of the pathogenic types failed to grow on agar where as some did not ferment carbohydrates (Alder *et al.*, 1958).

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

Mycoplasma isolates produced typical fried-egg appearance on the mycoplasma agar surface and were confirmed by Diene's method of staining (Madoff, 1960).

Clyde (1964) identified PPLO cultures isolated from humans based on growth inhibition test by specific antiserum, as the ability of the antiserum to produce growth inhibition was a stable property being retained after long periods of storage at 20^0C and 4^0C and after heating at 56^0C.

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

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

Aycardi *et al.* (1971) reported that in growth inhibition studies of avian mycoplasma using specific antiserum, the 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 and that immunodiffusion and growth inhibition appeared to correlate fairly well in the antigenic analysis of avian mycoplasma.

In the metabolic inhibition test with specific antiserum, the zones of complete or almost complete inhibition were wider with the shallower media (Jordan, 1973).

Woode and McMartin (1973) reported that in metabolic inhibition test with high dilution of antiserum, the lag phase of growth of *Mycoplasma gallisepticum* was significantly prolonged, but eventually growth commenced and proceeded normally and in low dilutions of antiserum the growth was permanently inhibited.

2.5.3 Haemagglutination

Study conducted by Adler and DaMassa (1967) found that formalin-treated *Mycoplasma gallisepticum* antigens haemagglutinated fresh and formalinized chicken erythrocytes and the antigens retained this haemagglutinating ability over an extended period of time.

An investigation of the influence of different factors affecting the sensitivity of the indirect haemagglutination test has been performed by Krogsgaard-Jensen (1971) and found that greatest sensitivity was obtained with fresh erythrocytes than with formalinized erythrocytes and also the sensitivity of the test while using formalinized erythrocytes could be increased by performing the sensitization at a lower pH.

2.5.4 Serological Tests

2.5.4a Serum Plate Agglutination test (SPA)

Avakian *et al.* (1988) found that during the first 1 to 3 weeks, antibodies induced by experimental infection with *Mycoplasma gallisepticum* were better detected by Serum Plate Agglutination test (SPA) test than Enzyme Linked Immuno Sorbent Assay (ELISA) and the Haemagglutination Inhibition (HI) test, thus confirming the importance of SPA test in diagnostic serology.

Serological tests as Rapid slide agglutination (RSA) test and ELISA, although useful for examining flocks, lacks specificity or sensitivity (Yoder, 1991).

Frey's medium supplemented with artificial liposomes substituting for serum was evaluated for *Mycoplasma gallisepticum* SPA antigen by Kleven *et al.* (1998) and found that all static-culture grown MG liposome antigens were highly

sensitive, specific and resulted in a greater yield compared with fermenter-grown liposome antigens.

2.5.4b Enzyme-linked immuno sorbent assay (ELISA)

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

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 was evaluated by Talkington *et al.* (1984) and found that the ELISA was more sensitive with the former mode of antigen preparation.

Selected immunogenic proteins of *Mycoplasma gallisepticum* R strain and *Mycoplasma synoviae* purified from SDS-PAGE gels were evaluated as potential antigens for an ELISA test by Avakian and Kleven (1990). They could observe that the *Mycoplasma gallisepticum* antigen p64 detected antibodies three days after the SPA test and seven days before the Haemagglutination-Inhibition test (HI).

Ewing *et al.* (1996) compared ELISA and HI test for their ability in detecting antibodies to *Mycoplasma gallisepticum* in broilers .Results from this study showed there were no differences between the two tests as confirmatory tests in populations with a low prevalence of *Mycoplasma gallisepticum* infection, however ELISA was superior to HI in a population with moderate levels of *Mycoplasma gallisepticum* infection.

May and Branton (1997) developed an antigen-based enzyme –linked immunosorbent assay for the rapid identification of *Mycoplasma gallisepticum*

and *Mycoplasma synoviae* and compared it with the conventional fluorescent antibody technique. They observed that the principal advantage of this ELISA was its ability to identify the strain of *Mycoplasma* although it did not identify as many isolates as the fluorescent antibody procedure.

2.5.4c Immunobinding assay

An Avidin-Biotin enhanced Dot-Immunobinding Assay for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* serum antibodies was evaluated by Cummins *et al.* (1990) and found that the immunobinding assay was at least 20 times more sensitive in detecting antibodies for *Mycoplasma synoviae* and 75 times more sensitive in detecting antibodies for *Mycoplasma gallisepticum* than the HI test.

2.5.4d Immunofluorescence

Immunofluorescence was used as a tool to demonstrate mixed species within *Mycoplasma* cultures by Bradbury and McClenaghan (1982). Out of the 67 field isolates studied, 26 were found to be mixed cultures of *Mycoplasma.*

Mycoplasma sturni was identified by indirect immunofluorescence from cases of conjunctivitis in Northern mocking birds and blue jays at a wildlife care facility in Florida (Ley *et al.*, 1998).

2.5.4e Haemagglutination-Inhibition (HI)

Lin and Kleven (1983) observed that although HI test had higher specificity than microagglutination and serum plate agglutination tests, its sensitivity was found to be lower in comparison to the latter tests.

Kleven *et al.* (1998) suggested that flocks infected with *Mycoplasma gallisepticum* strains may not give positive results with the HI test because of strain variation.

2.5.4f Other serological tests

Khokhar and Prasad (1983) found that single radial haemolysis test was at least as sensitive as HI test for the detection of antibodies to *Mycoplasma gallisepticum* in sera of chickens.

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

An avidin-biotin-immunoperoxidase diagnostic test using polyclonal primary antibodies raised in rabbits was developed to facilitate rapid identification of *Mycoplasma gallisepticum* in respiratory tissue of turkeys by Radi *et al.* (2000) and found that the test was less specific. They also opined that the use of multiple monoclonal antibodies directed against several different epitopes specific to the cell membrane of *Mycoplasma gallisepticum* would improve the specificity of the test.

2.5.5 Molecular Methods for the Detection and Differentiation of *Mycoplasma* spp

2.5.5a Polymerase chain reaction

Mycoplasma gallisepticum F-vaccine strain specific PCR was developed by Nascimento *et al.* (1991) and found that the MGF-PCR was 1000 to 10,000 times more sensitive than dot-blot assays using two MGF-strain specific probes.

Mycoplasma synoviae species-specific primers, MS-1 and MS-2, selected from the 16s r RNA sequence were evaluated by PCR and the MS-PCR was

found to have a specificity of 100 percent with 82 percent sensitivity (Lauerman *et al.*, 1993).

Study conducted by Ley *et al.* (1993) using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Restriction Endonuclease Analysis (REA), and Polymerase Chain Reaction (PCR) provided the first documentation of *Mycoplasma gallisepticum* infection in commercial breeder and meat turkeys resulting from F strain infection. They suggested that the DNA tests are highly specific, sensitive and rapid to identify the target organism even in the absence of viable organisms.

A polymerase chain reaction (PCR) method specific for *Mycoplasma gallisepticum* (MG PCR) was evaluated by Slavik *et al.* (1993). The PCR method was found to detect as few as two colour changing units (CCU) of *Mycoplasma gallisepticum* and did not give false positive reactions with other avian mycoplasmas. In chickens inoculated with either *Mycoplasma gallisepticum or Mycoplasma synoviae* (MS), the PCR method was found to closely correlate with *Mycoplasma gallisepticum* culture reisolation methods in chicken intranasally inoculated with *Mycoplasma gallisepticum.* All chickens inoculated with *Mycoplasma synoviae* tested negative using the MG PCR method.

Zhao and Yamamoto (1993) used the primers namely L and R, each consisting of twenty-five bases for the amplification of *Mycoplasma iowae* species-specific 299-base pair product. They reported that an annealing temperature of 58.5°C was critical for detecting all members of this species.

Geary *et al.* (1994) demonstrated that the arbitrary primed polymerase-chain-reaction-based DNA fingerprinting method (random amplified polymorphic DNA or RAPD) could be used to distinguish among strains of the avian pathogen *Mycoplasma gallisepticum.* Strain-specific arrays of DNA fragments were generated. Isolates of *Mycoplasma synoviae, Mycoplasma*

gallinarum and *Mycoplasma iners* yielded arrays of DNA fragments that differed markedly from those generated from the *M. gallisepticum* isolates using the same arbitrary primers. The results show that the RAPD fingerprinting method distinguishes genetically different strains of *M. gallisepticum* and indicates that it should be valuable for monitoring transmission of this pathogen.

Kempf (1994) regarded the selection of target, specimen collection, DNA preparation and detection of amplification reaction inhibitors as the major issues in the development of DNA-amplification tests for diagnosis and epidemiological investigations of avian mycoplasmosis.

The sensitive level of a PCR test can be as low as a single organism, enabling detection of mycoplasmas in patients treated with antibiotics and in asymptomatic patients (Razin, 1994).

PCR-based diagnostic tests using oligonucleotides specific to 16S rRNA were designed for the specific detection of the turkey pathogens *Mycoplasma meleagridis* and *M iowae* by Boyle *et al.* (1995) and the method was shown to be rapid, species specific, and unaffected by strain variation or the presence of other organisms. Definitive identification by culture and growth inhibition required up to 3 weeks, whereas positive results from PCR testing were obtained within a day.

Sidhu *et al.* (1995) developed a quantitative PCR with primers designed on the basis of the most conserved nucleotide sequences of the 16S rRNA gene of *Mycoplasma* spp. The PCR so developed could differentiate *Mycoplasma gallisepticum*, *M. synoviae*, *M.orale, M.hyorhinus* and *M. pnuemoniae*. The detection limits ranged from four to sixty genome copies per assay. The method was suggested to be much useful in monitoring the progression and significance of *Mycoplasma* in the disease process

Various diagnostic procedures for the detection of *Mycoplasma synoviae* in commercial multiplier-breeder farms and commercial hatcheries in Florida were tried by Ewing *et al.* (1996). They found that ELISA could be considered as a screening test *in lieu* of Serum Plate Agglutination (SPA), and PCR could be used as a confirmatory test.

Garcia *et al.* (1996) compared PCR-based assay to detect *M. gallisepticum, M. synoviae* and *M. iowae* with that of commercial *M gallisepticum* and *M. synoviae* DNA probe tests and found that the results obtained by PCR assay were in agreement with DNA probe kit results.

A polymerase chain reaction assay was developed for the specific detection of *Mycoplasma iowae* by Laigret *et al. (*1996) and they obtained amplification from culture medium samples of *M. iowae* by using Tth DNA polymerase instead of *Taq* DNA polymerase, and were able to detect as few as 10 organisms.

Mycoplasma gallisepticum was identified as the likely etiology of epornitic of conjunctivitis in house finches in USA during 1994 using DNA probe based Polymerase chain reaction by Ley *et al.* (1996).

Luttrell *et al.* (1996) tried isolation of *Mycoplasma gallisepticum* from house finches with or without conjunctival lesions and found that isolation was not possible from those finches with conjunctival lesions, although they gave positive results for polymerase chain reaction and for various serological tests.

Luttrell *et al.* (1996) propounded that, though PCR is regarded as a sensitive assay, it should be used in conjunction with other tests to assess the *Mycoplasma gallisepticum* status of wild bird populations.

Optimisation of a PCR for the diagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* was carried out by Silveira *et al.* (1996). They found

that DNA extracted by the non-phenolic method was suitable for *Mycoplasma* diagnosis by PCR.

The polymerase chain reaction (PCR) was evaluated to detect *Mycoplasma* contamination of avian live virus vaccines. The specificity of the primers showed that 34 strains belonging to nine species of avian *Mycoplasma* DNA could be detected. The sensitivity of PCR to detect *Mycoplasma* DNA was $10^{0.2}$colony forming units (cfu) of *Mycoplasma synoviae* and $10^{0.7}$cfu of *Mycoplasma gallisepticum* (Kojima *et al.*, 1997).

In separate trials, layer pullets were vaccinated with *Mycoplasma gallisepticum* strain 6/85 or strain ts-11 live vaccines by Ley *et al.* (1997). They found that the Random amplified polymorphic DNA analysis was capable of distinguishing each of the vaccinal strains 6/85 and ts-11 from each other by their distinct DNA banding patterns.

Percentages of infected birds detected by culture or PCR for samples collected prior to antibiotic treatment were almost identical but the percentage of positive samples detected after antibiotic treatment was much higher with the PCR test (Moalic *et al.*, 1997).

Use of an internal control in PCR amplifications for the detection of *Mycoplasma meleagridis* by Moalic *et al.* (1997) revealed that more than 35 per cent of turkey tracheal swabs and more than 45 per cent of turkey cloacal swabs contained inhibitors for PCR.

A multiplex polymerase chain reaction (PCR) was optimized to simultaneously detect four pathogenic species of avian mycoplasmas (Wang *et al.*, 1997). Four sets of oligonucleotide primers specific for *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* and *Mycoplasma iowae* were used in the test and the sensitivity of detection was between one

picogram for *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, 100 femtogram for *M. meleagridis* and 100 picogram for *M. iowae* after 35 cycles of PCR

In a study conducted by Ewing *et al.* (1998) it was revealed that culture and PCR were positive by three days postinoculation where as positive serum plate agglutination results were detected only three to four weeks later.

In a pen trial study conducted by Kleven *et al.* (1998) to assess the comparative efficiency of F strain, ts-11 or 6/85 vaccine strains of *Mycoplasma gallisepticum* to displace the virulent R strain, RAPD-PCR was utilized for determining the strain differentiation among the isolates obtained.

Results of the studies carried out by Salisch *et al.* (1998) confirmed that the PCR technique was as specific as culture. They found that the *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS)-PCR based DNA probe test kits were valuable additions to existing methods for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection, especially, in cases of concurrent infections.

In an experiment to study the potential for transmission of the Finch strain of *Mycoplasma gallisepticum* to chickens, Stallknecht *et al.* (1998), experimentally infected chickens with finch strain of *Mycoplasma gallisepticum* and the isolates obtained from chickens were identified to be the original finch strain using Arbitrary Primed polymerase chain reaction (AP-PCR).

Strain differentiation between F and ts-11 vaccine strains of *Mycoplasma gallisepticum* was performed by Turner and Kleven. (1998) using Random Amplified Polymorphic DNA (RAPD).

The oligonucleotide T12, corresponding to the conserved DNA sequence of the leader peptide of *pMGA* family members of *Mycoplasma* and the

oligonucleotide C13 complementary to the conserved trinucleotide repeat sequence (GAA)*n* preceding known pMGA genes were used as PCR primers by Markham *et al.* (1999).

The *pvpA* gene from *Mycoplasma gallisepticum* strains was amplified by PCR by Boguslavsky *et al.* (2000) for the molecular characterization of the gene that encodes a putative variable cytadhesin protein.

The use of PCR to show the presence of mycoplasmas in different clinical as well as environmental samples was described by Marois *et al.* (2000).

Successful detection of *Mycoplasma synoviae* (MS) by culture and PCR from samples collected in the environment of experimentally infected chickens and turkeys, or under field conditions, was described by Marios *et al.* (2000). The observations highlight the high disseminating capacities of this *Mycoplasma* and show the usefulness of the PCR method for epidemiological studies.

The polymerase chain reaction (PCR) was performed by Bencina *et al.* (2001) with genomic DNA isolated from pelleted cells of *Mycoplasma synoviae* strains using two sets of primers to amplify the *vlhA* gene region encoding *Mycoplasma synoviae* protein-B (MSPB) as a part of their work for determining the molecular basis of the length variation in the N-terminal part of *Mycoplasma synoviae* haemagglutinin.

In order to study horizontal transmission of *Mycoplasma synoviae* a reverse transcription-polymerase chain reaction (RT-PCR) assay was developed by Marois *et al.* (2002) to detect viable *Mycoplasma* in environment samples. The test was based on the RT-PCR of the 16S ribosomal RNA (rRNA) of *Mycoplasma* genus. Results confirmed the usefulness of RT-PCR in checking the efficiency of biosecurity measures and in improving cleaning and disinfection protocols.

A PCR to identify *Mycoplasma suis* infected pigs based on a novel DNA sequence was developed by Hoelzle et al. (2003).

Kleven *et al.* (2004) compared five turkey isolates of *Mycoplasma gallisepticum* from various outbreaks with that of 6/85 vaccine strain of *Mycoplasma gallisepticum* by RAPD analysis using four primer sets and by DNA sequence analysis and could obtain more than 99 per cent homology indicating that these isolates were closely related to the vaccine strain but they were not able to prove that these strains had originated from the vaccine strain.

Since the isolation of *Mycoplasma synoviae* is slow and laborious as a result of the fastidious nature of the species, Yang Hong *et al.* (2004) developed a PCR test to specifically detect *Mycoplasma synoviae* in commercial poultry.

The quinolone-resistance determining regions (QRDR) of the *gyrA* and *gyrB* genes for DNA gyrase and the *parC* and *parE* genes for topoisomerase IV were amplified by polymerase chain reaction and analyzed for the clones with a higher MIC of enrofloxacin. (Reinhardt and Bouchardton, 2005).

2.5.5b Restriction enzyme analysis

Electrophoretic patterns of *Mycoplasma gallisepticum* DNA digested with *Bam Hl, Eco Rl* and *Hind III* were found more sensitive than SDS-PAGE for differentiating the strains of *Mycoplasma gallisepticum* (Khan and Yamamoto, 1989).

The electrophoretic patterns of the DNA of *Mycoplasma gallisepticum* strains digested with restriction enzymes, *Bam Hl, EcoRl* and *HindIII* were useful for differentiating the vaccine F-strain from other strains of *M.gallisepticum* (Khan and Yamamoto, 1989).

Restriction enzyme analysis performed by Ley *et al.* (1993) on *Mycoplasma gallisepticum* isolated from cases of respiratory infections in commercial breeder and meat turkeys proved to be the vaccinal F-strain of *M.gallisepticum.*

Fourteen species of avian mycoplasmas were differentiated by Fan *et al.* (1995) based on the restriction patterns generated with six different restriction enzymes.

The polymerase chain reaction (PCR) with primers complementary to the 16s rRNA genes was used to detect avian mycoplasmas by Kiss et al. (1997) and found that *Mycoplasma iowae, Mycoplasma meleagridis* and *Mycoplasma synoviae* could be detected by PCR with this primer pair, and distinction could be made among them by restriction fragment length polymorphism (RFLP) assay with two restriction enzymes (*BamHI* and *RsaI*). For the detection of *Mycoplasma gallisepticum* by PCR, species-specific primers were needed.

Kleven *et al.* (1998) compared different strains of *Mycoplasma gallisepticum* by homologous and heterologous haemagglutination-inhibition tests and Restriction Enzyme Analysis (REN) to determine the relatedness between these strains. Results of the study indicated that REN could be a useful tool for determining strain relationships in epidemiological studies.

Liu *et al.* (2001) performed and evaluated a semi-nested PCR-RFLP procedure, which could detect and rapidly differentiate *Mycoplasma gallisepticum* strains present in infections of poultry flocks directly from tracheal samples. They had also concluded that PCR-RFLP was superior to PCR-based Random Amplified Polymorphic DNA (RAPD-PCR) and Arbitrarily Primed PCR (AP-PCR) for differentiation of *Mycoplasma gallisepticum.*

2.5.5c Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) analysis was developed and compared with random amplifed polymorphic DNA (RAPD) method to type 18 *Mycoplasma synoviae* (MS) strains. All analyzed strains were typeable by RAPD but only 89 per cent of *Mycoplasma synoviae* strains were typeable by PFGE because of DNA degradation. The discriminatory power of RAPD was greater than that of PFGE but the two techniques had a discriminatory index superior to 0.95, the threshold value for interpreting typing results with confidence but, the interpretation of RAPD patterns was complicated because of inconsistent band intensity (Marois *et al.*, 2002).

2.5.5d DNA sequencing and Nucleic acid hybridization methods

The use of DNA probes for the early and rapid detection of *Mycoplasma gallisepticum* infection which could replace laborious culture techniques and less effective serological methods had been reported by Hyman *et al.* (1989).

A portion of the putative *Mycoplasma gallisepticum* cytadhesin gene was identified and used as a diagnostic DNA probe by Dohms *et al.* (1993).

Sensitivity of *Mycoplasma* detection by the different probes ranged between 10^3 and 10^6 colony-forming units, a level which may not be sufficiently high for use in a clinical laboratory (Razin, 1994).

Laigret *et al.* (1996) described a dot blot hybridization test, using cold labelling and chemiluminescence, which is very convenient for routine detection of *M. iowae*.

Pillai *et al.* (2003) observed that DNA sequence analysis unveils more polymorphism among isolates than by PCR-RFLP.

Kleven *et al.* (2004) compared five turkey isolates of *Mycoplasma gallisepticum* with that of 6/85 vaccine strain by DNA sequence analysis and obtained more than 99 per cent homology.

Kleven *et al.* (2004) opined that DNA probes were a better method than serology and isolation procedures to detect *Mycoplasma gallisepticum* at earlier stages as detection by serology and isolation is difficult during the first few weeks of infection.

Typing of *Mycoplasma synoviae* strains by conducting DNA sequence analysis on PCR product was carried out by Yang Hong *et al.* (2004). The target gene for the study was *vlh*A which encodes the haemagglutinin, an abundant immunodominant surface lipoprotein in *Mycoplasma synoviae.*

2.6 PROTEIN PROFILES OF AVIAN MYCOPLASMA

2.6a Sodium-Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

A 64-kilodalton membrane protein was found to be expressed in higher amounts *in vitro* in virulent strains of *Mycoplasma gallisepticum* than in strains of low virulence by observing the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns by Avakian and Ley (1993). The p64 was found to be the major protein determining cytadherence by demonstrating the inhibition in growth and attachment to chicken tracheal rings by *Mycoplasma gallisepticum* after being exposed to antibodies directed to p64.

Immunoblotting experiments and SDS-PAGE were carried out by Noormohammadi *et al.* (1997) to detect the major membrane antigens of *Mycoplasma synoviae.*

Bencina *et al.* (1999) performed Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis and immunoblotting in an experiment to

characterize the EF-Tu protein of *Mycoplasma synoviae* and also other proteins involved in hemadherence.

In a study conducted to identify the *Mycoplasma gallisepticum* surface antigen recognizable by a monoclonal antibody capable of inhibiting the growth and metabolism, Yoshida *et al.* (2000) utilized SDS-PAGE at eight per cent gel concentration for the better separation of *Mycoplasma* proteins.

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

3. MATERIALS AND METHODS

3.1 MATERIALS

Molecular grade chemicals and biologicals procured from Integrated DNA Technologies Inc, USA; Alpha DNA, France; Genei, Bangalore; Vision Scientific, Ernakulam, and analytical grade chemicals and media purchased from Difco, France; Sigma, Germany; Sisco Research laboratory (SRL); Hi-Media, Mumbai were used, wherever the source is not mentioned. Glassware of Borosil and Riviera brands and Tarsons brand plastic ware were used in the study.

3.1.1 Samples for the Study

Samples were obtained from sick/apparently healthy birds of different age groups from the University Poultry Farm, Mannuthy; Regional Poultry Farms at Chathamangalam, Koovappady and Kudappanakkunnu and birds brought for disease diagnosis to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy. Birds showing ailments such as nasal discharges, sinusitis, laboured breathing, and conjunctivitis and / or with reported reduction in egg production were specifically included in the collection of samples for the study.

Tracheal swabs were invariably collected from all the birds subjected to the study. Apart from tracheal swabs, cloacal swabs and conjunctival swabs were also collected, depending on the type of symptoms exhibited by the birds. In case of necropsied birds, lung samples and airsac materials were also collected. Synovial swabs were obtained from a few necropsied birds.

3.1.2 Detection of Avian Mycoplasma 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' GGC AGC AAA CAG GAT TAG ATA CCC T3'

5' TGC ACC ATC TGT CAG TCT GTT AAC CTC3'

Primers were obtained as custom made 100 nM standard desalted oligonucleotides with OD-260 values of 17.2 and 18.1 respectively from IDT, Inc. USA.

3.1.2.1b PCR Reaction Buffer (10 X)

Contains 500 mM KCl, 100 mM Tris hydrochloride (pH 9) and 0.1 per cent gelatin in a total volume of one millilitre.

3.1.2.1c Magnesium Chloride

At a strength of 25mM.

3.1.2.1d. Taq DNA Polymerase

At a concentration of one unit per microlitre.

3.1.2.1e Deoxy Nucleotide Triphosphates

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

3.1.2.1f Template DNA

i) DNA separation from Clinical Samples

Clinical samples suspended in one milliliter of BHI broth were spun at 13,000 X g at 4° C for 20 minutes, supernatant discarded and the pellet was

resuspended in PBS (pH of 7.4) by agitating in a vortex mixer. The cell pellet was washed twice in PBS at 13,000 X g for 15 minutes at 4° C. Resuspended the pellet in a final volume of 20 microlitre of PBS, heated in a dry block at 110° C for 10 minutes, and placed at 70° C for at least 15 minutes. The lysate was then centrifuged at 13,000 X g for two minutes to remove the debris. Supernatant containing the DNA was collected, numbered and stored at – 70° C till use.

ii) DNA separation from other bacterial strains.

Pure colonies of bacterial strains namely *Escherichia coli*, *Staphylococcus aureus* (Reference strain MTCC-144) and *Pseudomonas cepacia* were inoculated separately into 5ml of BHI broth and incubated at 37° C for 18 hours. From this broth culture 1.5 ml was transferred to an Eppendorf tube and centrifuged at 3000 X g for 10 minutes, the supernatant was discarded, washed the pellet twice with sterile PBS and final pellet was resuspended in 100 µl of triple distilled water. Mixture was boiled for 10 minutes and immediately chilled on ice for 30 minutes. The samples were thawed and centrifuged at 3000 X g for five minutes and supernatant was stored at –20° C for further use as template for PCR reactions.

3.1.3 Species Differentiation of Avian Mycoplasma in Clinical Samples

3.1.3.1 Mycoplasma gallisepticum Species-specific PCR (MG-PCR)

3.1.3.1a Oligonucleotide Primers

Mycoplasma gallisepticum specific primers pvp AIF, pvpA2R, pvpA3F (Liu et al., 2001)

5' GCC AMT CCA ACT CAA CAA GCT GA 3'

5'GGA CGT SGT CCT GGC TGG TTA GC 3'

5'GGT AGT CCT AAG TTA TTA GGT C 3'

Primers were obtained as custom made 50 nM standard desalted oligonucleotides with OD-260 values of 24.5, 25.5 and 25.7 respectively from Alpha DNA, Notre-Dame.

The primers used in the present study pvpA1F, pvpA2R and pvpA3F were designed from the *pvpA* gene sequence of *M.gallisepticum.* The *pvpA* gene encodes for PvpA, the phase-variable putative cytadhesin protein of *M.gallisepticum.* The outer primers pvpA1F and pvpA2R amplify 662 bp regions from 417 to 1079 base positions of the *pvpA* gene. The semi-nested primer, pvpA3F attaches to the 583rd position and the maximum size of the amplified product is 497 base pair. Size variation of the gene due to deletions occurring in the segment encoding the proline-rich C terminal region of the protein occurs in different strains of *M.gallisepticum* and so amplification product size polymorphism is reported by the authors

3.1.3.2 Mycoplasma synoviae Species-specific PCR (MS-PCR)

3.1.3.2.1 Oligonucleotide Primers

Mycoplasma synoviae specific primers, MS1 and MS2 (Lauerman et al., 1993)

5' GAG AAG CAA AAT AGT GAT ATC A 3'

5' CAG TCG TCT CCG AAG TTA ACA A 3'

Primers were obtained as custom made 100 nM standard desalted oligonucleotides with OD-260 values of 15.3 and 13.8 respectively from IDT, Inc. USA.

3.1.3.3 Mycoplasma iowae Species-specific PCR (MI-PCR)

3.1.3.3.1 Oligonucleotide Primers

Mycoplasma iowae specific primers L, R, Li, Ri. (Zhao and Yamamoto, 1993)

5' GAA TTC TGA ATC TTC ATT TCT TAA A3'

5'CAG ATT CTT TAA TAA CTT ATG TAT C 3'

5' AAT GGC AAC TTT TGA GTC ATC ATC AA 3'

5' CTT ATG TAT CAA ACA ATA AAG AAG CAG 3'

Primers were obtained as custom made 100 nM standard desalted oligonucleotides with OD-260 values of 17.9, 16.2, 19.6 and 15.8 respectively from IDT, Inc. USA.

The primers were designed by Zhao and Yamamoto (1993) based on the sequence data of *M.iowae* recombinant DNA probe pMI-12, which contained an *M.iowae* strain I-695 DNA insert of approximately 300 bp. The outer primer sets, L and R amplify 299 bp of target DNA. The pMI-12 internal Li and Ri primers amplify 167 bp of the central region of the 308 bp fragment.

3.1.4 Detection of Amplified Products

3.1.4.1 Submarine Gel Electrophoresis

i) Agarose

ii) EDTA stock solution (0.5 M) pH 8.0

Sodium EDTA.$2H_2O$	186.1 g
Distilled water	800 ml

The pH was adjusted to 8.0 with 1 N NaOH. Distilled water was added to make up the volume to one litre. The solution was sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure.

iii) Tris Borate EDTA Buffer (TBE) pH 8.2

a) Stock solution (10 X)

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

b) Working solution (1 X)

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

iv) Tris Acetate EDTA Buffer (TAE)

Stock solution (50 X concentration) from Genei, Bangalore.

1 X solution prepared in distilled water was used for electrophoresis.

v) Ethidium Bromide stock solution

Ethidium Bromide	10 mg
Triple distilled water	1 millilitre

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

vi) Get Loading Buffer (6 X)

a) Sucrose solution

Sucrose	40 g
Triple distilled water	100 ml

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.

vii) DNA Molecular size Marker (330 microgram per milliliter)

a) pUC 19 DNA /*Msp* I Digest consisting of 9 double –stranded DNA segments of 34/ 34, 67, 111/110, 147, 242, 331, 404 and 489 bp.

b) pBR 322 DNA/*Alu* I Digest consisting of double – stranded DNA segments of 63/57/49, 100/90, 226, 257, 281, 403, 521, 659/656 and 908 bp.

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

3.1.5 Media for the Isolation of Avian Mycoplasma

3.1.5.1 Media Ingredients

3.1.5.1a Buffalo Heart Infusion

Buffalo heart infusion was prepared by boiling 500 g minced fat free buffalo heart muscle with 1000 ml distilled water for 45 minutes. It was filtered through a muslin cloth with cotton pad, distributed in small quantities, sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure and stored at -70° C until use.

3.1.5.1b Horse Serum

Blood was obtained observing aseptic precautions from horses maintained by 1 (K) R&V Sqr., KAU Unit, Mannuthy. Serum was separated and sterilized by passing through Seitz filter and stored at -70° C in 20 ml aliquots till use.

3.1.5.1c Swine Serum

Blood was obtained from pigs slaughtered at the Centre of Excellence in Meat Science and Technology, Kerala Agricultural University, Mannuthy. Serum was separated, treated at 56 ° C for 30 minutes, sterilized by passing through Seitz filter and stored at -70° C in 20 ml aliquots till use.

3.1.5.1d Yeast Extract

250 g baker's yeast was suspended in one litre of distilled water, heated to boiling point, cooled and centrifuged for 20 minutes at 3000 X g. The supernatant fluid was decanted and adjusted to pH 8.0 with 0.1 M NaOH. This was clarified by filtration through Whatman filter paper No.1 and then sterilized by passing through Seitz filter. The extract was stored in small aliquots of 10 ml at –70° C.

3.1.5.1e Glucose Solution

Fifty per cent of glucose stock solution was prepared in distilled water, sterilized by filtration and stored at -70 ° C in 10 ml aliquots.

3.1.5.1f Antibiotics

Penicillin G (10, 0000 1U/5ml) prepared in sterile distilled water and stored at -70 °C in 10 ml aliquots.

3.1.5.1g Thallium Acetate

Thallium acetate solution (2.5 per cent) was prepared in sterile distilled water and stored at –70°C in 10 ml aliquots.

3.1.5.1h Phenol Red

0.1g phenol red was ground in 2.8 ml of 0.1 M NaOH, and then made upto 100ml in sterile distilled water, sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure and stored at 4°C.

3.1.5.1i Cysteine Hydrochloride

One per cent stock solution was prepared in distilled water, sterilized by filtration and stored in 10 ml aliquots at -70° C

3.1.5.1j Nicotinamide Adenine Dinucleotide (NAD)

One percent stock solution was prepared in distilled water and stored in 10 ml aliquots at -70° C.

3.1.5.1k Buffered Peptone Water

20.07 g of buffered peptone water (Hi-media) was dissolved in1000 ml distilled water, sterilized by autoclaving at 121°C for 15 min at 15 lb pressure.

3.1.5.1l Glycerine

Analytical grade glycerin was sterilized in the hot air oven and stored at room temperature

3.1.5.2 Buffalo Heart Infusion Broth (BHI Broth) (Lecce and Sperling, 1954)

Buffalo heart infusion - 700 ml

Bactopeptone (Difco) - 11 g

Phenol red	-	10 ml
NaCl	-	5 g
Glucose	-	2 ml
Horse serum	-	200 ml
Yeast extract	-	20 ml
Thallium acetate	-	20 ml
Penicillin	-	10 ml

Bactopeptone was dissolved in buffalo heart infusion, sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure and supplemented with the remaining ingredients under aseptic conditions. The pH was adjusted to 7.8. About two to three millilitres of BHI broth were dispensed into screw-capped glass tubes of five millilitres capacity, kept for sterility checking at 37° C for 48 hours and after that stored at 4° C to be used within 14 days.

3.1.5.3 Buffalo heart Infusion agar (BHI Agar)

PPLO agar base (Difco) - 700 ml

Prepared by dissolving 13.7 g of the agar base in 700 ml distilled water and sterilized by autoclaving at 121° C for 15 min at 15lb.

Buffalo heart infusion	- 50 ml
Horse serum	- 150 ml
Yeast extract	- 100 ml
Glucose	- 20 ml
Penicillin	- 10 ml

Thallium acetate - 20 ml

pH adjusted to 7.8.

Mycoplasma agar base was allowed to cool to 45 to 50° C and supplemented with the remaining ingredients under aseptic conditions. Agar was then poured into 55 mm diameter Petri dishes at a depth of five to six mm, kept for sterility checking at 37° C for 48 hours and stored at 4° C to be used within 14 days.

3.1.5.4 Modified BHI Agar for Mycoplasma synoviae isolation

PPLO agar base (Difco) - 700 ml sterilized by autoclaving

Buffalo heart infusion - 150 ml

Swine serum - 150 ml

Yeast extract - 100 ml

Glucose - 20 ml

Penicillin - 10 ml

Thallium acetate - 20 ml

Nicotinamide Adenine Dinucleotide - 0.1 ml

L-Cysteine Hydrochloride - 0.1 ml

pH adjusted to 7.8

Mycoplasma agar base was allowed to cool to 45 to 50° C and supplemented with the remaining ingredients under aseptic conditions. Agar was then poured into 55 mm diameter Petri dishes at a depth of five to six mm, kept

for sterility checking at 37° C for 48 hours and stored at 4° C to be used within 14 days.

3.1.5.5 Transport media

i) Buffalo heart infusion broth (Lecce and Sperling, 1954)

ii) Modified Buffered Peptone water

Buffered peptone water	- 1000 ml
Glycerin	-12 ml
Penicillin	-10 ml
Thallium acetate	- 20 ml

pH adjusted to 7.8.

Two milliliter of the medium was dispensed in glass test -tubes and kept for sterility checking at 37° C for 24 hours and stored at 4°C to be used within 14 days.

iii) Phosphate Buffered Saline (PBS) Stock Solution (10 X)

Sodium Chloride	80 g
Potassium Chloride	2 g
Disodium Hydrogen Phosphate	11.32 g
Potassium dihydrogen Phosphate	2 g
Distilled Water	1000 ml

The pH was adjusted to 7.4 by I N NaOH and sterilized by autoclaving at 121° C for 15 min at 15 lb pressure. The stock solution was diluted to 1X before use.

3.1.6 Characterization of Mycoplasma Isolates

3.1.6.1 Diene's Staining Technique

3.1.6.1*a Diene's Stain*

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

Sterilized by filtration and stored at room temperature

3.1.6.2 Characterization of Mycoplasma Isolates by PCR

3.1.6.2.1 Template-DNA Preparation from the Isolates

Small agar blocks containing individual or homogenous colonies were cut out and placed in broth. The procedure was repeated with three separate colonies into three separate broth tubes. These broth media were incubated for a period of 24 hours or till a colour change was visible, whichever being the earliest. About one milliliter of the broth was spun at 13,000 X g at 4° C for 20 min. The supernatant was then discarded and the pellet was resuspended in PBS of pH of 7.4 by agitating in a vortex mixer. The cell pellet was washed twice in PBS at 13,000 X g for 15 minutes at 4° C. Resuspended the pellet in a final volume of 20 microlitre 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 X g for two min to remove the debris. Supernatant containing the DNA was collected, numbered and stored at – 70° C till use.

3.1.7 Strain Differentiation of *M. gallisepticum* Isolates

3.1.7.1 Restriction Enzyme Fragment Length Polymorphism (RFLP)

i) Restriction enzymes (RE)

Restriction enzymes were chosen based on published reports (Liu *et al.*, 2001).

RE	Concentration	Recognition sequence	Procured from
Acc I	10,000U/ml 500U	5'C.T ▼(A/C)T/G)AC3' 3' CA(T/G)A/C) ▲TG 5'	New England Biolabs, UK
ScrF I	10,000 U/ml 1000 U	5'CC ▼NGG3' 3' GGN ▲CC5'	New England Biolabs, UK
Pvu II	2500 U	5' CAG ▼ CTG 3' 3' GTC▲GAC 5'	Bioenzymes, Biogene, USA

ii) Amplified PCR products

iii) RE buffer (10 X)

It was supplied by the manufacturer along with RE

3.1.7.1.1 Separation of Restriction Fragments

3.1.7.1.1a Polyacrylamide Gel Electrophoresis (PAGE)

The PCR products digested separately by *Acc* l, *Pvu* II and *ScrF* 1 were separated by PAGE

i) Eight per cent acrylamide gel was prepared by mixing the following ingredients,

Acrylamide: Bisacrylamide 30: 8	3.72 ml
TBE (5 X)	2.8 ml
Distilled water	7.8 ml
10 per cent ammonium per sulphate	50 µl
TEMED	3 µl

3.2 METHODS

3.2.1 Sample Collection

All the samples were invariably collected in Buffalo heart infusion broth medium (3.1.5.2). Tracheal swabs from all the ailing birds and a few healthy birds were directly plated on to the solid medium also. A few of the tracheal swabs were transported in PBS/buffered peptone water with one per cent glycerine.

Sterile swabs made of cotton gauze were used for sampling. Just prior to sampling the cotton swab was soaked with BHI broth and the excess broth was extruded by pressing the swab against the sides of the tube.

3.2.1.1 Tracheal Swabs

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

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

3.2.1.2 Cloacal Swabs

Pre-soaked sterile cotton swab was gently inserted through the vent, the region swabbed and the swab replaced into the broth tube. Thrust out portion of the swab was snipped off and the tube screwed tightly.

3.2.1.3 Airsac Material

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

3.2.1.4 Lung

Lung was scooped out using blunt scissors and the portion of the lung showing lesions was cut and transferred carefully to the broth. Broth tube was then tightly closed.

3.2.1.5 Synovial Fluid

After cutting open the hock joint, the synovial cavity was swabbed with pre-soaked sterile cotton swab and the swab transferred to the broth, thrust out portion of the swab sniped off and the broth tube tightly closed.

3.2.1.6 Conjunctival swabs

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

All the samples were collected in BHI broth medium which was used as the primary transport medium. In order to assess the utility of buffered peptone water with glycerine as a transport medium, a few tracheal swabs were also collected in that medium. In an effort to understand the performance in PCR of samples collected in PBS, certain numbers of tracheal and cloacal swabs were also collected in PBS.

All the clinical samples subjected for the study were collected in BHI broth and then processed for *Mycoplasma* DNA detection/ isolation, while a few tracheal swabs were directly streaked onto solid medium for isolation.

Inoculated plates and broths were preserved and transported over ice to the laboratory. On reaching the laboratory, the plates and broths were transferred to a CO_2 incubator and incubated at 37° C under six per cent CO_2 tension.

3.2.2 Processing of Samples

All the samples obtained at the Department of Microbiology were processed immediately after collection. When the samples were collected from far away farms, the inoculated plates and broths were preserved and transported over ice to the laboratory. All the inoculated plates and broths were transferred without delay to a CO_2 incubator and incubated at 37° C under six per cent CO_2 tension. Broth tubes were shaken occasionally. The caps of the broth medium containers were tightly closed before incubation so as to avoid spurious changes in pH.

Swabs were removed from BHI broth after four hours and one millilitre of this broth was transferred aseptically into sterile Eppendorf tubes in laminar airflow so as to prepare DNA templates for PCR. The remaining broth media were placed back and further incubated till an appreciable colour change of the broth to orange or yellow was evidenced or upto 21 days, whichever was earlier.

The DNA template was prepared from the broth that was removed for the purpose, on the same day and the *Mycoplasma* genus-specific PCR was performed.

The swabs obtained in PBS and buffered peptone water was placed at 4° C and not in the incubator and then used for *Mycoplasma* DNA detection.

Broth media that did not show a significant colour change were plated onto agar depending upon a positive result by PCR. Serial ten fold dilutions of such broths were made and were inoculated onto agar plates and incubated. Broths that appeared to be contaminated were passed through 0.2 micrometer Millipore filter in an attempt to retain the contaminant bacteria in the filter and the filterate was used as the inoculum.

3.2.3 Polymerase Chain Reaction for the Detection of Avian Mycoplasma from Clinical Samples

3.2.3.1 Reconstitution of Primers

Primers obtained in the lyophilized form were reconstituted in sterile glass distilled water to a concentration of 200 picomoles per milliliter. 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 10 microlitre aliquots and stored at -70^{0}C. At the time of use, aliquots were thawed and working solutions of the primers were obtained by 10 fold dilution 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 the method elaborated by Marois *et al.*, (2000) with modifications.

Polymerase chain reaction was performed in a total volume of 25-microlitre reaction mixtures. A master mix was prepared before setting up the PCR reaction by combining the following reagents in 25-microlitre volumes.

10 X PCR assay buffer	2.5 µl
$MgCl_2$	0.5 µl
Forward primer, MGSO (20 pM)	1 µl
Reverse primer, GPO3 (20 pM)	1 µl
dNTPs (200 µM)	1 µl
Taq DNA polymerase	1 unit
Template DNA	5 µl
Triple distilled water	13. 7 µl

Depending upon the number of samples to be subjected for PCR, a master mix was be formulated with all the reagents excluding the template. Then to each PCR tube, 20 microlitre of master mix was distributed and five microlitre of respective template DNA were added separately and mixed. One negative control with triple distilled water substituting the template DNA was included to monitor contamination, if any. The tubes were placed in the thermal cycler (Eppendorf Master Cycler Gradient). The reaction protocol was as follows.

Initial denaturation		90° C for 1 minute
40 cycles	denaturation	95° C for 15 seconds
	annealing	58° C for 20 seconds
	elongation	75° C for 20 seconds
One cycle of	denaturation	95°C for 15 seconds
	annealing	58° C for 45 seconds
	extension	75° C for 5 minutes

3.2.3.3 Identification of PCR Product

Submarine Agarose Gel Electrophoresis was performed using one per cent agarose in 1X TBE buffer as the matrix. 0.25 g of agarose was dissolved in 25 ml of TBE buffer by heating and cooling to 50° C. To 25 ml of the hot gel three 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 and allowed to polymerize. Once the gel had set, comb was lifted gently and the adhesive tapes were removed and placed the gel tray into the buffer tank and filled the tank with buffer till it covered the top of the gel. Five microlitre of the amplified product with one microlitre of 6 X gel loading buffer was loaded into the wells of the submerged gel. Five microlitre of distilled water mixed with one microlitre of the 6 X gel loading buffer constituted the negative control and pBR 322 DNA/*Alu* I Digest was used as the DNA molecular weight marker. Gel was run until the blue dye had migrated the full length of the gel.

3.2.3.4 Recording of Result

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

3.2.4 Species Differentiation of Avian Mycoplasma from the Clinical Samples

Samples that tested positive by genus-specific PCR were further subjected sequentially for *M.gallisepticum, M.synoviae* and *M.iowae* specific reactions

3.2.4.1 Mycoplasma gallisepticum Specific Semi-nested PCR (MG-PCR)

The templates that tested positive by *Mycoplasma* genus-specific PCR were subsequently subjected for *Mycoplasma gallisepticum* species-specific PCR.

A 25 µl. reaction mixture was set up for the single PCR reaction consisting of

10 X PCR assay buffer	2.5 µl
$MgCl_2$	0.5 µl
Forward primer, $pvpA_1F$ (20 pM)	1 µl
Reverse primer, $pvpA_2R$ (20 pM)	1 µl
dNTPs (200 µM)	1 µl
Taq DNA polymerase	1 unit
Template DNA	3 µl
Triple distilled water	15 µl

Protocol: (Master gradient Thermal cycler, Eppendorf)

Initial denaturation		94° C for 3 minutes
20 cycles each of	denaturation	94° C for 30 seconds
	annealing	55° C for 30 seconds
	elongation	72° C for 1 minute
Final extension		72° C for I minute

The semi-nested PCR was performed using the similar reaction mixture as for the single PCR except that the amplified product of the single PCR formed the template for the semi-nested reaction and a varied reaction protocol.

Protocol: (Master gradient Thermal cycler, Eppendorf)

Initial denaturation		94° C for 3 minutes
40 cycles each of	denaturation	94° C for 30 seconds
	annealing	55° C for 30 seconds
	elongation	72° C for 1 minute
Final extension		72° C for I minute

3.2.4.2 Mycoplasma synoviae – Specific PCR (MS-PCR)

The templates from clinical samples that tested positive for *Mycoplasma* genus-specific PCR were then subjected for *M. synoviae* species-specific PCR.

A 25 µl reaction was performed

10 X PCR assay buffer	2.5 µl
$MgCl_2$	0.5 µl
Forward primer, MS1 (20 pM)	1 µl
Reverse primer, MS2 (20 pM)	1 µl
dNTPs (200 µM)	1 µl
Taq DNA polymerase	1 unit
Template DNA	3 µl
Triple distilled water	15 µl

Protocol: (Master gradient Thermal cycler, Eppendorf)

Initial denaturation		90° C for 1 minute
40 cycles	Denaturation	95° C for 15 seconds
	Annealing	58° C for 20 seconds
	Elongation	75^0 C for 20 seconds
One cycle	Denaturation	95° C for 15 seconds
	Annealing	58° C for 45 seconds
	Extension	75° C for 5 minutes

3.2.4.3 Mycoplasma iowae – Specific PCR (MI-PCR)

The templates from clinical samples that tested positive by *Mycoplasma* genus-specific PCR were finally subjected to *M. iowae* species-specific PCR.

A 25 µl. reaction mixture was set up for the single PCR reaction consisting of

10 X PCR assay buffer	2.5 µl
$MgCl_2$	0.5 µl
Forward primer, L (20 pM)	1 µl
Reverse primer, R (20 pM)	1 µl
dNTPs (200 µM)	1 µl
Taq DNA polymerase	1 unit
Template DNA	3 µl
Triple distilled water	15 µl

Protocol: (Master gradient Thermal cycler, Eppendorf)

Initial denaturation		95 ° C for 4 minutes
35 cycles	Denaturation	95° C for 1 minute
	Annealing	58.5° C for 2 minutes
	Elongation	72° C for 2 minute
Final extension		72° C for 10 minutes.

3.2.4.4 Identification of PCR Product

Procedure same as that of 3.2.3.1.4

3.2.4.5 Recording of Result

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

3.2.5 Isolation of Avian Mycoplasma

3.2.5.1 Samples Collected in Mycoplasma Broth Medium

All the tracheal swabs collected in *Mycoplasma* broth medium that tested positive by *Mycoplasma* genus-specific PCR were streaked on to the solid media, after 24 hours of incubation or a colour change of the broth to orange or yellow was evidenced, whichever was earlier. All the inoculated agar plates and broth media were incubated at 37^0C in a CO_2 incubator under six per cent CO_2 tension till visible colonies were evidenced or up to a maximum of 21 days.

Those broth media that were positive by PCR but did not show a colour change, were also streaked on to the solid media after 24 h of incubation and alongside the broth media were examined daily for the change in pH. Such broth media that yielded a positive result by PCR but evoked no indication of growth were serially diluted in fresh broth medium in an attempt to dilute out specific antibodies or extraneous antibiotics or other inhibitory substances in the sample that might inhibit the growth of *Mycoplasma*.

Even if no colour change was observed in the broth, subculturing of that sample on to solid medium was performed, provided the PCR result was positive.

A few negative samples by PCR were also streaked at random on to the solid media after 7 – 10 days of incubation.

For the isolation of *M. synoviae*, synovial swabs were streaked directly on to the *M. synoviae* -specific agar medium and incubated at 37° C under six per cent CO_2 tension.

3.2.5.2 Samples Directly Streaked on to Agar Medium

Agar plates inoculated directly with the samples at the site of collection of samples were incubated at 37° C in a CO_2 incubator under six per cent CO_2 tension irrespective of the PCR results.

Agar plates (directly streaked with samples or those subcultured from the broth) were examined daily under an inverted microscope for the presence of mycoplasmal 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 and the procedure was repeated with three separate broth tubes per isolate. The plates were examined under 10 X objective of the microscope and the colony characters were studied.

A clinical sample was considered negative for the presence of *Mycoplasma* mostly by a negative result by genus-specific PCR. PCR results were solely used as the guideline to decide whether the sample necessitated further isolation trials. Those samples, which yielded negative results, were not subjected for isolation trials except in a few cases.

Once colonies were obtained homogenous colonies were selected and cut out from the agar using a sterile straight wire and carefully transferred into BHI broth medium and incubated in the CO_2 incubator till a colour change was visible. The procedure was repeated with three such homogenous colonies and put separately into three broth tubes and kept in CO_2 incubator at 37° C. After the colour change was evidenced, a loopful of the broth would be streaked on to the agar medium and kept for incubation. MG-PCR was performed from these broths to ensure the presence of *M. gallisepticum* and the broths found positive by MG-PCR were subcultured. To counter check, individual colonies were selected from three different areas of the agar plate and again subjected to MG-PCR.

3.2.6 Characterization of Avian Mycoplasma Isolates

3.2.6.1 Diene's Staining Technique

Agar plates with *Mycoplasma* colonies were flooded with Diene's stain diluted 1:100 in normal saline, for one minute. Excess of stain was removed and incubated at 37° C for 30 to 60 minutes. Mycoplasma colonies stain an intense blue at the centre with light blue periphery. Colonies of *Mycoplasma* were examined for the characteristic fried egg appearance, colour, texture, size and for characteristic staining with Diene's stain.

All the isolates obtained were subjected to Mycoplasma genus-specific PCR (3.2.3.2). The isolates were further characterized by subjecting to species-specific PCR for *M. gallisepticum* (3.2.4.1), *M. synoviae* (3.2.4.2) and *M. iowae* (3.2.4.3).

3.2.7 Strain Differentiation of *M. gallisepticum* Isolates

3.2.7.1 Restriction Enzyme Analysis of M.gallisepticum – Specific PCR Amplified Product

The amplicon of the MG-PCR reaction with all the *M. gallisepticum* isolates were subjected for RFLP. The PCR products were digested with restriction enzymes according to the manufacturer's instruction. The restriction enzyme digestions were performed in 20µl reaction mixtures consisting of

PCR product	15 µ l
RE buffer (10 X)	2 µl
Restriction enzyme	1 µl
Sterile distilled water to make	20 µl

The mixtures were incubated at 37° C for 2 hours

3.2.7.2 Separation of Restriction Fragments by Poly Acrylamide Gel Electrophoresis (PAGE)

Discontinuous system of Polyacrylamide gel electrophoresis was employed. Hoefer Mighty Small (SE 245) Dual Gel Caster was used for casting the polyacrylamide gel. All the gel caster components (casting cradle with casting clamp assembly, silicone rubber gaskets, and cams) glass and alumina plates and spacers were washed with a mild detergent. Rinsed thoroughly with de-ionized water and allowed to dry. For constructing gel sandwich, one notched alumina plate and one rectangular glass plate (10 X 8 cm) and two 0.75 mm spacers were used. Aligned the plate bottoms on a flat surface, spacers were then inserted in between the plates and it was aligned with the sides of the plate. The sandwich was then slid into the casting clamp assembly, notched plate facing the back block, and plate bottom flushed with the flat surface. The sandwich was secured by tightening all the six screws, while holding the sandwich in place until they were finger-tight. To prevent leaking, the bottom of the plates and spacers was made to protrude slightly below the back block. The clamp assembly was placed in the casting cradle, screw side facing out. In this position, the gel would be visible through the rectangular glass plate.

Eight per cent acrylamide gel (3.1.7.1.1a) was prepared and poured between two glass plates using a pipette taking care not to introduce any air pockets. The solution was filled to just below the top of the notched plate. Air pockets, if formed, were removed with a syringe. Comb was introduced at a slight angle into the sandwich, taking care not to trap air bubbles under its teeth. Overlaid the gel with a thin layer of water using a syringe fitted with a 22 gauge needle to prevent exposure of the top surface of the gel solution to oxygen. Allowed the gel to polymerize for a minimum of one hour. The comb was removed after complete polymerization. The glass plates containing polyacrylamide gel was transferred to the vertical slab gel electrophoresis system (Hoefer, USA). The wells were washed with 1 X TBE buffer to remove unpolymerized particles and the wells were half-filled with 1X TBE. Five

microlitre of the amplified PCR product digested with restriction enzyme was mixed with one microlitre of 6 X gel loading buffer and carefully layered under the buffer column in the wells. DNA molecular size markers were loaded in separate wells. The upper and lower buffer tanks were filled with 1 X TBE buffer and electrophoresis was carried out at 70 V till the bromophenol blue dye reached the bottom of the gel.

When the electrophoresis was over, glass slab containing the gel was dismantled and the gel was separated out and stained with ethidium bromide.

3.2.7.3 Recording of Results

The gel was viewed in a transilluminator and photographed using a gel documentation system.

3.2.8 Preservation of the Isolates

3.2.8.1 Lyophilization

Forty-eight hours broth cultures of *Mycoplasma* were mixed with an equal quantity of sterile 10 per cent skim milk powder (sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure) and dispensed as 0.5 ml aliquots into freeze drying vials of 10 ml capacity. The cultures were frozen in a deep freezer (-70° C), and transferred to the freeze drier while frozen. Vacuum reading was usually 80μ and the vials were left for six hours. Vials were vacuum-sealed, and kept at –70° C. A sample vial was reconstituted with 350 μl of triple distilled water and was inoculated on to buffalo heart infusion agar plate for checking the viability. Stored vials were reconstituted after 1 ½ months period and checked for viability.

Results

4. RESULTS

4.1 SAMPLES FOR THE STUDY

A total of 225 birds including 156 apparently healthy birds and 69 ailing birds belonging to different age groups were sampled. From these birds, a total of 415 clinical samples including 225 tracheal, 58 cloacal, one conjunctival and 23 synovial swabs and 54 airsac and lung materials were investigated in the present study. Ninety-four tracheal swabs (69 ailing and 25 healthy birds) were directly streaked on to BHI agar for *Mycoplasma* isolation while forty-six tracheal swabs (25 ailing and 21 healthy birds) were transported in PBS/ modified buffered peptone water.

4.2 DETECTION OF AVIAN MYCOPLASMA IN CLINICAL SAMPLES

4.2.1 *Mycoplasma* Genus-specific Polymerase Chain Reaction

The primers, GPO3 and MGSO when used to amplify the DNA prepared from *Leptospira icterohaemorrhagiae, Escherichia coli, Staphylococcus aureus and Pseudomonas cepacia* did not evidence any amplification, indicating the specificity of the primers and hence the primer pairs were selected for the amplification of *Mycoplasma* DNA from clinical samples.

The presence of a 270 bp fragment in electrophoresed gel under UV transillumination indicated a positive test and no amplification product indicated a negative test. In the negative control, amplification product was not detected (Fig.1).

Out of the total 415 samples (225 tracheal swabs, 58 cloacal swabs, 54 airsac materials, 54 lung samples, 23 synovial swabs and one conjunctival swab)

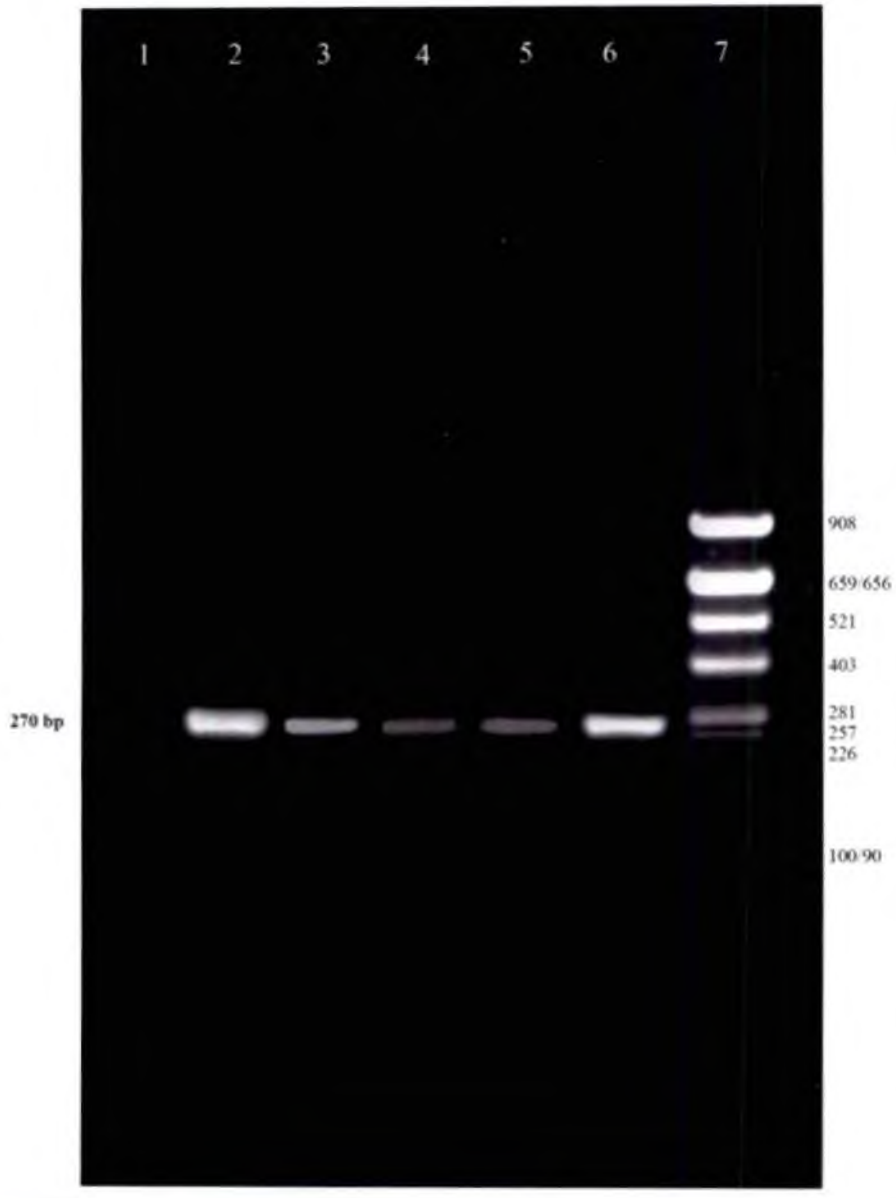

Fig. 1. Agarose gel (1 per cent) electrophoresis of *Mycoplasma* genus-specific PCR products

Lane 1: Negative Control
Lane 2: *M. gallisepticum* isolate
Lane 3: Tracheal sample in BHI broth
Lane 4: Tracheal sample in buffered peptone water
Lane 5: Tracheal sample in PBS
Lane 6: Non -*M. gallisepticum* isolate
Lane 7: pBR 322 DNA/*Alu* I Digest

transported in BHI broth when subjected for *Mycoplasma* genus-specific PCR employing the primers GPO3 and MGSO, 30 samples including 25 tracheal swabs, three cloacal swabs, one conjunctival swab and one airsac material were positive (Table 1). The 30 clinical samples that were positive by genus-specific PCR were from 25 birds and the tracheal swabs from all these birds were positive (Table 1 & 2). A bird reared at RPF, Koovappady tested positive for genus-specific PCR with all the three samples (tracheal swab, cloacal swab and conjunctival swab), whereas two birds maintained at UPF, Mannuthy gave positive result from tracheal and cloacal swabs. Airsac material from a bird necropsied at the Dept. of Microbiology was positive for genus-specific PCR in addition to its tracheal swab.

Out of the total 25 birds positive for *Mycoplasma* genus-specific PCR, 21 were ailing birds and the rest were apparently healthy birds (Table 3 and Table 4).

Of the total 225 birds, 163 were older than one year whereas 62 were below one year. Out of the 163 birds, 12 were positive by PCR and 13 of the 62 tested positive for genus-specific PCR (Table 5 and Table 6).

Tracheal swabs from 46 birds were transported separately in PBS and buffered peptone water in addition to BHI broth. Tracheal swabs from six out of this 46 birds tested positive by genus-specific PCR and these birds had similar results when samples were collected in BHI broth as well.

The overall amplification band intensity presented on gel electrophoresis with the amplified product was more with samples collected in BHI broth when compared to PBS or modified buffered peptone water while no difference could be noted between buffered peptone water and PBS.

4.3 SPECIES DIFFERENTIATION OF AVIAN MYCOPLASMA FROM CLINICAL SAMPLES

4.3.1 *Mycoplasma gallisepticum* Species-specific PCR (MG-PCR)

Primers, pvpA1F, pvpA2R and pvpA3F could not amplify the DNA prepared from *Leptospira icterohaemorrhagiae, Escherichia coli, Staphylococcus aureus* and *Pseudomonas cepacia* and were selected for this study.

Out of the 25 birds positive by genus-specific PCR, only five birds were positive by *M. gallisepticum* species-specific PCR (Table. 7). When 30 samples from the above 25 birds (25 tracheal swabs, three cloacal swabs, one airsac material and one conjunctival swab) were subjected for MG-PCR, nine samples were positive for MG-PCR. This included five tracheal swabs, three cloacal swabs, and one conjunctival swab.

Polyacrylamide gel electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (pUC19 DNA/ *Msp* 1 Digest) in 1x TBE buffer. Amplified product was indicated by a band at or between the 267 and 497 bp region. Analysis of the electrophoresed gel under UV transilluminator revealed the presence of three distinct bands at the regions of 497 bp, 404 bp, and 380 bp. All the nine samples yielded the same result (Fig.2).

Four out of the five birds that tested positive by MG-PCR were ailing birds, of which one was an 18 months old ailing bird with conjunctivitis reared at Poultry farm, Koovappady and three were ailing birds of nine to ten weeks of age maintained at UPF, Mannuthy. The fifth one was an apparently healthy bird of 24 weeks of age reared at UPF, Mannuthy (Table 8). Tracheal swab, cloacal swab and conjunctival swab taken from the bird reared at RPF, Koovappady were

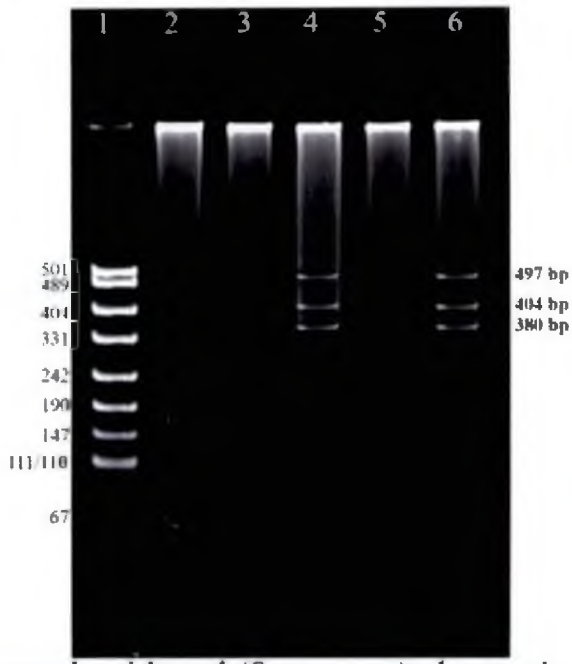

Fig. 2. Polyacrylamide gel (8 per cent) electrophoresis of *M. gallisepticum* species-specific PCR product of representative clinical samples

Lane 1: pUC 19 DNA/*Msp1* Digest
Lane 2: Negative Control
Lane 3: Tracheal sample positive by genus-specific PCR
Lane 4: Tracheal sample in BHI broth
Lane 5: Tracheal sample negative by genus-specific PCR
Lane 6: Tracheal sample in buffered peptone water

Fig. 3. Polyacrylamide gel (8 per cent) electrophoresis of *M. gallisepticum* species-specific PCR product of representative isolates

Lane 1&3: *M. gallisepticum* isolate
Lane 2: Non-*M. gallisepticum* isolate
Lane 4: Lung sample of MG positive bird
Lane 5: Negative control
Lane 6: pUC 19 DNA/*Msp*1 Digest

positive for MG-PCR. Cloacal swabs from two birds maintained at UPF, Mannuthy gave positive result for MG-PCR in addition to their tracheal swabs.

4.3.2 *Mycoplasma synoviae* Species-specific PCR

The primers, MS1 and MS2 when used to amplify the DNA prepared from *Leptospira icterohaemorrhagiae, Escherichia coli, Staphylococcus aureus* and *Pseudomonas cepacia,* no amplification could be obtained.

Of the 30 samples positive by genus-specific PCR when subjected for *M. synoviae* species-specific PCR, none of them yielded a positive result. Amplified product at the region of 169 bp could not be obtained with any of the samples subjected for MS-PCR. Twenty-three synovial samples negative by genus-specific PCR were also subjected to MS-PCR but were negative.

4.3.3 *Mycoplasma iowae* Species-specific PCR

The primers L, R, Li and Ri when used to amplify the DNA prepared from *Leptospira icterohaemorrhagiae, Escherichia coli, Staphylococcus aureus* and *Pseudomonas cepacia* failed to yield any amplified product and hence the primers were selected for the amplification of *M. iowae* DNA from clinical samples.

None of the 30 samples positive for genus-specific PCR when subjected for MI-PCR yielded a positive result. A band at the region of 299 base pair as described by Zhao and Yamamoto (1993) was not observed with any of the samples.

4.4 MEDIUM FOR THE ISOLATION OF AVIAN MYCOPLASMA

Broth and agar media formulated as per Lecce and Sperling (1954) were found to support and maintain the growth of *Mycoplasma* upon primary isolation and subsequent subcultures. All the isolates obtained, except one could be successfully subcultured and maintained using the prescribed medium.

4.5 ISOLATION OF AVIAN MYCOPLASMA

Out of the 94 tracheal swabs (from 69 ailing birds and 25 healthy birds) when directly streaked on to BHI agar, 15 were positive for isolation. On the other hand, the 30 BHI broth samples that tested positive by genus-specific PCR, when subcultured on to BHI agar medium, only 13 of them were positive for isolation and all the isolates were obtained from tracheal swab alone (Table 1).

It was observed that out of the 15 samples positive for isolation, those that have been streaked directly on to the agar yielded isolates. But with the same samples that had been collected simultaneously in the broth and later subcultured on to the solid medium following a positive result in PCR /change in the colour of the broth, only 13 samples yielded colonies on the agar. Out of the two samples which did not yield growth, the colour change produced in the broth was not conspicuous in one case. A turbid yellow colouration was observed in the other.

Twelve of the isolates were from ailing birds and three were from apparently healthy birds.

No isolates could be obtained from any of the airsac materials or cloacal swabs subjected for the study.

Isolation was not successful when samples transported in PBS and buffered peptone water (positive by genus-specific PCR) were streaked on to BHI agar.

Thirty six samples that were negative by *Mycoplasma* genus-specific PCR were also tried for isolation but did not yield any growth even upto 21 days of incubation and were discarded there after.

Of the 15 samples positive for isolation upon direct streaking on to the agar medium, five of them were *M. gallisepticum* isolates. Out of the 30 broth samples positive for genus-specific PCR, only nine were positive by *M. gallisepticum* species-specific PCR. MG-PCR positive broths when subcultured on to BHI agar, five yielded colonies suggestive of *Mycoplasma* on the agar but only two of them were *M. gallisepticum* isolates and these two isolates were from tracheal swabs.

Four out of the five *M. gallisepticum* isolates were from ailing birds, of which one was from an 18 months old ailing bird with conjunctivitis maintained at Poultry farm, Koovappady. Three were from ailing birds of nine to ten weeks of age maintained at UPF, Mannuthy. Another isolate obtained was from an apparently healthy bird of 24 weeks of age from UPF, Mannuthy. All the isolates obtained could be maintained except one which failed to grow on further subculturing.

When the broths tested positive for *M. gallisepticum* by MG-PCR were plated on to the solid medium following the result of the PCR, only two of them yielded MG colonies, whereas with three other broths the colonies obtained tested negative for MG-PCR but were positive by genus-specific PCR. Agar plates directly inoculated with the tracheal swabs in these five cases yielded MG colonies, although interspersed with non-MG colonies.

The isolation trial from an MG positive case with clinical presentation of conjunctivitis yielded isolate from tracheal swab only. The conjunctival swab although positive by PCR did not yield any isolate. Similarly attempts to isolate *M. gallisepticum* from the cloacal swab of three birds that tested positive by *M. gallisepticum* species-specific PCR were unsuccessful.

4.5.2 Isolation of *M. synoviae*

Synovial swab collected from 23 birds necropsied at the Department of Microbiology were streaked onto *M. synoviae* specific media. Thirty two tracheal swabs and cloacal swabs obtained from apparently healthy and ailing birds were also subjected for isolation. None of the samples yielded colonies on the agar following 21 days of incubation at 37° C in a CO_2 incubator under six per cent CO_2 tension.

4.6 CHARACTERIZATION OF AVIAN MYCOPLASMA ISOLATES

4.6.1 Colony Characters

Growth was obtained from the second day onwards. It was seen that all *M. gallisepticum* isolates evinced growth from the third day onwards.

Firm colonies were visible to the naked eye as minute dew drops along the streak line which could not be scooped out with inoculation loop.

Under the 10X objective of the microscope, typical colonies with a dense centre and a less dense periphery, giving a fried-egg appearance were appreciated (Fig.6 and 7). Texture of the colonies initially was smooth and dense. Umbonate colonies increased in size gradually and after sixth day of incubation, a typical fried-egg appearance was observed. Coalesced colonies, less raised colonies and atypical elongate colonies but with raised central region were also observed (Fig.11 and 12). On storage at 4° C, the colonies appeared more friable, rough

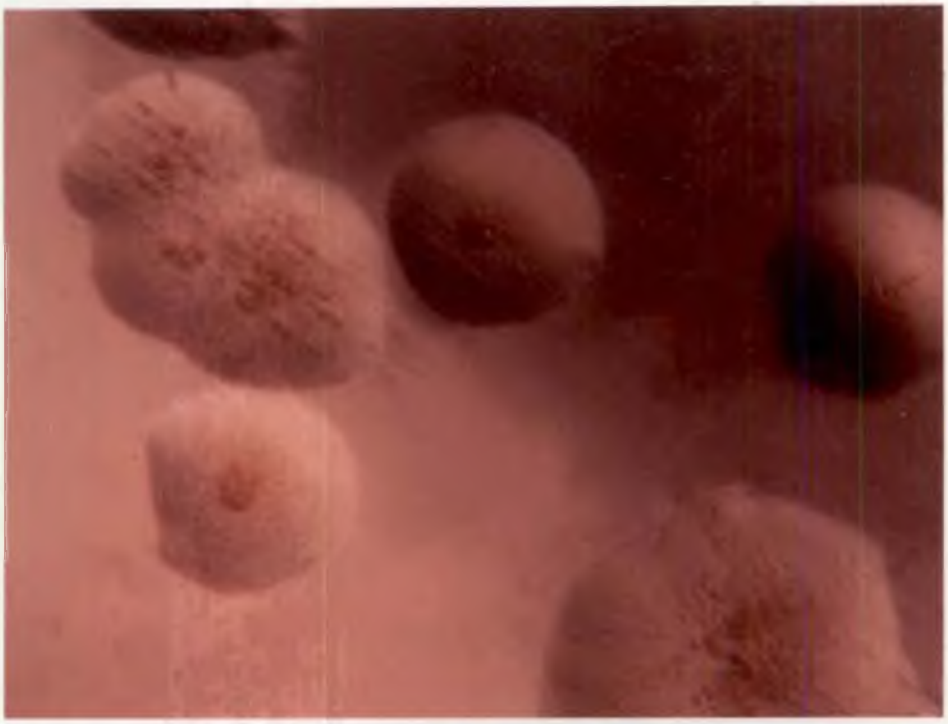

Fig. 6. Colonies of *M. gallisepticum* showing fried-egg appearnce (10 X magnification)

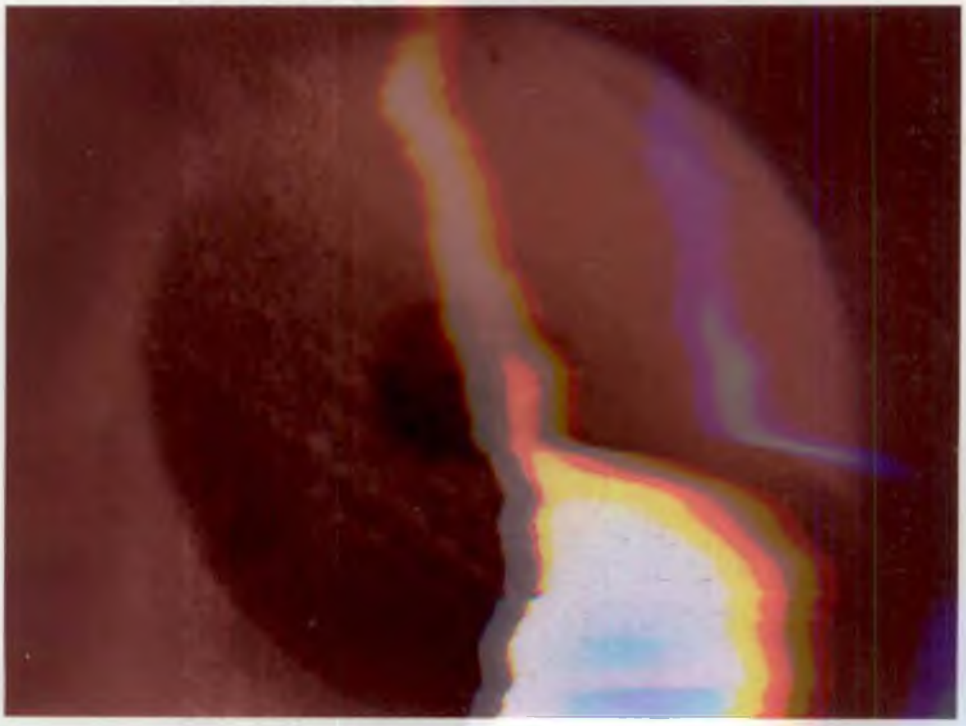

Fig. 7. *M. gallisepticum* - single colony (10 X magnification)

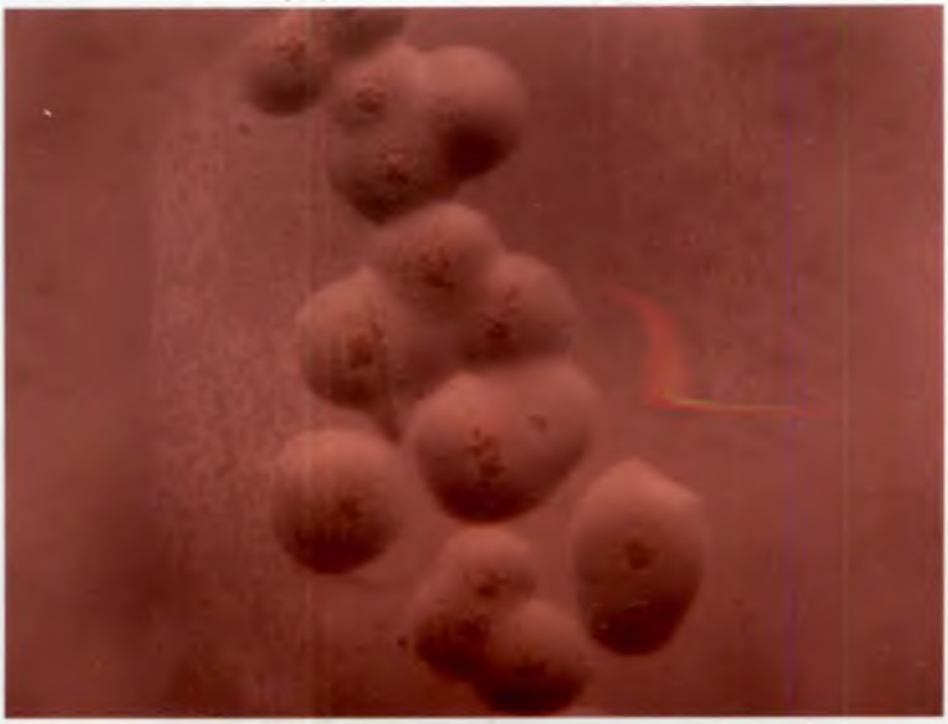

Fig. 11. Coalesced colonies of *M. gallisepticum* (10 X magnification)

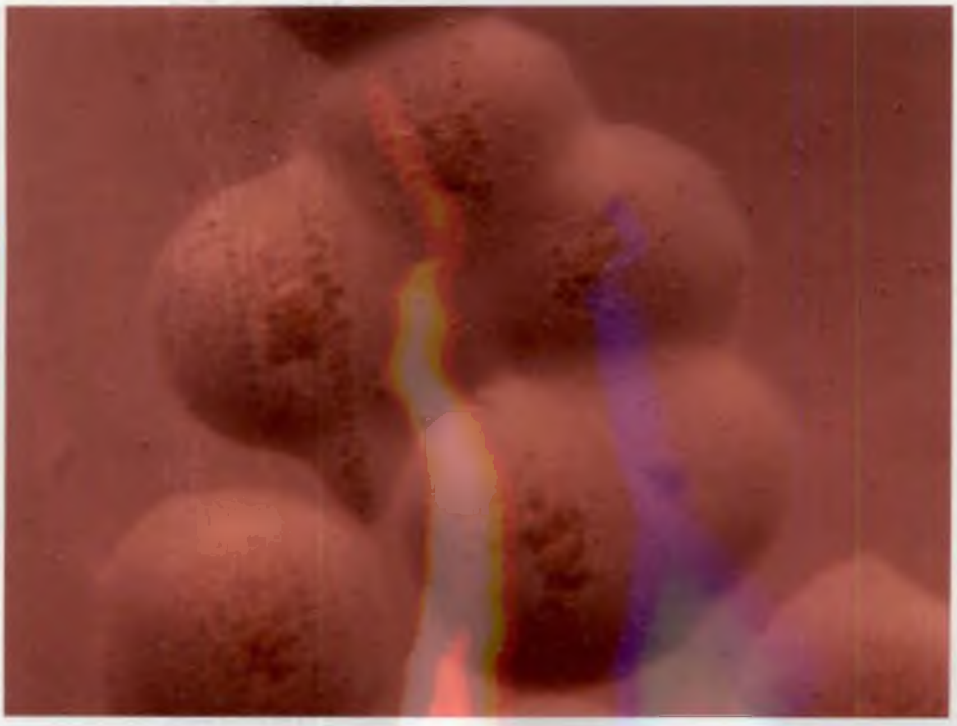

Fig. 12. Coalesced colonies of Non-*M. gallisepticum* (10 X magnification)

and the central denser region was more conspicuous. Colonies obtained on subsequent subculturing lacked a prominent fried-egg appearance.

4.6.2 Diene's Staining Technique

Mycoplasma colonies stood out distinctly with dense blue centres and less blue periphery. Colonies were never decolourized. They were easily distinguishable from that of the L-forms obtained along with the *Mycoplasma* colonies in two cases by their gross appearance. Colonies of L-forms were flat, transluscent and often more shiny than mycoplasmal colonies which were also transluscent but firm and raised at their centres (Fig.8, 9 and 10).

4.6.3 Characterization of Isolates by PCR

4.6.3.1 Mycoplasma Genus-specific PCR

All the 15 isolates obtained were subjected for *Mycoplasma* genus-specific PCR. All of them yielded an amplified product of 270 bp, similar to that obtained with the clinical samples. Intensity of the amplified fragment was more with that of the isolate than with clinical sample (Fig.1).

4.6.3.2 Mycoplasma gallisepticum Species-specific PCR

Out of the 15 isolates, only five of them tested positive for *M. gallisepticum* by MG-PCR. These five isolates were same as the five MG positive clinical samples (Fig.3).

Polyacrylamide gel electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (pUC19 DNA/ *Msp* I Digest) in 1x TBE buffer. Analysis of the electrophoresed gel under UV transilluminator revealed the presence of three distinct bands at the regions of 497 bp, 404 bp, and 380 bp. All the five isolates yielded the same result and were

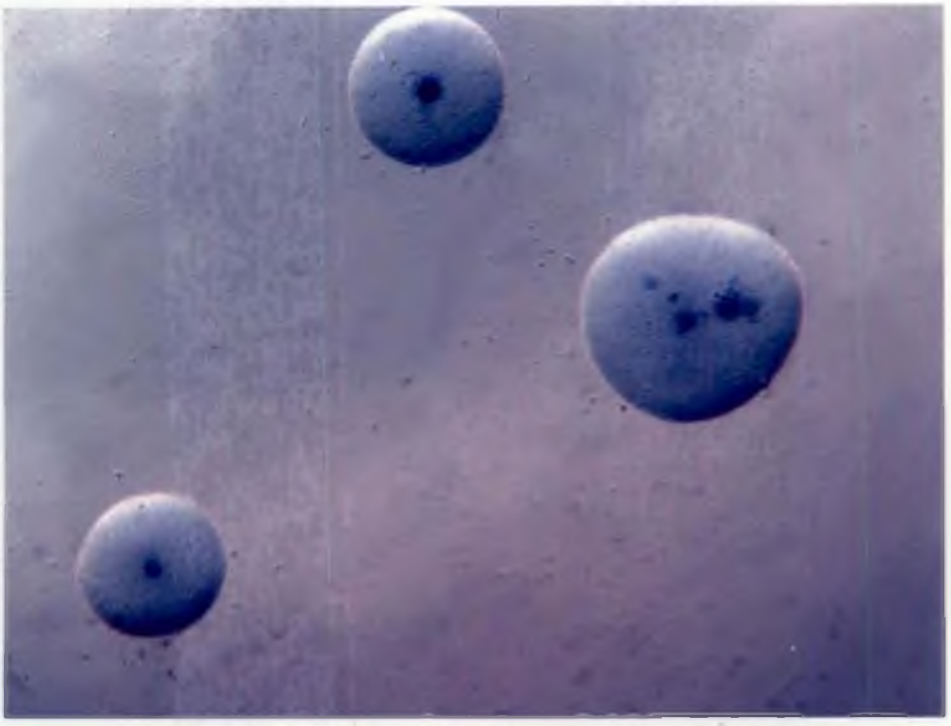

Fig. 8. Colonies of *Mycoplasma* stained by Diene's method of staining (10 X magnification)

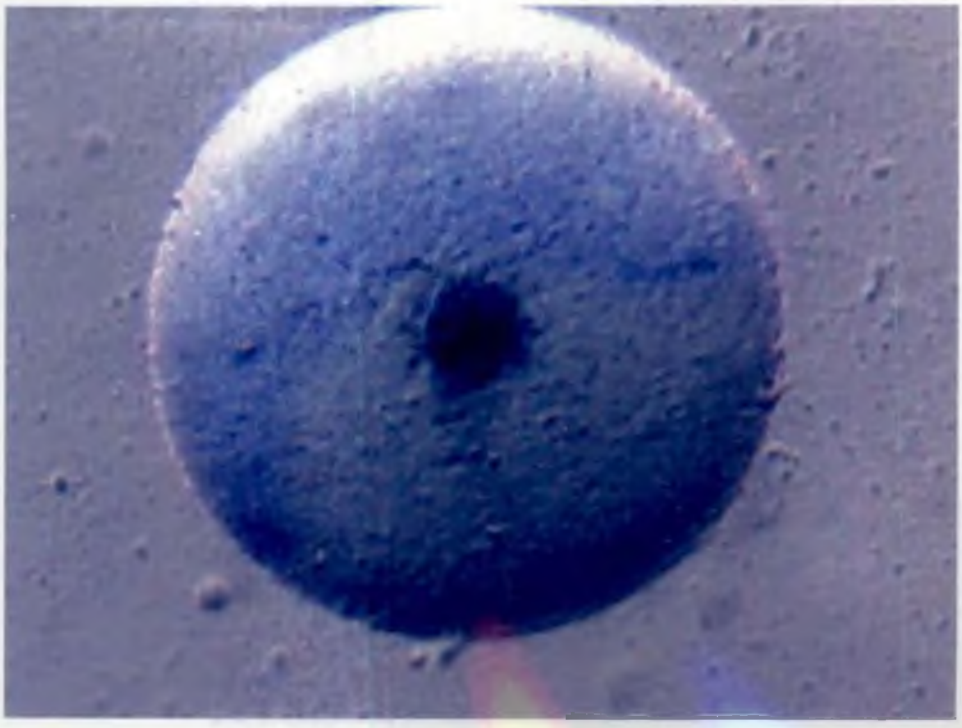

Fig. 9. Single colony of *Mycoplasma gallisepticum* stained by Diene's method of staining (10 X magnification)

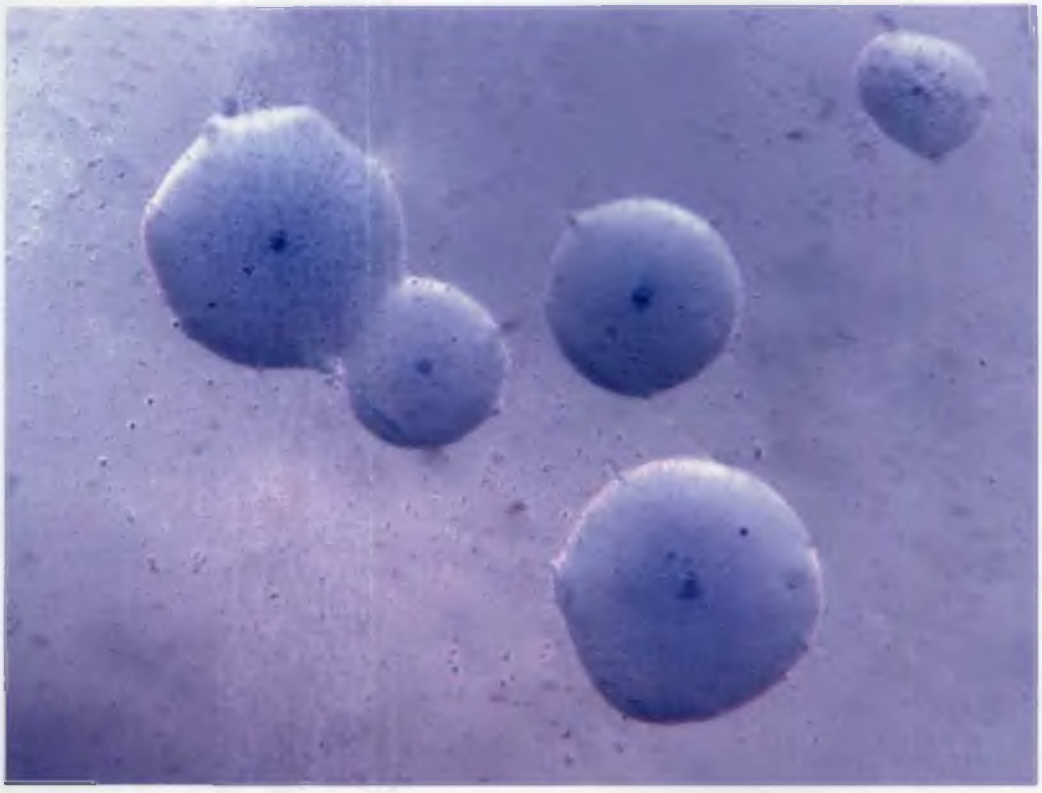

Fig. 10. Colonies of *Mycoplasma gallisepticum* stained by Diene's method of staining (10 X magnification)

exactly similar to that of the naïve MG-PCR profiles obtained with the clinical samples.

Interestingly, not all the colonies in *M.gallisepticum* plate were *M.gallisepticum* colonies. Templates prepared from certain colonies from *M.gallisepticum* positive plates were negative by MG-PCR, but were positive by *Mycoplasma* genus specific PCR.

4.7 STRAIN DIFFERENTIATION OF *M. gallisepticum* ISOLATES

4.7.1 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

When the PCR products amplified using the primers pvpA1F and pvpA2R and pvpA3F, were digested separately by restriction enzymes *Pvu* II, *Acc* I and *Scr*F I, all the isolates yielded similar restriction profiles. When digested with *Pvu* II, seven bands were obtained which ranged from 111 to 404 bp. The five bands obtained on digesting with *Acc* I ranged from 150 bp to 404 bp. Digestion with *Scr*F I yielded five bands and they ranged from 170 bp to 404 bp (Fig.4 and 5).

Based on the results of RFLP, it could be assumed that the five isolates of MG were of the same strain.

4.8 PRESERVATION OF THE ISOLATES

Agar plates with Mycoplasmal colonies were stored at 4° C. Log phase broth cultures and lyophilized cultures were stored at -70° C. Growth were obtained upon revival of the lyophilized cultures and broth cultures stored at –70°C upto 13 months, but revival of isolates from all the agar plates stored at 4° C were found to be unsuccessful after four months of their storage.

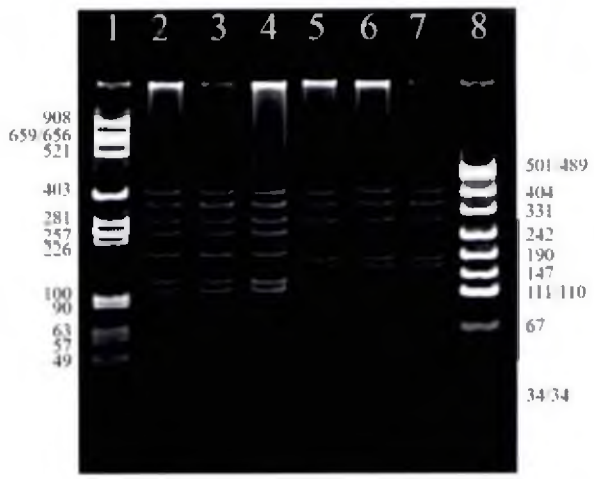

Fig. 4. Polyacrylamide gel (8 per cent) electrophoresis of *Pvu* II and *ScrF* I digested *M. gallisepticum* species-specific PCR product

Lane 1: pBR 322 DNA/*Alu* I Digest
Lane 2,3 & 4: *Pvu* II digested *M. gallisepticum* species-specific PCR product
Lane 5,6 & 7: *ScrF* I digested *M. gallisepticum* species-specific PCR product
Lane 8: pUC 19 DNA/*Msp* I Digest

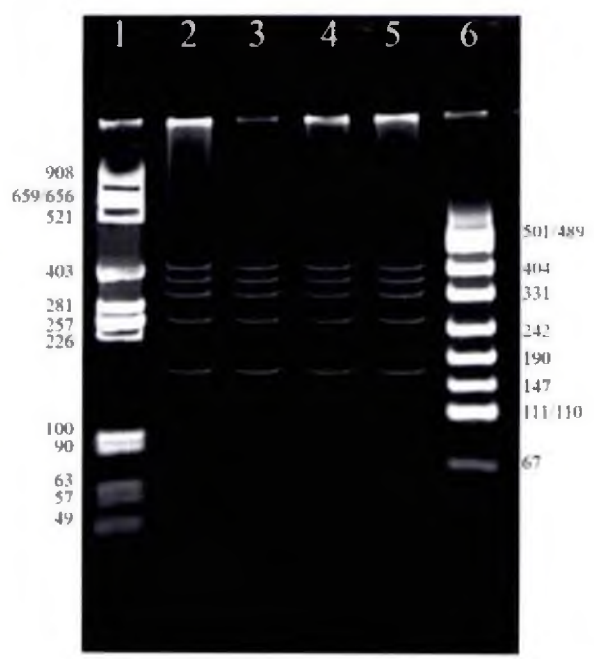

Fig. 5. Polyacrylamide gel (8 per cent) electrophoresis of *Acc* I digested *M. gallisepticum* species-specific PCR product showing similar profiles

Lane 1: pBR 322 DNA/*Alu* I Digest
Lane 2,3,4 & 5: *Acc* I digested *M. gallisepticum* species-specific PCR product
Lane 6: pUC 19 DNA/*Msp* I Digest

Table 1. Overall summary of the result

Source of Birds		Birds sam-pled	No. of samples subjected to PCR						No. of samples positive by PCR						No. of samples tried for isolation							No. of samples positive by isolation						
															DI	S/c from broth after the result of PCR						DI	S/c from broth after the result of PCR					
			TS	CS	AS	L	SS	JS	TS	CS	AS	L	SS	JS	TS	TS	CS	AS	L	SS	JS	TS	TS	CS	AS	L	SS	JS
RPF, Chathamangalam	Ailing	3	3	3	-	-	-	-	3	-	-	-	-	-	3	3	-	-	-	-	-	-	-	-	-	-	-	-
	Healthy	25	25	3	-	-	--	-	1	-	-	-	-	-	4	1	-	-	-	-	-	-	-	-	-	-	-	-
RPF, Kudappanakunnu	Ailing	3	3	3	-	-	-	-	1	-	-	-	-	-	3	1	-	-	-	-	-	1	1	-	-	-	-	-
	Healthy	28	28	3	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-
RF, Koovappady	Ailing	5	5	5	-	-	-	1	5	1	-	-	-	1-	5	5	1	-	-	-	1	5	5	-	-	-	-	-
	Healthy	30	30	4	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	--	-
Birds brought to Microbiology	Ailing	54	54	26	54	54	23	0	8	-	1	-	-	-	54	8	-	1	-	-	-	3	2	-	-	-		-
	Healthy	0	0	0	-	-	-	-	-	-		-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-
UPF, Mannuthy	Ailing	4	4	4	-	-	-	-	4	2	-	-	-	-	4	4	2	-	-	-	-	3	3	-	-	-	-	-
	Healthy	58	58	2	-	-	-	-	2	-	-	-	-	-	10	2	-	-	-	-	-	2	2	-	-	-	-	-
Vadakkevila Panchayat, Kollam	Ailing	0	0	0	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-
	Healthy	15	15	5	-	-	-	--	1	-	-	-	-	-	4	1	-	-	-	-	-	1	0	-	-	-	-	-
Total		225	225	58	54	54	23	1	25	3	1	-	-	1	94	25	3	1	-	-	1	15	13	-	-	-	-	-

DI- direct inoculation on to BHI agar, TS- tracheal swab, CS- cloacal swab, AS- airsac material, L- lung, SS- synovial swab, JS- conjunctival swab

Table 2. Farm-wise categorization of results

Source of birds	Number of birds	Number positive	
		PCR	Isolation
Regional Poultry Farm, Chathamangalam	28	4	-
Regional Poultry Farm, Kudappanakkunnu	31	1	-
Poultry Farm, Koovappady	35	5	5
Birds brought to the Department of Microbiology	54	8	3
UPF, Mannuthy	62	6	6
Vadakkevila Panchayat, Kollam	15	1	1
TOTAL	225	25	15

Table 3. Results in comparison with the health status of the birds

Number of ailing birds	Number positive		Number of healthy birds	Number positive	
	By PCR	By isolation		By PCR	By isolation
69	21	12	156	4	3

Table. 4. Farm-wise analyses of the results in respect to the health status of the birds

Source of birds	Total No. of birds	No. of ailing birds	Number positive		Number of healthy birds	Number positive	
			PCR	Isolation		PCR	Isolation
Regional Poultry Farm, Chathamangalam	28	3	3	-	25	1	-
Regional Poultry Farm, Kudappanakkunnu	31	3	1	1	28	-	-
Poultry Farm, Koovappady	35	5	5	5	30	-	-
Birds brought to the Department of Microbiology	54	54	8	3	-	-	-
UPF, Mannuthy	62	4	4	3	58	2	2
Vadakkevila Panchayat, Kollam	15	-	-	-	15	1	1
TOTAL	225	69	21	12	156	4	3

Table. 5. Age-wise analysis of the result.

Age of birds	Total sampled	Number of birds positive	
		PCR	Isolation
1 year and above	163	12	6
Below 1 year	62	13	9

Table. 6. Farm-wise and age-wise analyses of the PCR and Isolation results

Source of birds	No. above 1 year	Number positive		Number below 1 year	Number positive	
		By PCR	By isolation		By PCR	By isolation
Regional Poultry Farm, Chathamangalam	20	4	-	8	-	-
Regional Poultry Farm, Kudappanakkunnu	27	1	-	4	-	-
Poultry Farm, Koovappady	25	5	5	10	-	-
Birds brought to the Department of Microbiology	28	1	-	26	7	3
UPF, Mannuthy	48	-	-	14	6	6
Vadakkevila Panchayat,Kollam	15	1	1	-	-	-

Table. 7 PCR Vs Isolation

Number of birds positive by genus-specific PCR	Number of birds positive by isolation	Number of birds positive for *M.gallisepticum* by MG-PCR.	Number of birds positive for *M. gallisepticum* by isolation
25	15	5	5

Table 8. *Mycoplasma gallisepicum*- Age & health status of positive birds

Source of birds	Age of birds	Health status of birds	Number of MG positive birds
PF, Koovappady	Above 1 year	Ailing	1
UPF, Mannuthy	9 to 10 weeks	Ailing	3
UPF, Mannuthy	24 weeks	Healthy	1

Discussion

5. DISCUSSION

Avian mycoplasmosis is one of the economically significant diseases affecting the poultry (Jordan, 1979). The pathogenic avian mycoplasmas include the *M. gallisepticum* and *M. synoviae* in chickens and turkeys, *M. iowae* which can kill chicken and turkey embryos and *M meleagridis* in the turkeys. *M. gallinarum* although regarded non-pathogenic, induction of fatty liver haemorrhagic syndrome in birds experimentally inoculated with them (Branton *et al.*, 2003) and the repeated isolation from cases of airsacculitis had put the status of this *Mycoplasma* in ambiguity. Reports on the pathogenic significance of *M. pullorum* as a cause of embryo mortality, incorporates it to the league of pathogenic mycoplasmas (Moalic *et al.*, 1997).

For many years, diagnosis of avian mycoplasmosis was based on serological assays to detect antibody production and / or on isolation and identification of the organism. Serological tests as Rapid slide agglutination (RSA) test and ELISA, although useful for examining flocks, lack specificity or sensitivity (Kleven and Fletcher, 1983 and Yoder, 1991). Isolation techniques are laborious, slow and expensive and require sterile conditions. Problems posed due to the overgrowth of faster-growing mycoplasma species, or no growth in subculture are predictable difficulties underlying the isolation of pathogenic mycoplasmas (Kempf, 1994).

Rapid, sensitive and specific tests that detect nucleic acids from pathogenic mycoplasmas are very attractive for the laboratory detection of infected flocks and PCR assay as an effective tool for the detection of avian mycoplasmas have already been published (Nascimento *et al.*, 1991; Lauerman *et al.*, 1993; Zhao and Yamamoto, 1993; Geary *et al.*, 1994; Silveira *et al.*, 1996).

Identification of isolates usually involves cloning subcultures, immunofluorescence and determination of biochemical and serological characters. The whole procedure is time consuming and requires specific hyperimmune sera which are not commercially available. In these context, PCR-based diagnostic tools aids in the identification as well in the strain differentiation of *Mycoplasma*. Lauerman *et al.* (1993) could distinctly differentiate nine mycoplasma species using the RFLP analysis of the PCR amplicon. Fourteen species of avian mycoplasmas were differentiated by Fan *et al.* (1995) based on the RFLP patterns generated with six different restriction enzymes.

5.1 SAMPLES FOR THE STUDY

The samples collected for the isolation of *Mycoplasma* were tracheal swabs, airsac materials, cloacal swabs, conjunctival swab, lung material and synovial swabs. Tracheal swabs were collected from all the birds sampled. Cloacal swabs were collected from birds reported to have lowered egg production records or otherwise at random. Airsac materials and lungs were collected from all the birds brought and necropsied at the Department of Microbiology. Joint fluids were collected and subjected for study from a certain number of birds necropsied at the Department of Microbiology. Swabs are advised to be taken from trachea, chaonal cleft, oesophagus, cloaca and phallus in live birds and from nasal cavity, infraorbital sinus, trachea and airsacs in the case of dead birds (OIE, 2000).

Conjunctival swabs yielded good isolation rates of MG from house finches with conjunctivitis (Ley *et al.*, 1996). Air sac materials were collected from those birds, which showed specific lesions on air sac. The swabs were pre-wet in mycoplasma broth before collecting samples. Wet swabs yielded significantly greater number of mycoplasmas than dry swabs (Zain and Bradbury, 1995). Conjunctival swab was taken form a bird which was presented with conjunctivitis.

5.2 DETECTION OF AVIAN MYCOPLASMA IN CLINICAL SAMPLES

5.2.1 *Mycoplasma* Genus-specific PCR

A total of 30 samples were positive when 415 samples collected in BHI broth from 225 chickens were subjected for *Mycoplasma* genus-specific PCR. Positive samples yielded a band of 270 bp. *Mycoplasma* DNA if present in the sample would give an amplified product of 270 bp by using the primer MGSO and GPO3 (Marois *et al.*, 2000).

As far as PCR was concerned, the band intensity was more with samples collected in broth. There was no significantly visible variation in the band intensity with samples collected in buffered peptone water or PBS. Out of the 46 tracheal swabs collected in PBS and modified buffered peptone water, six were positive for genus-specific PCR.

5.2.2 *M. gallisepticum* Species-specific PCR (MG-PCR)

Five tracheal samples were positive by *M. gallisepticum* species-specific PCR out of the total 25 tracheal samples positive by genus-specific PCR. Analysis of the *M. gallisepticum* species-specific amplified product revealed the presence of three distinct bands at the regions of 497 bp, 404 bp, and 380 bp.

Amplification product size polymorphism was reported due to the size variation of the gene due to deletions occurring in the segment encoding the proline-rich C terminal region of the protein (Liu *et al.*, 2001). In this study all the five isolates showed similar band patterns.

5.2.3 *M. synoviae* Species-specific PCR (MS-PCR)

None of the samples tested positive by *M. synoviae* species-specific PCR. None of the isolates were found to be *M. synoviae* by MS-PCR. Amplified product of 169 bp was obtained by Lauerman *et al.* (1993) upon amplification of *M. synoviae* DNA using the primers MS-1 and MS-2.

5.2.4 *M. iowae* Species-specific PCR (MI-PCR)

None of the samples tested by MI-PCR were positive in the present study. Zhao and Yamamoto (1993) used the primers L and R to amplify *M. iowae* DNA from clinical samples and could obtain a 299 bp amplified product.

5.2.5 Medium for the Isolation of Avian Mycoplasma

The critical requirement in isolation of avian mycoplasmas is to have a good media base, which could be used for the isolation of all the pathogenic avian mycoplasmas. The medium used in the present study was supplemented buffalo heart infusion broth (Lecce and Sperling, 1954). The medium was found ideal for the isolation and propagation of avian mycoplasmas. Similar results have been obtained 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.

PPLO broth containing 20 per cent horse serum was used by Alder *et al.* (1954), whereas Fabricant (1958) used chicken embryo for the isolation of avian mycoplasmas. Availability of chick embryos free from mycoplasmas limits the routine use of the latter mode of isolation. In the present study all the inoculated media were incubated at 37° C under six per cent CO_2 tension in a CO_2 incubator.

Increased humidity and CO_2 tension between 5-10 per cent have been reported to enhance growth of mycoplasma (OIE, 2000).

5.2.6 Isolation of Avian Mycoplasma

Out of the 94 tracheal swabs directly streaked on to BHI agar, 15 were positive by isolation. Avian mycoplasma had been isolated from chickens by various scientists (Nelson, 1939; Markham and Wong, 1952; Alder *et al.*, 1954; Yoder and Hofstad, 1964; Frey *et al.*, 1968; Jain *et al.*, 1971; Power and Jordan, 1976; Shimizu *et al.*, 1979; Branton *et al.*, 1984).

It was observed that out of the 15 samples positive upon isolation, all were from tracheal swabs that have been streaked directly on to the agar. But with the same samples that had been collected simultaneously in the broth and later subcultured onto the solid medium following a positive result in PCR/ change in the colour of the broth, only 13 samples yielded colonies on the agar. For the isolation of avian mycoplasma solid medium was found to be more effective than liquid medium by Ronglian *et al.* (1996). With the two samples which did not yield growth, the colour change produced in the broth was not conspicuous in one case whereas with the other a turbid yellow colouration was observed.

One conjunctival swab although positive by PCR, did not yield any isolate. Airsac material also did not yield any isolate. Airsac materials and conjunctival swab collected from advanced stages of infection showed heavy contamination with other bacteria so that isolation of mycoplasmas was unsuccessful. Heavy contamination with bacteria was reported by Majid (1986) when specimen from fowl was directly inoculated on to mycoplasma medium for isolation.

Out of the 225 birds sampled, only 15 isolates were obtained although 25 of the sampled birds were positive for the presence of *Mycoplasma* by genus-specific polymerase chain reaction. 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 as 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 that DNA based tests as PCR were capable of highly specific and sensitive identification of the target organism without reliance on the viability of the organism.

Out of the four PCR positive tracheal samples from apparently healthy birds (out of a total 156 birds), only three were positive by isolation. Out of the 21 PCR positive tracheal samples obtained from ailing birds (from a total of 69 birds), only 12 of them were positive for isolation. Of the total nine PCR positive samples that failed to yield any colonies, four were from Chathamangalam farm and these could not be isolated because of the heavy contamination of the media due to the omission of thallium acetate from that batch of media. The significance of bacterial inhibitors as thallium acetate and penicillin in *Mycoplasma* media had been well documented (Yoder and Hofstad, 1964; Lecce and Sperling, 1954; Frey *et al.*, 1968; Branton *et al.*, 1984).

The contaminated broths were passed through 0.2 micrometer Millipore filter in an attempt to selectively retain other bacteria in the filter and the filterate was used as the inoculum (OIE, 2000). Filtrate was streaked on to fresh thallium acetate supplemented agar. But the isolation trials were unsuccessful and no colonies were obtained on repeated passage of the filterate in broth and agar.

Broth media which did not show any colour change were also streaked on to agar if the results by PCR were positive. The presence of arginine hydrolyzing

(alkali producing) mycoplasma species if present may mask the acid colour change produced (OIE, 2000).

All the broth media which did not yield any isolates despite being positive by PCR were serially diluted in fresh broth. Serial dilutions of the specimens were reported to be of value as the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth unless they are diluted out (OIE, 2000). In the present study no growth could be obtained on the agar upon plating of serially diluted broth.

Thirty-six samples negative by PCR were tried for isolation but did not yield any growth upto 21 days of incubation. Results of the study conducted by Moalic *et al.* (1997) showed that PCR could detect *M. meleagridis* in cloacal swabs from a known infected turkey flock at 13 and 7 weeks before point of lay whereas attempts at culture were negative. Similar results were obtained with tracheal swabs at week 0 and week 8 after point of lay. In the present study also four tracheal samples positive by PCR could not be isolated. Although one of the cloacal swabs was tested positive by MG-PCR, the same could not be isolated.

Of the 15 isolates obtained, five were found to be MG isolates by MG-PCR. Out of this five MG isolates obtained one was from a healthy bird of 24 weeks of age maintained at the University Poultry Farm, Mannuthy. Chickens if exposed to MG infection at a younger age may get partially recovered and remain as carriers of infection without any obvious clinical signs (Yoder and Hofstad, 1964). Rest four was obtained from ailing birds. Similarly only one out of the five MG positive birds showed positive results by PCR from cloacal swab but did not yield growth in BHI agar.

It was observed that all the clinical samples obtained from chickens positive for *M.gallisepticum* yielded isolates when the tracheal swabs were directly plated on to the solid media. Following the results of MG-PCR, when the

broths were plated on to the solid media, the colonies obtained gave a negative result in MG-PCR but was positive by genus PCR in three of the cases. It might have happened due to the overgrowth of non-pathogenic species of avian mycoplasma in the broth overwhelming the growth of *M.gallisepticum*. Isolation of mycoplasmas become technically complicated in cases in which nonpathogenic mycoplasma species might overgrow the virulent mycoplasmas (Liu *et al.*, 2001). Problems experienced with culture of mycoplasmas include overgrowth by faster–growing mycoplasma species or other organisms (Kempf, 1997).

It was seen that all *M. gallisepticum* isolates did not show any growth before the third day. Pathogenic mycoplasmas are usually more fastidious than the less pathogenic ones (Yoder, 1991). The colour change of the broth from red to yellow indicated the growth of mycoplasma. Inclusion of phenol red with sufficient dextrose provides a growth indicator system as the fermentation of dextrose by mycoplasmas change phenol red to a yellow colour as medium becomes acidic (Yoder, 1991).

All the isolates obtained could be subcultured and maintained except one *M. gallisepticum* isolate. Kempf (1997) reported lack of growth in subculture as one major problem encountered during the culture of mycoplasma

Garcia *et al.* (1996) compared PCR-based assay to detect *M. gallisepticum*, *M. synoviae* and *M. iowae* with that of commercial *M. gallisepticum* and *M. synoviae* DNA probe tests and Mycoplasma cultural trials. They found that the results obtained by PCR assay were in agreement with DNA probe kit results, whereas, for a few of the tested flocks only *M. gallinaceum* and *M. gallinarum*, two non-pathogenic mycoplasmas, could be isolated by culture and inhibited the isolation of *M. gallisepticum* or *M. synoviae*. In the present study inhibition of the growth of *M. gallisepticum* by other non-pathogenic mycoplasmas could not be appreciated on agar plates although colonies of other

mycoplasmas were obtained along with *M. gallisepticum*. But the inhibition of *M. gallisepticum* by other non-pathogenic mycoplasmas seems to be true with broth cultures. Three of the samples collected in broth that gave a positive result on MG-PCR, upon plating on to the solid media after the colour change of the broth was appreciated, yielded *Mycoplasma* colonies that tested negative by MG-PCR whereas the same samples that had been plated directly on to the agar yielded *M. gallisepticum* colonies although interspersed with non MG colonies.

5.2.7 Isolation of *Mycoplasma synoviae*

In the present study no isolates of *M. synoviae* could be obtained. Isolation of *M. synoviae* requires extreme care and is difficult to isolate. Frey *et al.* (1968) reported that avian meat infusion base medium supported isolation and propagation of *M. gallisepticum* and *M. meleagridis* but not *M. synoviae*.

M. synoviae is highly exacting in its nutrient requirements and need the supplementation of NAD, L-cysteine hydrochloride and swine serum (Kempf, personal communication). None of the birds under the present study had been presented with signs of synovitis. None of the isolates from birds with respiratory ailments were proved to be *M. synoviae*.

Throughout the study period, chickens with synovitis could not be encountered. *M. synoviae* mainly causes infectious synovitis in chickens, where as *M. gallisepticum* is the major respiratory pathogen of chickens.

5.3 CHARACTERIZATION OF AVIAN MYCOPLASMA ISOLATES

5.3.1 Colony Characters

The colonies obtained with mycoplasma isolates showed in general typical fried-egg appearance. Apart from typical colonies, elongate and coalesced

colonies were also obtained. Mycoplasma colonies frequently occur in ridges along the streak line, since closely adjacent colonies readily coalesce (Yoder and Hofstad, 1964).

The fried egg appearance of the mycoplasma colonies had been well documented (Markham and Wong, 1952; Alder *et al.*, 1954; Yoder and Hofstad, 1964; Frey *et al.*, 1968; Jain *et al.*, 1971; Power and Jordan, 1976). There were no significantly appreciable differences between the colonies of *M. galllisepticum* and other mycoplasmal colonies obtained. Variation in colonies of isolates representing numerous species of avian mycoplasma had been noted, but the species designation of mycoplasmas could not be determined by their colony characters (Yoder and Hofstad, 1964).

5.3.2 Diene's Staining

In the present study the isolates obtained were identified as mycoplasma based on their morphology. Mycoplasma isolates produced typical fried-egg appearance on the mycoplasma agar surface and were confirmed by the Diene's method of staining (Madoff, 1960).

5.3.3 Characterization of the isolates by PCR

5.3.3.1 Mycoplasma genus-specific PCR

All the 15 isolates were positive for Mycoplasma genus-specific PCR. The PCR profiles obtained were similar to that obtained with the naïve results with clinical samples. *Mycoplasma* DNA if present in the sample will give an amplified product of 270 bp by using the primer MGSO and GPO3 (Marois *et al.*, 2000).

5.3.3.2 M. gallisepticum species-specific PCR

Five among the 15 isolates tested positive for MG-PCR and the results were exactly similar to that obtained with the MG positive clinical samples. Amplification product size of *M. gallisepticum* can range from 267 to 497 bp (Liu *et al.*, 2001).

Interestingly, not all the colonies in *M.gallisepticum* agar plate were *M.gallisepticum* colonies. Templates prepared from certain colonies from *M.galliespticum* positive plates were negative by MG-PCR, but were positive by Mycoplasma genus-specific PCR. This may be due to the presence of other species of mycoplasmas in the tracheal mucosa of the birds from which the samples were procured. More than one species of *Mycoplasma* infecting the same tissue was reported (Jordan and Amin, 1980). Bradbury and McClenaghan (1982) reported that out of 67 field isolates of *Mycoplasma* subjected for study, 26 of them were found to be mixed cultures.

Usually *M. gallinarum* may be present in the trachea of birds, without any clinical significance. *M.gallinarum* has not been considered to be one of the pathogenic avian mycoplasma species and they are commonly isolated as a contaminant during attempts to isolate *M. gallisepticum* or *M. synoviae* (Kleven, 1998). So selective isolation of the *M.gallisepticum* colonies prior to subculturing is mandatory. Thus it is important that individual colonies be selected while cutting out the agar blocks and validated as pure MG before proceeding with the subculturing.

5.3.3.3 M. synoviae species-specific PCR

None of the 15 isolates tested positive by MS-PCR. Similarly none of the clinical samples did yield any positive result by MS-PCR. Primer set MS-1 and MS-2 were evaluated by MS-PCR (Lauerman *et al.*, 1993) and could obtain 100

per cent specificity. Amplified product of 169 bp was obtained by Lauerman *et al.* (1993) upon amplification of *M. synoviae* DNA using the primers MS-1 and MS-2.

5.3.3.4 M. iowae species-specific PCR

None of the isolates could be attributed the identity of *M. iowae* due to a negative result evidenced by all the 15 isolates for MI-PCR. Zhao and Yamamoto, (1993) used the primers L and R to amplify *M. iowae* DNA from clinical samples and could obtain a 299 bp amplified product.

5.3.3.5 Strain differentiation of M. gallisepticum isolates

5.3.3.5a M. gallisepticum Species-specific PCR-RFLP

Digestion of the MG-PCR amplicon with restriction enzymes *Acc*1, *Pvu* 11 and *Scr* F1 yielded distinct but similar restriction profiles for all the five isolates. Rapid differentiation of various strains of *M. gallisepticum* by digestion with restriction enzymes *Acc* 1, *Scr* F1 and *Pvu* 11 was reported by Liu *et al.*, 2001.

Based on the result of PCR-RFLP, it could be inferred that the five isolates obtained in the present study might be of the same strain. Most avian mycoplasma species can be differentiated, according to their unique restriction fragment length polymorphism patterns generated after digestion of PCR products with different restriction enzymes (Kempf *et al.*, 1997).

5.4 STORAGE OF THE ISOALTES.

Agar plates with mycoplasmal colonies were stored at 4° C, log phase broth cultures were stored at -70° C and the isolates were lyophilized and stored

at -70° C. Growth was obtained upon revival of the lyophilized cultures and broth cultures stored at -70° C, but not all the isolates stored in agar plates at 4° C could be revived after four months of their storage. Kelton (1964) reported that lyophilized cultures of mycoplasma were preserved for upto four years where as Harry, (1964) could obtain viable mycoplasmas after fourteen years of storage in the freeze-dried state at 4° C. Broth cultures of *Mycoplasma* were viable when subcultured after three to four years of storage at -30° C (Harry, 1964)

Thus under the light of the present study, it could be inferred that PCR can be considered as an efficient tool in the early and rapid detection of mycoplasmas. The PCR assay was found to be more sensitive than isolation technique. Species and strain differentiation of *Mycoplasma* is a definite advantage of molecular-based techniques as PCR and PCR-RFLP when compared to isolation techniques. For isolation of pathogenic mycoplasma species, solid medium was found suitable. Transit of clinical samples in PBS or buffered peptone water served the purpose for PCR but the same could not be relied for isolation, thus making PCR-based detection of mycoplasmas a much sought after method especially when dealing with samples obtained from field cases and those which require long transit time.

Summary

6. SUMMARY

Poultry production in India has achieved a remarkable growth from age-old backyard farming to dynamic and most sophisticated agro-based industry in recent times, 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 increase the production have greatly contributed to higher incidence of several infectious diseases.

Avian mycoplasmosis is one of the economically significant diseases affecting poultry. Disease transmission occurs directly through contact, egg transmission, genital route and secondary transmission through aerosols and fomites, food, water, insect vectors or carriers had been documented. When a chicken is infected with *M. gallisepticum*, the infection is of longer duration and the infection persists in the flock indefinitely and the chickens may shed the organism intermittently, especially following a period of stress. This peculiarity makes the elimination of *M. gallisepticum* extremely difficult in multi-age breeder and laying complexes. Thus, taking into account the immense economic significance and persistence of avian mycoplasmas, it is imperative to detect the infection in a flock at the earliest. Antigen detection methods are superior when the aim is for an earlier, accurate and definite diagnosis. The present study was undertaken to understand the utility of molecular based methods for the early detection and differentiation of avian mycoplasma.

A total of 225 birds including 156 apparently healthy and 69 ailing birds belonging to different age groups were obtained from UPF, Mannuthy; Regional Poultry Farms at Chathamangalam, Koovappady, Kudappanakkunnu and birds brought for disease diagnosis to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy. From these birds a total of 415

biomaterials including 225 tracheal, 58 cloacal, 23 synovial swabs, 54 airsac and lung materials and one conjunctival swab were subjected for investigation. Sterile cotton swabs pre-soaked with BHI broth were used for sampling.

Of the total 415 samples, 30 samples including 25 tracheal , three cloacal, one conjunctival and one air-sac material were positive for Mycoplasma genus-specific PCR. These 30 samples were obtained form 25 birds of which 21 were ailing and 4 were apparently healthy. Tracheal swabs form all these birds were positive. Cloacal swab was positive from two birds reared at UPF, Mannuthy and a bird reared at RPF, Koovappady along with its conjunctival swab.

Out of the 25 birds positive for genus-specific PCR, only 5 birds were positive for *Mycoplasma gallisepticum* species-specific PCR. Nine samples including 5 tracheal, three cloacal and one conjunctival swab were positive for MG species-specific PCR out of the 30 genus-specific PCR positive samples. These nine samples were obtained form five birds and the tracheal swabs of all these birds were positive in addition to three cloacal and one conjunctival swabs. Four out of these five birds were ailing birds.

When the 30 genus-specific PCR positive samples were subjected for *M. synoviae* species-specific and *M. iowae* species-specific PCRs, none of them yielded a positive result. Randomly sampled 23 synovial swabs although subjected for MS-PCR did not yield any positive result.

Out of the 94 tracheal swabs form 69 ailing and 25 healthy birds when streaked directly on to BHI agar, 15 were positive for isolation. Tracheal swabs from these 15 birds collected in BHI broth and later subcultured onto BHI agar after a positive result in PCR, only 13 yielded colonies in the agar where as two samples failed to yield any colonies. Isolates could be obtained only from tracheal swabs from live birds.

Out of the 15 isolates obtained, five were confirmed as *M. gallisepticum* by MG species-specific PCR. Nine broth samples (five tracheal, three cloacal and one conjunctival) positive by MG-PCR when subcultured onto BHI agar only five yielded colonies suggestive of *Mycoplasma* in the agar but only two of them were *M. gallisepticum* colonies and these isolates were from tracheal swabs alone.

When the broths tested positive for *M. gallisepticum* by MG-PCR were plated on to BHI agar following the result of the PCR, only two of them yielded MG colonies, whereas with three other broths the colonies obtained tested negative for MG-PCR but were positive for genus-specific PCR.

Agar plates directly inoculated with tracheal swabs in these five cases yielded MG colonies but were interspersed with non-MG colonies as well. Thus the selective isolation of the MG colonies was found to be important while attempting subculturing form these original plates. Attempts to isolate *Mycoplasma* from conjunctival, cloacal and airsac swabs were unsuccessful.

Mycoplasmal colonies were obtained from second day of incubation. Typical umbonate colonies with fried-egg appearance were obtained form sixth day onwards. Colonies of *M. gallisepticum* evidenced growth only after the third day of incubation. Coalesced, elongate and less raised colonies were also observed. Diene's staining technique was used to study the colony morphology.

To chart out the possible strain differences among the obtained *M. gallisepticum* isolates, PCR-RFLP was performed. All the isolates yielded similar restriction patterns. All the obtained isolates were lyophilized and stored at –70° C.

In order to assess the utility of PBS and buffered peptone with glycerine as a transport medium and to understand the performance in PCR of samples

collected in them, 46 tracheal swabs were collected in them separately. Six of the samples were positive by genus-specific PCR but none could be successfully cultivated. Thus PBS and buffered peptone water could be a substitute for the costly BHI broth for molecular methods based detection of *Mycoplasma* but not definitely for isolation trials.

Findings of the present study suggest that PCR could be regarded as an effective means for the early and rapid detection of mycoplasmas. In comparison with isolation techniques PCR claims a higher sensitivity. Species and strain differentiation of *Mycoplasma* is a definite advantage of molecular-based techniques over isolation techniques. Polymerase chain reaction based detection of avian mycoplasma proved to be more cost and labour effective based on the present study as compared to isolation trials. For the isolation of pathogenic mycoplasma species, the utility of the solid medium was appreciated. Substitution of the costly BHI broth with PBS or buffered peptone water for the transit of clinical samples for PCR could be another advantage of molecular methods based detection of mycoplasmas. Moreover the relative speed and accuracy with which a definite diagnosis could be made makes PCR-based detection of mycoplasmas the most meaningful, especially in disease control programmes and with samples involving long transit time.

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MOLECULAR METHODS BASED DETECTION OF PATHOGENIC MYCOPLASMAS OF CHICKEN

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ABSTRACT

A study was undertaken for the detection of *Mycoplasma* DNA from the specimens by Polymerase Chain Reaction (PCR), differentiate the three significantly pathogenic mycoplasmas of chicken namely *M. gallisepticum*, *M. synoviae* and *M. iowae* from the other less pathogenic ones based on the result of polymerase chain reaction. Attempts were also carried out to isolate *Mycoplasma* from clinical samples testing positive by polymerase chain reaction and from a few randomly selected negative samples and to comprehend the different strains of *M. gallisepticum*, if any, among chicken of various age groups. Out of a total of 225 birds subjected for the study 25 were found positive for the presence of avian Mycoplasma by genus-specific PCR. Thirty samples from these twenty-five birds were positive. Tracheal swabs form all these birds were positive. Fifteen isolates were obtained from these twenty five birds when the tracheal swabs were directly streaked onto BHI agar whereas when these tracheal swabs were collected in BHI broth initially and later subcultured onto BHI agar following a positive result in PCR / colour change of the broth, only 13 of them yielded colonies. Five of the isolates were found to be *M. gallisepticum* by MG-PCR and these were obtained upon direct inoculation of BHI agar with tracheal swabs from birds. Those samples collected in BHI broth yielded MG colonies only in two cases. Non-MG colonies were found interspersed with MG colonies in agar plates and thus the selective isolation of MG colonies must be performed prior to subculture. The utility of PBS and buffered peptone water supplemented with glycerol as transit media for clinical samples intended for PCR was evidenced. The isolates obtained were successfully lyophilized and stored at –70°C throughout the span of the study. The PCR-RFLP pattern of the obtained MG isolates revealed uniformity among the isolates. None of the samples yielded positive result in MS-PCR and MI-PCR. The sensitivity and usefulness of molecular methods based detection of pathogenic mycoplasmas of chicken over isolation techniques could be appreciated.