MICROBIAL AGENTS ASSOCIATED WITH EYE INFECTION IN CHICKEN

By

JAISON GEORGE

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

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Kerala Agricultural University

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

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DECLARATION

I hereby declare that the thesis entitled **"MICROBIAL AGENTS ASSOCIATED WITH EYE INFECTION IN CHICKEN"** is a bonafide record of research work done by me during the course of research and that this 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.

15/7/2003

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CERTIFICATE

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Dedicated to

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Jaison George

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Introduction

1. INTRODUCTION

Poultry production in India has achieved a remarkable growth from an age-old backyard farming into a dynamic and most sophisticated agro-based industry in recent times. Today, the Indian poultry industry can be considered to be operating on a full-fledged commercial basis.

Although India has made a major break-through in poultry production during last 25 years, the problems facing this industry are many and diverse, the major problem being lack of adequate facilities for the diagnosis and prevention of emerging poultry diseases. Disease still takes a heavy toll from all types of poultry enterprises. Intensive breeding and management programmes to increase the production have greatly contributed to higher incidence of several infectious diseases.

During the past two decades, primary breeders of broiler and layer strains have eliminated vertically transmitted diseases from their elite great-grand parent stocks. Unfortunately, infection of grand parents and parent flocks occurs in many developing countries, resulting in dissemination of diseases such as mycoplasmosis and salmonellosis. Angara disease, virulent infectious bursal disease, highly pathogenic influenza and swollen head syndrome are examples of emerging diseases affecting flocks in Asia, Africa and Latin America. In addition, chronic low-intensity infections such as

infectious coryza, pasteurellosis and salmonellosis continue to erode profit margins.

Recently, from the year 1998 onwards, unilateral conjunctivitis of unknown etiology in chicken was reported extensively in many parts of Kerala, with low mortality and a morbidity of five to ten per cent. The condition was characterized by irritation, lacrymation and congestion of the conjunctiva in the beginning, followed by purulent discharge, accumulation of caseous materials and sticking of eyelids leading to bulging of the affected eye.

A similar condition with mild respiratory signs was recently reported in Andamans (Shome *et al.*, 2000). They conducted a preliminary study on this condition and suggested mycoplasma as the possible etiological agent. Reports from all over the world show mycoplasma as the most important single etiological agent of conjunctivitis in a number of species of birds and animals.

The affected birds respond to timely treatment with antibiotics. This indicates possible involvement of secondary microbial invaders complicating the condition, leading to loss of vision. For a complete understanding of the disease process, it is necessary to conduct investigations on both primary and secondary organisms involved in the disease process and correlate the findings with the managemental and

environmental parameters. This will also facilitate to chalk out control measures to curb the incidence of conjunctivitis in chicken.

In view of the above factors, the present work was undertaken, with the following objectives

(i) The isolation of the microbial agents associated with conjunctivitis in chicken.

(ii) The identification of the isolates

(iii) The ability of the isolates to reproduce the infection

(iv) The antibiogram of the bacterial isolates

Review of Literature

2. REVIEW OF LITERATURE

Infectious or non-infectious agents can cause conjunctivitis in birds and this condition can occur as the sole lesion or one among several other lesions. Various workers have attributed many factors as the cause of conjunctivitis in birds including chicken. The infectious causes among them include numerous viruses, some bacteria and *Chlamydia psittaci* (Williams, 1994; Swayne, 1996).

Among bacteria, *Mycoplasma gallisepticum* (MG) is the most important etiological agent that is reported to cause conjunctivitis in Japanese quails, chukar patridges and golden pheasants (Wills, 1955; Wichman, 1957; Osborn and Pomeroy, 1958 and Reece *et al.*, 1986a). It is also reported to cause typical unilateral eye infection in chickens (Power and Jordan, 1976). *Chlamydia psittaci* was found to cause conjunctivitis in ducks (Strauss, 1967; Farmer *et al.*, 1982). Other agents encountered in concurrent infection with MG were Newcastle Disease virus (Gross, 1961), Adeno virus (Schmidt *et al.*, 1970), Infectious Bronchitis virus (Timms, 1972), Influenza A virus (Alexander, 1995), *Escherichia coli* (Gross, 1956) and *Haemophilus gallinarum* (Kato, 1965). Unilateral eye lesions in chicks were caused by *Salmonella typhimurium* (Cantini and Rossi, 1972). Ocular lesions with facial oedema were reported in chronic fowl cholera (Rhoades and Rimler, 1991). Cases of catarrhal conjunctivitis and subcutaneous

oedema of face and wattles were reported in Infectious coryza caused by *Haemophilus gallinarum* (Yamamoto, 1991).

Unilateral ophthalmic lesions in chickens were caused by *Aspergillus fumigatus* (Reis, 1940). This fungus caused swelling of the eye in chicks (Sperling, 1953). Infectious Laryngotracheitis virus produced haemorrhagic conjunctivitis in chicken (Cover and Benton, 1958). Marek's disease virus caused blindness due to the involvement of iris (Calnek and Witter, 1991).

Blepharo-conjunctivitis of unknown etiology was reported as a disease of breeder turkeys (Bierer, 1956 and 1958; Sanger *et al.*, 1960). Ammonia burn was the most important among the non-infectious causes leading to Kerato-conjunctivitis in poultry (Peckham, 1984). Widespread lesions of unknown etiology in the eyelid known as "Eye-Notch syndrome" was described in caged layers (Peckham, 1984).

2.1. Mycoplasmal conjunctivitis

Mycoplasma gallisepticum is one of the pathogens causing heavy economic loss to the poultry industry and is reported to have worldwide distribution. Nelson (1936) isolated avian mycoplasma for the first time and described it as cocco-bacillary form bodies in coryza of chicken and he was able to cultivate these organisms in cell free media (Nelson, 1939). 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.

Van Roekel *et al.* (1952) propagated CRD agent in chick embryos. They found that streptomycin, aureomycin, chloromycetin and terramycin exerted an inhibitory effect on CRD agent.

Johnson and Domermuth (1956) reported the presence of Pleuro-Pneumonia Like Organism (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 "S_6" strain.

Marked unilateral ocular enlargement in chicks was seen when MG (S_6) of low passage was inoculated (Varley and Jordan, 1978).

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

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

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

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

2.1.1. Incidence

Natural infections of MG leading to conjunctivitis have been described in pheasants, chukar patridges, peafowl and quail (Wills, 1955; Wichman, 1957; Osborn and Pomeroy, 1958 and Reece *et al.*, 1986b).

Organisms that have been encountered in concurrent infection with MG were *Haemophilus gallinarum* (Kato, 1965), New castle Disease virus (Gross, 1961), Adeno virus (Schmidt *et al.*, 1970), Infectious Bronchitis virus (Timms, 1972), Influenza A virus (Alexander, 1995) and *Escherichia coli* (Gross, 1956).

Jain *et al.* (1969) reported that wild birds harboured MG and played an important role in the flock-to-flock transmission of this disease.

Power and Jordan (1976) reported unilateral enlargement of eye in chicks infected with a strain of MG.

Tylosin in the feed was found to improve egg production and feed consumption and slow down the rate of spread of infection in MG exposed birds (Ose *et al.*, 1978).

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.

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

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

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

Natural infection of MG in pheasants, Chukar patridges and peafowl characterised by foamy eyes, swollen infra orbital sinuses, respiratory distress and death was described by Cookson and Shivaprasad (1994).

Nunoya *et al.* (1995) reported natural cases of Kerato-conjunctivitis in pullets, first appearing around 30 days of age, characterized by unilateral or bilateral swelling of the facial skin and eyelids, increased lacrymation, congestion of conjunctival vessels and respiratory rales. Morbidity reached 27.8 per cent and mortality was ten per cent, mainly due to reduced feed intake due to impaired vision.

Mycoplasma gallisepticum was described as the etiologic 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; Pendleton *et al.*, 1997)

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

Mac Martin *et al.* (1996) reported an outbreak of conjunctivitis and severe respiratory disease caused by MG in an integrated chukar patridge

facility in San Diego county, California, leading to severe drop in egg production and increased culling rates.

Mycoplasmal conjunctivitis in wild house finches, spreading to an additional species, the American gold finch was first observed by Fisher *et al.* (1997). The outbreaks of conjunctivitis and its transmission to American gold finches increased the awareness of MG as a cause of conjunctivitis (Ley *et al.*, 1997).

Frasca *et al.* (1997) described conjunctivitis and episcleritis in an adult European starling, caused by *Mycoplasma sturni,* and the organism was isolated in pure culture from both the eyes.

Ley and Yoder (1997) found that although MG 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).

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 and the finch population decreased throughout winter season in areas with cold winter and high conjunctivitis prevalence, suggesting significant mortality associated with the disease.

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

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).

Naturally occurring mycoplasmal conjunctivitis was reported among wild-caught and initially sero-negative house finches maintained in captivity for 12 weeks (Luttrell *et al.*, 1998). Severe bilateral or unilateral ocular swelling, conjunctivitis and often death characterized the disease. They opined that infected birds without lesions might act as asymptomatic carriers in the wild.

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

2.1.2. Isolation procedures of avian mycoplasma

Avian mycoplasma was first isolated by Nelson (1936) and he succeeded in growing them in serum-enriched cell free media (Nelson, 1939).

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-pneumonia like organism.

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 MG.

Grumble *et al.* (1953) described a medium composed of fermentable carbohydrate and phenyl 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 MG.

Lecce and Sperling (1954) proposed heart infusion agar with thallium acetate as selective medium suitable 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 isolation and detection of avian mycoplasma.

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).

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 nocotinamide 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.

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 MG 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 MG from commercial layers.

Antibiotics effective in treating the MG 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 percentage of the total numbers of colony forming units of MG 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 MG 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 plates for the isolation of MG from captive flock of house finches with conjunctivitis.

Manohar (2001) compared six media for isolation of MG 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.

2.1.3. 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 whereas 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.

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 MG 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. The broth cultures of avian mycoplasma were viable when subcultured after three to four years of storage at -30^0C, while lyophilised preparations contained viable mycoplasma when subcultured after five to fourteen years of storage at 4^0C.

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

Aycardi *et al.* (1970) 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 Mc Martin (1973) reported that in metabolic inhibition test with high dilution of antiserum, the lag phase of growth of MG was significantly prolonged, but eventually growth commenced and proceeded

normally and in low dilutions of antiserum the growth was permanently inhibited.

2.1.4. Mycoplasmal conjunctivitis in animals

Mc Cauley *et al.* (1971) observed an epizootic of kerato-conjunctivitis in a herd of 300 milking goats that lasted approximately two months.

Haesebrouk *et al.* (1991) reported conjunctivitis in cats caused by *Mycoplasma felis.*

Hill (1991) isolated *Mycoplama oxoniensis* from cases of conjunctivitis in Chinese hamster.

2.2. Chlamydial conjunctivitis

2.2.1. Incidence

Strauss (1967) observed lacrymation, conjunctivitis, rhinitis, occasionally panophthalmitis, bulbar atrophy and inflammation of infraorbital sinuses in ducks affected with *Chlamydia psittaci.*

Conjunctivitis and rhinitis caused by *Chlamydia psittaci* in apparently healthy flock of ducks was reported by Farmer *et al.* (1982).

Mohan (1984) reported psittacosis in pet birds with signs of anorexia, weight loss, diarrhoea, yellowish droppings, sinusitis and respiratory distress.

Mixed infection with *Chlamydia psittaci*, fowl pox virus and *Haemophilus gallinarum* resulted in scab formation on the skin of the head and legs and a severe blepharo-conjunctivitis, leading to partial or complete closure of eyelids in ten month-old-broiler breeder chickens (Malkinson *et al.*, 1987).

Acute chlamydiosis in pigeons was accompanied by anorexia, unthriftiness, diarrhoea and emaciation (Grimes and Wyrick, 1991). Some developed conjunctivitis, swollen eyelids and rhinitis and recovered birds acted as asymptomatic carriers of the infection. After analysing the epidemiological and laboratory evidence, they found that chickens appeared to be relatively resistant to disease caused by *Chlamydia psittaci.*

Chlamydia psittaci infection was associated with stunting, ruffled feathers, serous to sero-sanguineous nasal discharge, and conjunctivitis with swollen eyelids in Bobwhite quail and Chukar partridge (Erbeck and Nunn, 1999).

2.2.2. Isolation of chlamydia using chicken embryo

The most favourable system for cultivation and isolation of chlamydial agent is yolk sac of developing chicken embryo and this property was first identified by Rake *et al.* (1940).

Page (1971) reported that the eggs inoculated with *Chlamydia psittaci* should be incubated at 37-39^{0}C at 60 per cent humidity and he found that the rate of multiplication of *Chlamydia psittaci* strains was enhanced at higher temperature.

Farmer *et al.* (1982) isolated *Chlamydia psittaci* from cases of conjunctivitis in ducks using McCoy cell monolayers.

The chicken embryos that died due to chlamydial infection might have hyperaemia; cyanotic legs and the toes might be deep red in colour with patchy haemorrhages in the skin (Collier, 1984).

The identification of chlamydiae during isolation is a difficult task unless the clinical specimens contain a heavy concentration of organisms. Most of the clinical specimens might require two to three passages in chick embryo before chlamydia could be identified (Idtse, 1984).

2.3. Eye lesions of viral etiology

Alexander (1991) reported conjunctival lesions associated with haemorrhage in Newcastle disease in chicks. Congestion and petechiae of the comb and wattle were common.

Blindness might result from involvement of iris in Marek's disease and the affected eye gradually loses its ability to accommodate light intensity. Clinical examination revealed changes varying from concentric annular or spotty depigmentation or diffuse bluish fading to diffuse grayish opacity of iris (Calnek and Witter, 1991).

Reduction in egg production, unthriftiness, watery eyes, swelling of infraorbital sinuses, persistant nasal discharge and haemorrhagic conjunctivitis were the clinical manifestations in mild forms of Infectious Laryngotracheitis (ILT) in chicken (Hanson and Bagust, 1991).

The clinical manifestation of Influenza A viral infection in chicken were oedema of head and face and increased lacrymation, along with mild to severe respiratory signs (Easterday and Hinshaw, 1991).

Tripathy (1991) reported that in fowl pox, lesions might be seen at the corners of mouth, eyelids and oral membranes along with other parts of head in chicken.

Droual and Woolcock (1994) mentioned cases of swollen head syndrome associated with *Escherichia coli* and Infectious bronchitis virus.

Linares *et al.* (1994) observed gasping, wheezing, swollen eyes and cheesy material in trachea of broiler birds infected with ILT virus. They could also isolate *Escherichia coli* from trachea in three cases and *Pasteurella haemolytica* from one case.

Turkey rhinotracheitis (TRT) virus, a pneumovirus, has been strongly implicated as a factor in swollen head syndrome in chickens (Naylor and Jones, 1993). *Escherichia coli* was thought to be a secondary complicating factor and the first invasive site of bacteria in the subcutaneous tissue of head was estimated to be the eyelid or nasal membrane.

2.4. Eye lesions of Eubacterial etiology

Blindness in chicks associated with *Salmonella pullorum* infection was described by Evans *et al.* (1955).

Nillo (1959) reported keratitis in chicken caused by *Pseudomonas aeruginosa.*

Salmonella arizonae infection produced caseous material covering the retina, leading to blindness in poults and chicks (Kowalski and Stephens, 1968).

Cantini and Rossi (1972) reported that about five per cent of 4000 female chicks infected with *Salmonella typhimurium* exhibited a lesion in one eye only.

Multiple outbreaks of acute, severe fibrino purulent lesions of eyelids, in chickens and turkeys were caused by *Staphylococci sp.*, *Escherichia coli* and *Streptococcus sp.* and they were isolated only during severe stages (Cheville *et al.*, 1988).

In chronic fowl cholera affecting chickens the signs were related to localised infections such as swelling of wattles, sinuses, wing joints, foot pads and sternal bursa, with exudative conjunctivitis and pharyngeal lesions (Rhoades and Rimler, 1991).

Garcia *et al.* (1992) observed bilateral conjunctivitis as the most significant manifestation of *Aeromonas hydrophila* infection in a three-year-old parrot, which was anorectic for three months. Both the conjunctivae were hyperaemic and left eye was closed by strong oedema.

Gooderham (1996) reported oedema of the face, swelling of the infraorbital sinuses and nasal discharge in *Ornithobacterium rhinotracheale* infection in turkeys.

Granulomatous conjunctivitis in two young female commercial emus (*Dromacus novaehollandiae*) caused by *Mycobacterium avium* was reported by Pocknell *et al.* (1996).

Pasteurella multocida infection reported by Gustafson *et al.* (1998) showed cranial air space involvement in White Leghorn chickens. The clinical signs included depression, torticollis, swollen eyelids, conjunctivitis and sinusitis. Complicating the condition was paramyxo virus-1 affecting the trachea.

Shome *et al.* (2000) isolated coagulase positive Staphylococci and *Escherichia coli* from acute ophthalmitis leading to blindness in chickens.

The infection was seen among the birds of different age groups and breeds in Andaman and the incidence was more during the rainy season. They were not successful in reproducing the disease even when the isolates of *Escherichia coli* and *Staphylococus sp.* were inoculated intra conjunctivally. They noticed that high humidity, temperature, vitamin A deficiency and poor sanitation were the predisposing factors and use of antibiotics in feed or as eye drops drastically reduced the severity of respiratory signs and conjunctivitis and suggested mycoplasma as the probable causative organisms.

2.5. Conjunctivitis caused by fungus

Reis (1940) reported unilateral ophthalmic lesions in chickens due to *Aspergillus fumigatus.*

Moore (1953) described eye infection in turkey poults that had respiratory aspergillosis with changes occurring in the posterior eye.

Sperling (1953) opined that, the swelling of the eye in *A. fumigatus* infection resembled coryza or vitamin A deficiency in chicks.

Yellow caseous plaque could become adherent to the cornea in superficial type of the eye infection caused by *A.fumigatus* (Itakura and Goto, 1973).

2.6. Experimental infection

Reis (1940) succeeded in isolating *A. fumigatus* from cases of unilateral conjunctivitis in chicken and were able to reproduce superficial eye infection in chickens by inoculating conidia of *A.fumigatus* into the eye.

Conidia of *Aspergillus fumigatus* when inoculated into the abdominal air sacs of chickens produced a plaque on the surface of one eye (Hudson, 1947; Chute and O'Meara, 1958). There was involvement primarily of the conjunctiva and external surfaces of the eye with the development of cheesy exudates or plaque, forming beneath the nictitating membrane. The fungus could be isolated readily from cultured plaque material.

Richard *et al.* (1984) experimentally exposed turkeys to aerosols of *A. fumigatus* conidia and found that they developed a cloudy eye with retinitis and iridocyclitis with secondary involvement of the remainder of the eye.

Staphylococcus sp., Escherichia coli and *Streptococcus sp.* isolated from cases of acute fibrino-purulent lesions of eyelids in chickens and turkeys failed to reproduce the natural disease when inoculated alone or in combination by intra-dermal scarification into the epithelium of the eyelid (Cheville *et al.*, 1988).

Mc Martin *et al.* (1996) could reproduce natural eye infection in chukar partridges when 48-hour culture of a field isolate of MG having a titre of $1x10^7$ CFU/ml was experimentally inoculated into the eye. The same

isolate when inoculated intra-abdominally to six-week-old chicks produced air sacculitis at 21 days post-inoculation. In both the experiments the birds were sacrificed 36 days post-inoculation and they were successful in re-isolating the organism from experimentally infected birds.

Coagulase positive Staphylococci and *Escherichia coli* isolated from acute ophthalmitis in chickens failed to reproduce the disease even when the isolates of *Escherichia coli* and Staphylococus were inoculated intra-conjunctivally. The infection was seen among the birds of different age groups and breeds in Andaman and the incidence was more during the rainy season (Shome *et al.*, 2000).

Materials and Methods

3. MATERIALS AND METHODS

General considerations

The glassware used in the study were Borosil, Mumbai make. The culture media, the Analar /Excellar grade chemicals and other reagents were obtained from Hi-media, Mumbai; Nice, Kochi and SRL, Mumbai. The media and reagents required were prepared in glass-distilled water. The glassware, screw caps with rubber linings were thoroughly cleaned and sterilized by standard procedures. Plasticware like syringes and centrifuge-tubes were procured from M/s. Tarson Ltd., Mumbai.

Unused glassware were soaked in four per cent Hydrochloric acid solution for at least 24 hours. Glassware were then rinsed, boiled in tap water for half an hour, hand washed and rinsed three times each in tap water and then with single, double and triple distilled water. They were dried in hot air oven (80^0C) and suitably wrapped before being sterilized either by autoclaving at 121^0C for 15 to 20 minutes or by dry heat at 160^0C for one hour. Stainless steel instruments were sterilized either by autoclaving or alcohol flaming.

Specimens collected

The birds utilised for the study were obtained from University Poultry Farm, Kerala Agricultural University, Mannuthy; Regional Poultry Farm, Kudappanakunnu and Central Hatchery, Chengannoor. The clinical stages of

infection in birds of different age groups from which samples were collected are given in table 1. Of these, one bird was two days old, two were four days old, 29 were 40 to 70 days old, 24 were 100 to 130 days old, 19 were 150 to 180 days old and eight were 270 to 290 days old (Table 2). Biomaterials collected from 83 cases of conjunctivitis in chicken included conjunctival swab samples, air sac materials and inflamed conjuntival tissue.

3.1 Isolation of avian mycoplasma

3.1.1 Media for the isolation of avian mycoplasma

In the present study, two media were employed for the isolation of avian mycoplasma. The media were pre-tested for their ability to support the growth of mycoplasma by growing Mycoplasma *mycoides* subsp. *capri* culture obtained from IVRI, Izatnagar.

3.1.1.1. Materials

Media ingredients

Horse serum: Horse serum was obtained from the horses maintained by 1(K) R&V Sqn., KAU unit, Mannuthy and was sterilized by passing through Seitz filter and stored at -20^0C in 20 ml aliquots until use. It was not inactivated by heat.

Yeast extract: Yeast extract was prepared by suspending 250 g of fresh baker's yeast in 1000 ml of distilled water and brought it to boil. The supernatant was filtered through Whatmann No.1 filter paper and then

Table 1. Details of sample taken at different stages of infection

Stages of infection	No. of samples taken at different stages of infection
Beginning of infection (Within 12 hours of onset of infection)	24
Mid-infection (two days after the onset of infection)	42
Advanced stage of infection (Eye bulges due to accumulation of caseous material and eyelids stick together)	17

Table 2. The details of age group of the birds utilised for the study

Age of bird (days)	Conjunctivitis		Total samples collected (Age wise)
	Unilateral	Bilateral	
2	1	-	1
4	2	-	2
40-70	28	1	29
100-130	21	3	24
150-180	18	1	19
270-290	8	-	8

sterilized using Seitz filter, dispensed in 10 ml aliquots and stored at –20^{0}C until use.

Glucose: Fifty per cent of glucose stock solution was prepared in distilled water, sterilized by filtration and stored at –20^{0}C in 10 ml aliquots.

Penicillin and Thallium acetate: Stock solution of Pencillin 10,000 IU/ml and Thallium acetate 2.5 per cent were prepared separately in sterile distilled water and stored at –20^{0}C in 10 ml aliquots.

Cysteine Hydrochloride: One per cent stock solution was prepared in distilled water, sterilized by filtration and stored in 10 ml aliquots at 4^{0}C.

Nicotinamide Adenine Dinucleotide (NAD): One per cent stock solution was prepared in distilled water and stored in 10 ml aliquots at –20^{0}C.

Buffalo heart infusion: Buffalo heart free from fat was minced and weighed, 500 gm of this was boiled in 1000 ml of distilled water for 45 minutes. It was filtered through a muslin cloth with cotton pad, distributed in small quantities, sterilized by autoclaving at 121^{0}C for 15 min and stored at –20^{0}C until use.

Phenol red: Stock solution of 0.2 per cent was prepared, sterilized by autoclaving and stored at room temperature.

Different types of media

Buffalo heart infusion broth (Lecce and Sperling, 1954)

Buffalo heart infusion	- 700 ml	}
Bactopeptone	- 11 gm	} Sterilized by
Phenol red	- 10 ml	} autoclaving
NaCl	- 5 gm	
Glucose	- 2 ml	
Horse serum	- 200 ml	
Yeast extract	- 100 ml	
Thallium acetate	- 20 ml	
Penicillin	- 10 ml	

pH adjusted to 7.8

Buffalo heart infusion agar (BHI Agar)

For buffalo heart agar medium, the following composition was used.

Mycoplasma agar base	- 700 ml	} Sterilized by
(Hi-media)		autoclaving
Buffalo heart infusion	- 50 ml	
Horse serum	- 150 ml	
Yeast extract	- 100 ml	
Glucose	- 2 ml	
Penicillin	- 10 ml	
Thallium acetate	- 20 ml	

ph adjusted to 7.8

The mycoplasma agar base was sterilized by autoclaving, allowed to cool at 45 to 50^{0}C and supplemented with the remaining ingredients. Mycoplasma agar was poured into 55 mm diameter petridishes at a thickness of five to six mm and stored at 4^{0}C until further use.

French Medium 4th formula broth (FM_4 broth) (Frey et al., 1968)

NaCl	- 5 gm	}	
Kcl	- 0.4 gm	}	
$MgSO_4$ $7H_2O$	- 0.2 gm	}	
Na_2HPO_4	- 1.6 gm	}	
KH_2PO_4	- 0.1 gm	}	
Glucose	- 10 gm	}	Sterilized by
Peptone CS	- 10 gm	}	autoclaving
Yeast autolysate	- 5 gm	}	
Phenol red indicator	- 10 ml	}	
Distilled water	- 1000 ml	}	
Nicotinamide Adenine Dinucleotide	- 0.1 ml		
L-Cysteine Hydrochloride	- 0.1 ml		
Penicillin	- 1000 IU/ml		
Thallium acetate	- 20 ml		
Horse serum	- 20 ml		

pH adjusted to 7.6 to 7.7

French Medium 4th formula agar (FM_4 agar)

FM_4 agar medium was prepared by adding 1.5 per cent Bacto agar (Difco) to the FM_4 broth and phenol red indicator. The agar base was sterilized by autoclaving, allowed to cool at 45 to 50^{0}C and supplemented with the remaining ingredients of FM_4 broth, poured into 55 mm diameter petridishes at a thickness of five to six mm and stored at 4^{0}C until further use.

3.1.2. Collection of samples

Conjunctival swab samples and air sac materials were tried for isolation of mycoplasma. Air sacs materials were collected from infected birds, specifically from areas with specific lesions and were directly cultured in appropriate media. Sterile swabs made of gauze were soaked with mycoplasma broth just before collection of samples.

3.1.2.1. Processing of samples

The swabs and air sac materials were immediately inoculated on to BHI agar and FM_4 agar. The same swabs after inoculation on the solid media were then placed in BHI broth and FM_4 broth and incubated at 37^{0}C with occasional shaking for three hours. After three hour the swabs were removed from the broth media and broth were further incubated at 37^{0}C for seven to fourteen days. The media were examined daily for the change in pH. The medium with indication of growth was sub cultured in broth lacking

penicillin and thallous acetate and was further incubated for a period of seven to fourteen days. When there was contamination, two to three drops of the contaminated medium was filtered through Millipore membrane filter of 450 nm diameters and was again inoculated to broth with microbial inhibitors.

The media with indication of growth but without contamination were streaked on to mycoplasma agar plates. The agar plates were incubated at 37^0C for three to five days in a carbon dioxide incubator adjusted to 10 per cent carbon dioxide tension with 50 per cent relative humidity. The plates were examined daily under an inverted microscope for the presence of mycoplasma colonies. When growth was observed on the agar plates, the subculture was made into a fresh broth by cutting out a small block of agar from plates containing a single colony. If no colonies were observed, the plates were further incubated for a period of eight to ten days and again examined for visible colonies before they were discarded. At least three subcultures were made into broth tubes with antibiotics and each time they were plated onto solid media and examined for visible colonies. When no colonies were observed on the third subculture, the clinical sample was considered as negative for the presence of mycoplasmas. The agar plates into which the samples were directly inoculated were also incubated at 37^0C as described earlier in carbon dioxide incubator for seven to ten days.

3.1.3. Characterization of avian mycoplasma

3.1.3.1. Colony characters

Diene's stain

Methylene blue	- 2.5 gm
Azur II	- 1. 25 gm
Maltose	- 10 gm
Na_2CO_3	- 0.25 gm
Distilled water	- 100 ml

Sterilized by filtration and stored at room temperature.

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 37^0C 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.

3.1.3.2. Differentiation of mycoplasma from acholeplasma

3.1.3.2.1. Growth at 22^0C

Mycoplasma agar plates inoculated with suspected cultures were incubated at 22^0C and another set of similar plates was kept for incubation at

37^0C. After three to four days of incubation, both the sets of plates were examined for the presence of mycoplasma colonies.

3.1.3.2.2. Growth on media without serum

The suspected cultures were inoculated into the buffalo heart infusion broth without horse serum and incubated at 37^0C for 48 hours. Two to three subcultures in similar broth were performed and finally the broth was plated onto mycoplasma agar plates without horse serum and incubated both at 37^0C and 22^0C. After three to four days of incubation, plates were examined for mycoplasma colonies.

3.1.3.3. Differentiation of mycoplasma from L- phase of bacteria

Suspected culture were sub cultured two to three times in buffalo heart infusion broth without bacterial inhibitors. After third subculture, the broth was inoculated on to mycoplasma agar plates to know the presence of contaminating bacterial growth.

3.1.3.4.Biochemical test (Aluotto *et al.*, 1970)

3.1.3.4.1. Glucose utilization

The glucose breakdown by the organism was tested in buffalo heart infusion broth containing a final concentration of one per cent glucose with phenol red as indicator. The pH of the medium was adjusted to 7.8. The positive result was indicated by change in colour of the medium from pink to yellow. No change in colour of the medium was taken as negative.

3.1.3.4.2. Arginine decarboxylation

The ability of the organism to hydrolyse arginine was tested in buffalo heart infusion broth containing a final concentration of one per cent arginine with phenol red as indicator. The pH of the medium was adjusted to 6.8. The positive result was indicated by colour change of medium from yellow to pink. No change in colour was taken as negative.

3.1.3.4.3. Urea hydrolysis

The property of hydrolysing urea was tested in BHI broth containing a final concentration of one per cent urea with phenol red as indicator. The pH of the medium was adjusted to 6. The positive result was indicated by change in colour of the medium from yellow to pink. No change in colour was taken as negative.

3.1.3.4.4. Tetrazolium reduction

Tetrazolium reduction was conducted in BHI broth containing a final concentration of 0.02 per cent of 2-3-5 triphenyl tetrazolium salt without phenol red indicator. The pH of the medium was adjusted to 7.6. The positive result was indicated by change in the colour of medium to brick red colour and no change in colour was taken as negative.

3.1.3.5 Production of film and spots (Aluotto *et al.*, 1970)

Production of film and spots was tested by inoculating the broth culture onto mycoplasma agar plates and incubated for 14 days at 37^0C. Plates were examined for the presence of film and spots.

3.1.3.6 Production of haemolysis (Somerson *et al.*, 1963)

The suspected cultures were inoculated on to mycoplasma agar plates and incubated for two to three days. When colonies could be observed, the plates were overlaid with an erythrocyte mycoplasma agar mixture and then incubated for a further period of three to four days. The plates were examined daily for haemolysis around the colonies.

Erythrocyte agar mixture was prepared by mixing mycoplasma agar cooled to 45^0C with washed erythrocytes in the final concentration of five per cent (v/v). Chicken erythrocytes were used in the present study. Only a clear cell free zone around the colonies was recorded as positive for haemolysins. Other types were recorded as negative.

3.1.3.7 Filtration

A two to three day old broth culture was passed through 450 nm diameter Millipore membrane filter. The resultant filtrate was plated separately to detect growth of mycoplasmas.

3.1.3.8 Serological tests

Although the present isolates were characterized initially by biochemical tests, precise identification was done by serological test.

3.1.3.8.1 Growth inhibition test (Clyde, 1964)

Growth inhibition (GI) was performed on the mycoplasma agar plates inoculated with two-day-old broth culture by the flood plate technique. After uniform spreading excess of the culture was removed. Sterile 6 mm filter paper discs (Whatmann filter paper No.1) (Moorthy, 1977) were saturated with 0.02 ml of undiluted antiserum (obtained from Department of Microbiology, Veterinary college, Bangalore) placed on the centre of the inoculated surface of agar plate and pressed gently. The plates were then incubated in carbon dioxide incubator as described earlier for two to three days. Then the plates were examined with an inverted microscope and zones of inhibition of growth around the discs were measured in millimetres by micrometry.

3.1.3.8.2 Metabolic inhibition test

A two-day-old broth culture of mycoplasma (0.25 ml) was inoculated into two sets of buffalo heart infusion broth (5 ml) each containing glucose, arginine, urea and 2-3-5 triphenyl tetrazolium salt in the same concentration used in

biochemical tests. To one set of inoculated media 0.25 ml of undiluted artiserum was added, incubated at 37°C for 48 h and observed for the alteration in pH indicated by colour change. Uninoculated media were kept as controls.

3.2. Isolation of Chlamydia

3.2.1. Sample collection

The birds were sacrificed and the inflamed conjunctiva were collected aseptically in sucrose phosphate glutamate (SPG) buffer with antibiotics (Batta *et al.*, 1995). If it was not possible to transport the specimens to the laboratory within a period of one hour, they were transported over ice in thermocole box.

3.2.2. Staining of smears from clinical samples

Giemsa staining, Modified Ziehl Neelsen staining and Gimeñez staining were done as per Cruickshank *et al.*(1975).

3.2.3. Storage of samples for isolation of chlamydia

The samples collected were washed two to three times in SPG buffer containing antibiotics. The specimens were kept in sterile vials with SPG supplemented with antibiotics and stored at 4^0C. When the conjunctival tissue could not be processed within 24 hours, they were preserved at -20^0C in SPG.

3.2.4. Processing of samples

The piece of conjunctiva was homogenized with sterile silica gel in a sterile mortar and pestle in SPG containing antibiotics to obtain 20 per cent(W/V) suspension. The tissue homogenate was transferred to properly labelled test tubes and kept at 4^0C for 20 to 30 minutes to facilitate settling of coarse particles and silica gel. The supernatant was then centrifuged in three steps at 600 x g, 1100 x g and 1700 x g for 30 min each. In the first two steps the supernatant colleted was stored for six to seven hours at 4^0C and the final supernatant after storing at 4^0C for overnight was tested for sterility by inoculation on to blood agar plates. After overnight incubation, of the inoculated plates at 37^0C, they were examined for bacterial contamination. The emulsion established to be free of bacterial contamination was used for inoculation into embryonated eggs.

3.2.5. Chick embryo inoculation

3.2.5.1. Materials

1.Processed samples

2. six to seven-day-old embryonated eggs

Hatching eggs from hens fed on antibiotic free ration were procured from University Poultry Farm, Mannuthy and were pre-incubated for six to seven days in a humid chamber at 37^0C.

3.2.5.2. Method

Yolk sac inoculation was done as per Rake *et al.*(1940). Each sample was inoculated on three eggs.

3.2.5.3. Harvesting of inoculated embryonated egg

Done as per Rake *et al.* (1940). Small pieces of Yolk sac (YS) blotted dry on a blotting paper were used to prepare impression smear on clean grease free glass slides. Haemorrhagic lesions on YS and over embryo were considered as typical for chlamydial infection. The YS so obtained was then suspended in SPG with antibiotics, stored in sterile vials, labelled and kept at -20^0C until further use.

The YS smear was stained as described earlier for detecting chlamydial elementary body (EB) or inclusion. Even when the YS material failed to reveal typical chlamydial bodies, the YS materials were subjected to three serial blind passages before they were discarded as negative. For repeated passaging, the YS was processed as mentioned earlier in the method of processing of samples.

3.3 Isolation of virus

3.3.1. Sample collection

3.3.1.1. Materials

Dry cotton swabs were used for the collection of conjunctival samples from 83 birds utilised in this study.

3.3.1.2. Method

The swab samples collected were soon soaked in 1.5 ml phosphate buffered saline with pH 7 containing 1000 IU of penicillin and 1000 microgram of streptomycin per ml and stored at -20^0C until used.

3.3.2.Processing of sample

At the time of chick embryo inoculation the swabs were thawed at room temperature and squeezed for about five times with a sterile pipette. The fluid so separated from the swabs were collected and centrifuged at 1000 x g for about 10 to 15 minutes. The supernatant was collected and incubated at 37^0C for one hour prior to inoculation.

3.3.3. Embryonated egg inoculation

Hatching eggs from hens fed on antibiotic free ration were procured from University Poultry Farm, Mannuthy and were pre-incubated in a humid chamber at 37^0C. The embryonated eggs were candled to check the viability. Viable and healthy eggs were selected for inoculation. Three eggs were inoculated with each sample in each of the routes. Yolk sac inoculation, allantoic cavity inoculation and Chorio-allantoic membrane (CAM) inoculation were done as per Bishai *et al.* (1974).

3.3.4. Harvesting of inoculated embryonated eggs

Collection of YS, CAM and Allantoic fluid were done as per Betts (1967). The YS and CAM collected were looked for lesions and the

Allantoic fluid was examined for the presence Newcastle disease and Avian Influenza virus by haemagglutination.

3.4. Isolation of Eubacteria

3.4.1. Sample collection

3.4.1.1. Materials

1. Dry cotton swabs
2. Peptone water

3.4.1.2. Method

Cotton swabs soaked in peptone water were used for collecting conjunctival swab samples.

3.4.2. Media inoculation

3.4.2.1. Materials

1. Blood agar (BA) } Prepared as per
2. Chocolate agar (CA) } Barrow and Feltham, 1993).
3. Trypticase soy agar (TSA) } Sterilized by
4. Mc Conkey agar (MA) } autoclaving.

3.4.2.2. Method

The eye samples collected were directly inoculated first on BA, followed by CA, TSA and lastly on MA and incubated aerobically at 37^0C up to 96 hours and the negative plates were then discarded. Two sets of

plates were similarly inoculated and one set was incubated for aerobic growth and the other for micro-aerophilic growth. Based on colony morphology and cell characteristics, colonies were separately sub cultured on BA and subsequently on TSA to make pure cultures.

3.4.3. Identification of Eubacterial isolates

3.4.3.1. Genus level characterization

Isolates were subjected to the following preliminary tests for genus level characterization (Barrow and Feltham, 1974).

1. Gram's reaction
2. Spore staining
3. Capsule staining
4. Motility
5. Aerobic growth
6. Anaerobic growth
7. Catalase test
8. Oxiase test
9. Glucose fermentation
10. Oxidation fermentation test (O/F)

3.4.3.2. Species level characterization

Species level characterization of the Eubacterial isolates were done as per Barrow and Feltham (1993) as shown in table 3.

Table 3. Details of secondary tests done for the species level identification of the Eubacterial isolates

Eubacterial isolates belonging to the genus/family			
Bacillus	*Staphylococcus*	*Enterobacteriaceae*	*Pseudomonas*
Growth at 45^0C	VP	Citrate utilization	Citrate utilization
Growth at pH 5.7	Nitrate reduction	Gas from glucose	Lactose fermentation
Growth at 7 per cent	Lactose	MR	Maltose fermentation
NaCl	fermentation	VP	Mannitol fermentation
Citrate utilization	Maltose	Indole	Xylose fermentation
Glucose fermentation	fermentation	Urease	Starch hydrolysis
Arabinose	Mannitol	H_2S production.	Gelatin hydrolysis
fermentation Mannitol	fermentation	Lysine	Tween 80 hydrolysis
fermentation	Sucrose	decarboxylation	Arginine hydrolysis
Xylose fermentation	fermentation	Ornithine	Nitrate reduction
VP	Xylose	decarboxylation	Urease
Starch hydrolysis	fermentation	Arginine hydrolysis	Lysine decarboxylation
Gelatin hydrolysis		Metallic sheen on	Ornithine
Nitrate reduction	Arginine	EMB	decarboxylation
Indole	hydrolysis	Eijkman's test	
Urease	Gelatin		
	hydrolysis		
	Urease		
	Coagulase		

3.5. Isolation of Fungus

3.5.1. Sample collection

The conjunctival swabs from cases of conjunctivitis in birds were used to inoculate fungal media.

3.5.2. Media inoculation

3.5.2.1. Materials

1. Sabouraud's dextrose agar (SDA) with cycloheximide
2. SDA without cycloheximide

3.5.2.2. Method

Sample collected using cotton swabs were inoculated on to SDA with and without cycloheximide and incubated at 37^0C for two to three days and were examined for yeast. Another set of inoculated plates was kept at room temperature for up to 30 days and was examined for fungal colony.

3.5.3. Characterization

Genus level identification of fungal isolates was done on the basis of colony morphology and microscopical morphology, by examination after Lactophenol cotton blue (LCB) staining (Hazen, 1973).

3.5.3.1. Colony morphology

Colony size, colour and texture were examined for classification.

3.5.3.2. Staining with Lactophenol cotton blue

The mycelial forms were stained with Lactophenol cotton blue for three hours and examined first under low power and then under high power objective of the microscope.

3.6. Pathogenicity study

3.6.1. Materials

1. Chicks: - MG free eight week old chicks protected against Ranikhet disease and Infectious Bursal disease procured from University Poultry Farm, Mannuthy were utilised for the study.

2. Ninety six hour old culture of MG isolate with a titre of 10^7 CFU/ml

3. Eighteen-hour-old culture of one loop full of *Escherichia coli* in one ml of the nutrient broth.

4. Eighteen-hour-old culture of one loop full of *Pseudomonas aeruginosa* in one ml of the nutrient broth.

5. Eighteen-hour-old culture of one loop full of *Bacillus coagulans* in one ml of the nutrient broth.

6. Eighteen-hour-old culture of one loop full of *Staphylococcus aureus* in one ml of the nutrient broth.

7. Eighteen-hour-old culture of one loop full of *Staphylococcus epidermidis* in one ml of the nutrient broth.

8. Eighteen-hour-old culture of one loop full of *Allescheria sp.* in one ml of the Sabouraud's dextrose (SD) broth.

9. Eighteen-hour-old culture of one loop full of *Penicillium sp.* in one ml of the Sabouraud's dextrose (SD) broth.

10. Eighteen-hour-old culture of one loop full of *Scopulariopsis sp.* in one ml of the Sabouraud's dextrose (SD) broth.

3.6.2. Method

Nineteen groups, each group comprising of 4 chicks were utilized for the study. Details of the pathogenicity test are shown in Table 4.

All the birds inoculated with MG were housed separately from other groups and were maintained up to 36 days post inoculation. Other groups except control were maintained only up to 14 days. Reisolation of the organisms was tried from conjunctival swabs and air sacs.

3.7. Sensitivity to chemotherapeutic agents

3.7.1. Materials

1. Eubacterial isolates
2. Antibiotic discs (Beacon Diagnostics Pvt. Ltd., India)
3. Blood agar

Table 4. Details of the pathogenicity test

Group	Inoculum used	Quantity of inoculum used(ml)	Route of administration	No. of birds inoculated
1	Sterile distilled water	0.2	i/ocular	4
2	MG	0.2	i/ocular	4
3	MG	0.2	i/abdominal	4
4	EC	0.2	i/ocular	4
5	EC	0.1	i/conjunctival	4
6	PA	0.2	i/ocular	4
7	SA	0.2	i/ocular	4
8	SE	0.2	i/ocular	4
9	BC	0.2	i/ocular	4
10	AS	0.2	i/ocular	4
11	SS	0.2	i/ocular	4
12	PS	0.2	i/ocular	4
13	MG,EC,PA,SA,SE, BC,AS,SS,PS	0.2 ml each	i/ocular	4
14	MG,EC	0.2 ml each	i/ocular	4
15	MG,PA	0.2 ml each	i/ocular	4
16	MG,SA	0.2 ml each	i/ocular	4
17	MG,SE	0.2 ml each	i/ocular	4
18	MG,BC	0.2 ml each	i/ocular	4
19	MG,AS,SS,PS	0.2 ml each	i/ocular	4

MG – *Mycoplasma gallisepticum*
EC – *Escherichia coli*
PA – *Pseudomonas aeruginosa*
SA – *Staphylococcus aureus*
SE – *Staphylococcus epidermidis*
BC – *Bacillus coagulans*
AS – *Allescheria sp.*
PS – *Penicillium sp.*
SS – *Scopulariopsis sp.*

3.7.2. Method

Disc diffusion method described by Bauer *et al.* (1966) was employed for studying the sensitivity of different bacterial isolates to a number of commonly used chemotherapeutic agents and antibiotics. Sensitivity tests were carried out on nutrient agar containing five per cent sheep blood.

Five colonies from each pure culture were picked up with sterile platinum loop and incubated in five millilitre of nutrient broth at 37^0C for two hours. The bacterial suspension thus obtained was adsorbed into a sterile cotton swab and uniformly inoculated on blood agar and allowed to dry for ten minutes. The different antibiotic discs were then placed on the medium suitably spaced with the help of a flamed and cooled forceps. These plates after 18 to 24 h of incubation at 37^0C were examined for the sensitivity pattern.

The diameters of the inhibition zones were measured in mm (including the diameter of the disc) and recorded for interpretation as per the zone size interpretation chart of Bauer *et al.* (1966). Commercial antibiotic discs from Beacon Diagnostics Pvt. Ltd., India were used for this study. They included erythromycin (15 mcg), ciprofloxacin (5 mcg), tetracycline (30 mcg), Chloramphenicol (30 mcg), streptomycin (10 mcg), penicillin (10 U), gentamicin (10 mcg) and cephalexin (30 mcg).

Results

4. RESULTS

Biomaterials collected from 83 birds of different age groups belonging to different breeds having unilateral/bilateral conjunctivitis were subjected to detailed microbial investigation. Occurrence of unilateral/bilateral conjunctivitis was more in the age groups of 40 to 70 days (Table 2).

Incidence of eye infection was seen through out the year, but was more during the period from August to December. The early symptoms of the disease included increased lacrymation and irritation of the affected eye. As a result the birds rubbed the affected eye against the body and the feathers on that part became soiled (Fig.1 and 2). The condition slowly progressed, the conjunctiva became inflamed, congested and the bird kept the eye closed. After three to four days there was purulent discharge from the eye and accumulation of caseated materials at the junction of bulbar conjunctiva and palpebral conjunctiva on the lower aspect of the eye (Fig.2). The eyelids stuck together and the eye bulged on its lower part as more and more caseated materials accumulated (Fig.3 and 4). The condition was mostly unilateral, with approximately 99 per cent of cases affecting either right/left eye. Most of the birds which received proper treatment with antibiotics recovered. Spontaneous recovery was found to occur in a few

Fig.1 Initial stage of unilateral conjunctivitis in White Leghorn

Fig.2 Unilateral conjunctivitis in White Leghorn with accumulation of caseous material

Fig.3 Advanced stage of unilateral conjunctivitis in a Naked Neck Desi fowl (front view)

Fig.4 Advanced stage of unilateral conjunctivitis in a Naked Neck Desi fowl (side view)

birds without any treatment. In the case of bilateral involvement, death occurred due to starvation.

Based on the clinical signs at the time of the collection of samples, the condition was differentiated as beginning ,mid, and advanced stages of infection (Table 2).

4.1 Isolation of mycoplasma from conjunctival swabs and air sac materials

Conjunctival swabs collected from 83 live birds with conjunctivitis were used for isolation of avian mycoplasma. After collecting the samples all the birds were sacrificed and examined for lesions in internal organs. Air sac lesions were found in nine out of 83 birds. Air sac materials were collected from these nine birds and isolation of avian mycoplasma was tried. Among the samples collected, conjunctival swabs collected at the beginning stage of infection showed minimum contamination resulting in isolation of mycoplasma, whereas air sacs and swabs collected at mid and later stages of infection showed heavy contamination even in the presence of bacterial inhibitors in the media.

In the present study four isolates of mycoplasma could be obtained and all these isolations were from conjunctival swabs (Fig. 5, 6, 7 and 8). Out of these four isolates two were from University Poultry Farm, Mannuthy (UPF-7 & UPF-13) and other two from Central Hatchery, Chengannoor (CH-2 & CH-25).

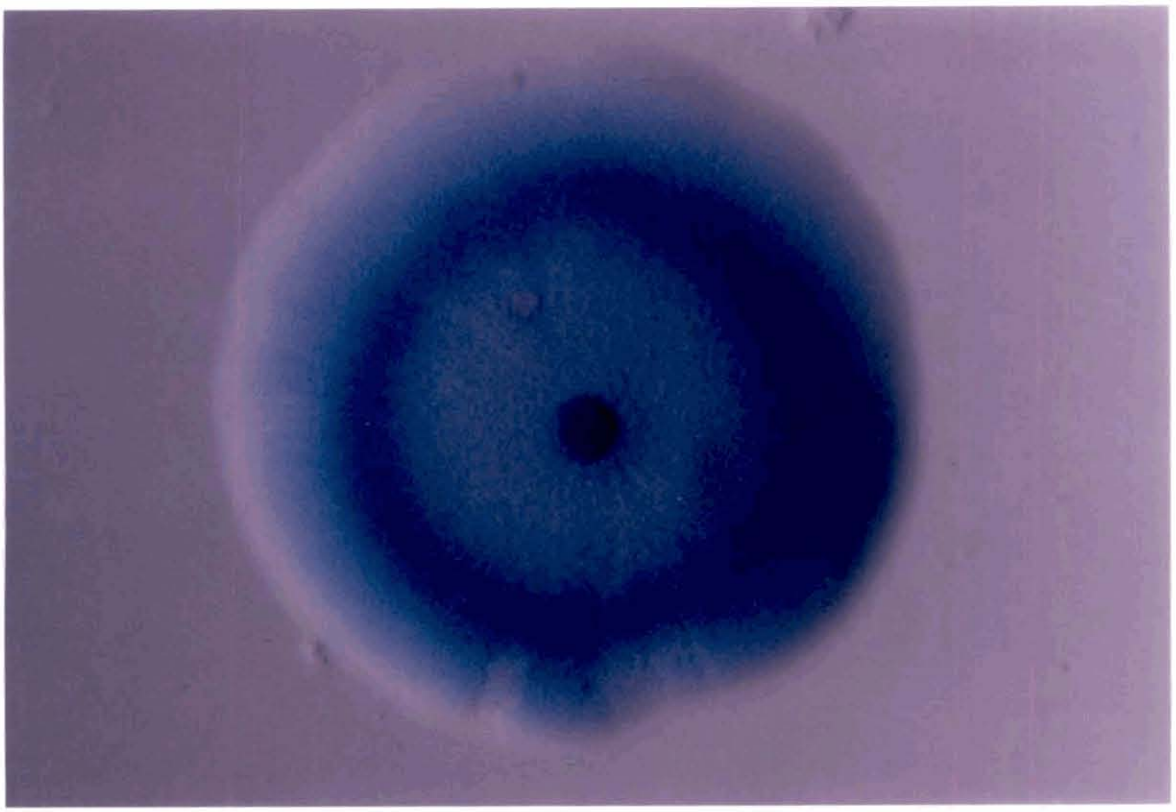

Fig.5 Colony of Mycoplasma isolate UPF 7 stained by Dien's staining technique (10 x magnification)

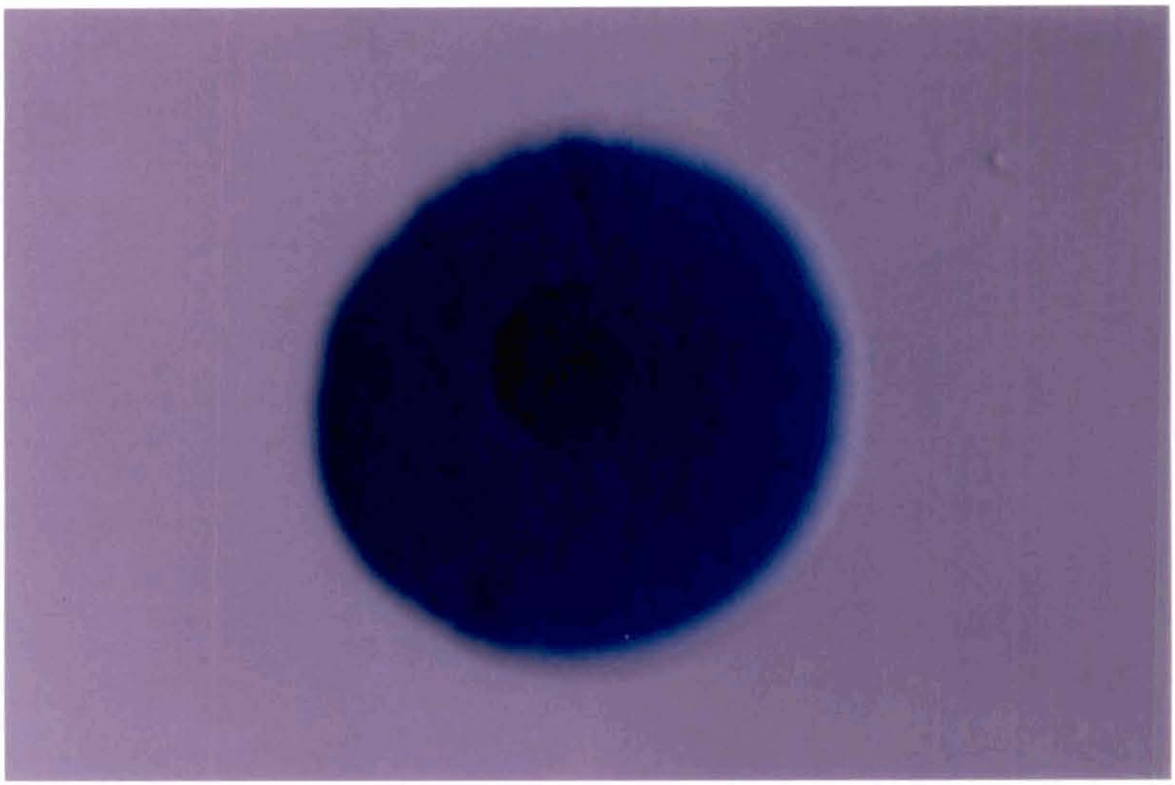

Fig.6 Colony of *Mycoplasma gallisepticum* isolate UPF 13 stained by Dien's staining technique (10 x magnification)

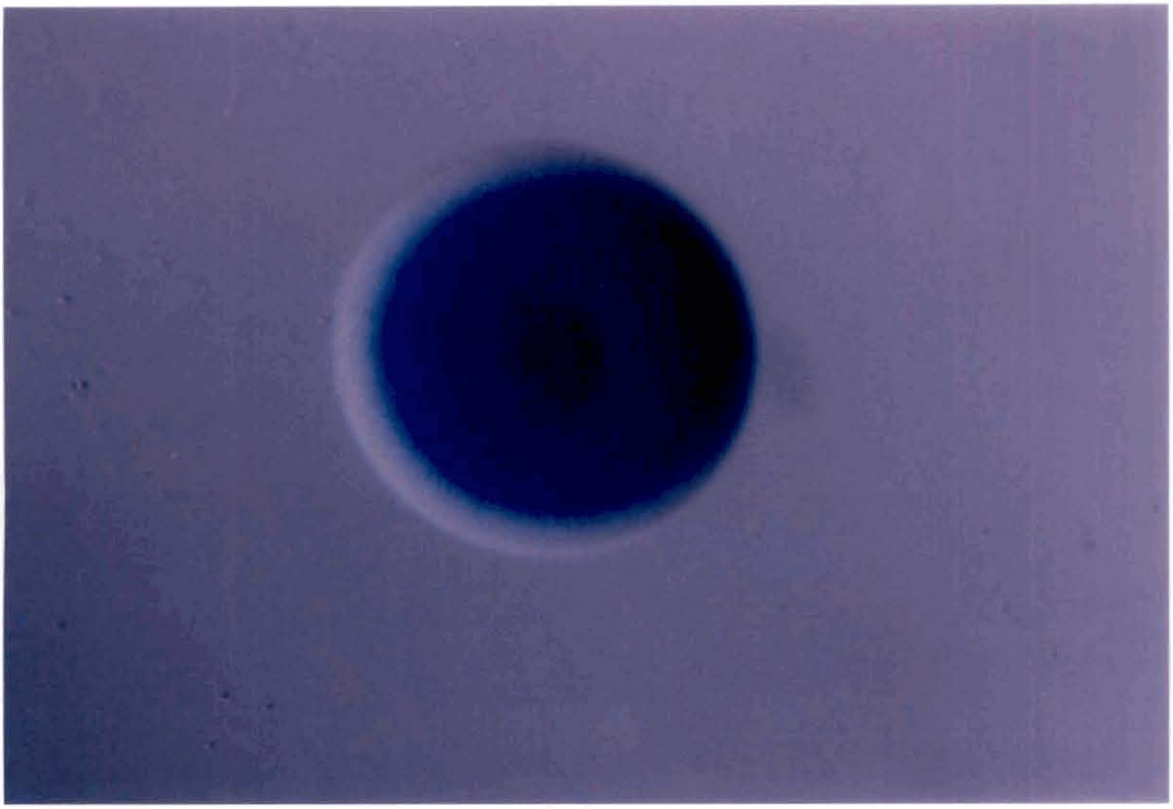

Fig.7 Colony of *Mycoplasma gallisepticum* isolate CH 2 stained by Dien's staining technique (10 x magnification)

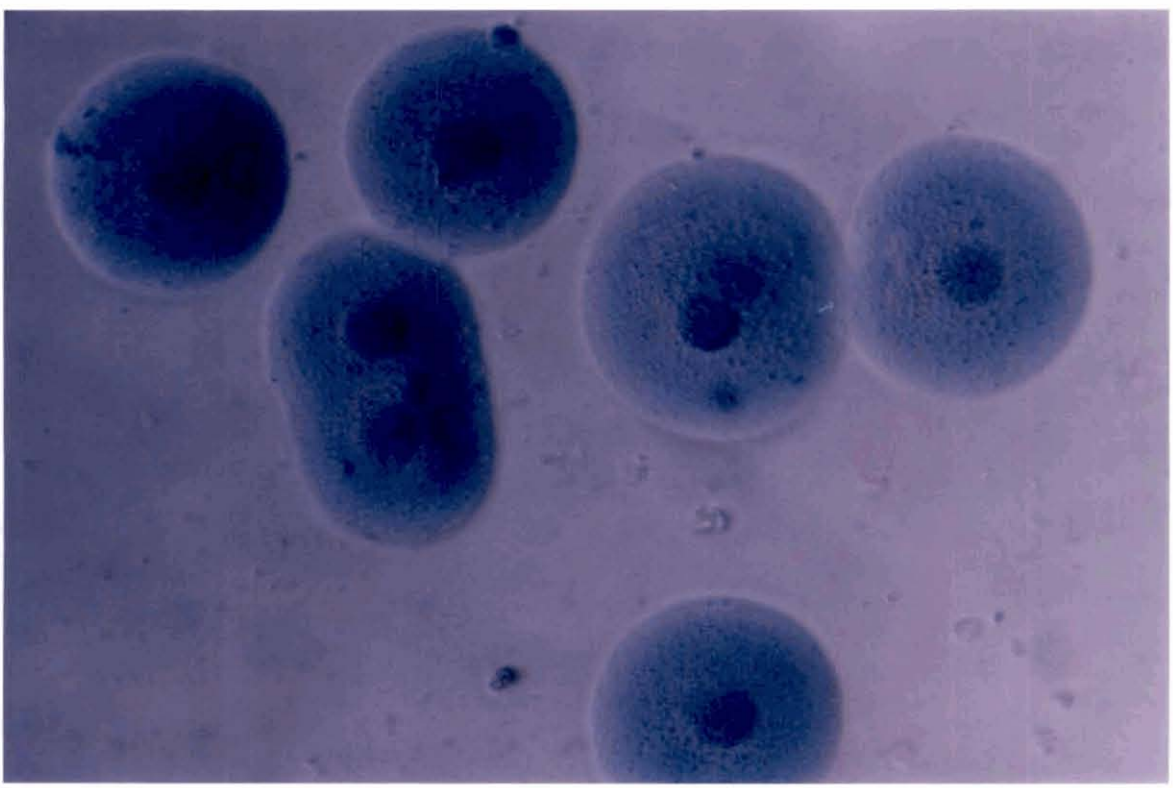

Fig.8 Colony of *Mycoplasma gallisepticum* isolate CH 25 stained by Dien's staining technique (10 x magnification)

4.1.1 Standardisation of medium for the isolation and propagation of avian mycoplasma

Of the two media tested for supporting the growth of avian mycoplasma, only Buffalo Heart Infusion (BHI) broth was found to be good. It supported growth, even on subsequent sub culturing at regular intervals of four days. French Medium Fourth formula (FM_4) broth supported the growth in initial subculture (one to two subcultures) only.

4.1. 2. Characterization of avian mycoplasma isolates

Only three out of four isolates were subjected to characterization since isolate UPF 7 was lost during the study.

4.1.2.1. Colony characters

On examination under 10 x objective of the microscope typical mycoplasma colonies were observed, with a dense centre and a less dense periphery, giving a fried egg appearance (Fig.5). The texture of the colonies varied with the age of the colonies and growth conditions. With Diene's method of staining, the colonies stood out distinctly with dense blue centres and light blue periphery (Fig. 8). The mycoplasma colonies were never decolourised.

4.1.2.2 Differentiation of mycoplasma from acholeplasma

4.1.2.2.1 Growth at 22^0C

None of the isolates showed growth at 22^0C but grew only at 37^0C, indicating that optimum temperature for growth was 37^0C for the present mycoplasma isolates. This suggested that the present isolates were not acholeplasmas.

4.1.2.2.2. Growth on media without serum

In the present study none of the isolates grew on mycoplasma agar plates without serum, indicating that all the present isolates were mycoplasmas which required serum for their growth and they were not acholeplasmas.

4.1.2.3. Differentiation of mycoplasma from L-phase of bacteria.

None of the three isolates reverted to L forms of bacteria on repeated sub culturing in media free of bacterial inhibitors, which suggested that the present isolates were not L-forms of bacteria. The colonies were not destained after 15 minutes, confirming that all the isolates were mycoplasmas.

4.1.2.4. Biochemical characteristics

The isolates 2, 3 and 4 showed similar biochemical reactions with that of the *M. galliseptium* i.e., they fermented glucose, reduced tetrazolium

salt and produced film and spots. All the three isolates did not utilize urea and none produced haemolysis of chicken RBC (Table 5).

4.1.2.5. Serological tests

4.1.2.5.1. Growth inhibition test

The growth of the isolates 2, 3 and 4 were inhibited by the antiserum against MG reference strain (S_6) (Table 6).

4.1.2.5.2. Metabolic inhibition test

The isolates 2, 3, and 4 did not ferment glucose, decaroxylate arginine and reduce tetrazolium salt in the presence of specific antiserum against MG (Table 7).

Of the three isolates tested by various biochemical and serological tests, isolate 2 (Fig. 6), 3 (Fig. 7) and 4 (Fig. 8) were identified as MG and the isolate 1 (Fig. 5) was not subjected to characterization since it was lost during the study.

4.2. Isolation of chlamydia

4.2.1. Staining of smears from samples

The conjunctival tissues collected from the sacrificed birds with conjunctivitis were used for preparing impression smears. The smears were subjected to Giemsa, Modified Ziehl Neelsen and Gimenez methods of

Table 5. Biochemical characterization of the mycoplasma isolates recovered from birds with conjunctivitis

Isolate No.	Sample No.	Growth at 22°C	Growth without serum	Glucose	Arginine	Urea Hydrolysis	Tetrazolium reduction	Film and spots	Haemolysis of chicken RBC
2	UPF 13	-	-	+	-	-	+	+	-
3	CH 2	-	-	+	-	-	+	+	-
4	CH 25	-	-	+	-	-	+	+	-

+ = Fermentation/decarboxylation/reduction/utilization

- = No changes observed

Table 6. Results of growth inhibition test on mycoplasma isolates using antiserum raised against MG (S_6) strain

Isolate No.	Sample No.	Zone of inhibition (mm)
2	UPF 13	5.7
3	CH 2	5.2
4	CH 25	4.8

Table 7. Results of metabolic inhibition test of mycoplasma isolates using antiserum raised against MG (S_6) strain

Isolate No.	Glucose	Arginine	Urea	Tetrazolium
2 (UPF 13)	+	-	-	+
2 + MGHS	-	-	-	-
3 (CH 2)	+	-	-	+
3 + MGHS	-	-	-	-
4 (CH 25)	+	-	-	+
4 + MGHS	-	-	-	-

MGHS – *Mycoplasma gallisepticum* Hyperimmune sera

\+ = Fermentation/decarboxylation/reduction

\- = No changes observed

staining. The stained smears on examination were found to be negative for elementary bodies (EBs) of chlamydia.

4.2.2. Chick embryo inoculation

The conjunctival tissues were processed and were used for chicken embryo inoculation through YS route at the rate of 0.25 ml per egg after ruling out bacterial contamination. Each sample was inoculated in four embryos and were kept for incubation at 37^0C for 12 days. Out of the 83 samples none was able to kill the embryo or produce lesions in the embryo or YS even after three blind passages. Impression smears prepared from YS also did not present EBs by the various staining methods.

4.3. Isolation of virus

The conjunctival swabs collected from the affected birds were processed and were used for chick embryo inoculation. Out of the 83 samples collected only 42 samples were subjected to chicken embryo inoculation after ruling out bacterial contamination. Processed samples were inoculated through YS, allantoic cavity and CAM routes in four embryos each and were kept at 37^0C for incubation for up to seven days. Of the 42 samples inoculated via the three routes, none was able to kill the embryo or produce any lesions in the embryo, yolk sac or chorio allantioc membrane, even after three blind passages. The allantoic fluid collected from the embryos of any passage failed to agglutinate chicken RBC.

4.4. Isolation of Eubacteria

Conjunctival swabs from 83 birds were used for the isolation of eubacteria. The swabs collected were directly inoculated on to two sets of plates with blood agar, chocolate agar, TSA and Mc Conkey agar and incubated at 37^0C upto 96 h, in both aerobic and microaerophilic condition and the negative plates were then discarded.

The bacterial colonies presented on blood agar, choclate agar and TSA were of similar morphology. On Mc Conkey agar lactose fermenting colonies were obtained from 45 samples and non lactose fermentors obtained from eight samples after 48 h of incubation. One among the identical colonies in different plates were selected and were separately sub cultured to produce pure cultures. They were subjected to Gram's staining and examined for cell morphology. Based on this, the isolates obtained were classified into gram-positive bacilli, gram-positive cocci, gram-negative bacilli and gram-negative cocco-bacilli (Table 8).

The gram-positive isolates were subjected to various primary tests for genus level identification (Table 9) and found that all the gram-positive bacilli belonged to the genus *Bacillus* and all the gram-positive cocci belonged to the genus *Staphylococcus*.

The gram-negative isolates were also subjected to various primary tests (Table 10) and found that all the gram-negative non lactose fermenting

Table 8. Results of Gram's staining and cell morphology of eubacterial isolates

Staining technique	Gram's reaction	Cell morphology	No. of isolates
Gram's staining	Gram-positive	Bacilli	11
		Cocci	30
	Gram-negative	Bacilli	8
		Cocco-bacilli	45

Table 9. Results of primary test for Genus level identification of Gram-positive bacteria

Cell morphology	Acid fast	Spores	Motility	Growth in air	Growth anearobically	Catalase	Oxidase	Glucose	O/F test	Genus of the isolate
Bacilli	-	+	+	+	-	+	-	+	F+	*Bacillus*
Cocci	-	-	-	+	+	+	-	+	F+	*Staphylococcus*

+ = Fermentation/growth/ production/utilization
- = No changes observed

Table 10. Results of the primary tests for Genus level identification of Gram Negative bacteria

Cell morphology	Motility	Growth in air	Growth anaerobically	Catalase	Oxidase	Glucose	O/F test	Genus/family of the isolate
Bacilli	+	+	+	+	+	+	O+	*Pseudomonas*
Cocco-bacilli	-	+	+	+	-	+	F+	*Enterobacteriaceae*

+ = Fermentation/growth/ production/utilization

- = No changes observed

bacilli which grew on Mc Conkey agar belonged to the genus *Pseudomonas* and all the gram-negative lactose fermenting cocco-bacilli which grew on Mc Conkey agar belonged to the family *Enterobacteriaceae.*

4.4.1. Characterization of Eubacteria

4.4.1.1. Species level identification of isolates belonging to the genus *Bacillus*

The results of the second stage biochemical tests of genus *Bacillus* are presented in Table 11. The test results of all the eleven isolates were uniform and comparable to that of *Bacillus coagulans* except for growth in seven per cent NaCl in case of UPF 2, UPF 11 and CH 23; Voges-Proskauer (VP) test in case of UPF 7 and CH 1. The isolates showed varied carbohydrate fermentation pattern except for glucose.

4.4.1.2. Species level identification of isolates belonging to the genus *Staphylococcus*

The results of the second stage biochemical tests of genus *Staphylococcus* are presented in Table 12. The test results of the two isolates UPF 2 and UPF 19 were uniform and comparable with *Staphylococcus epidermidis* (SE) while that of other 28 isolates were comparable with *Staphylococcus aureus* (SA) except for VP test in case of UPF 3, UPF 10, UPF 18, UPF 24, RPF 21, CH 4, CH 19, CH 24 and CH 26; nitrate reduction in CH 5, CH 8 and CH 21; fermentation of sucrose in UPF 3, UPF 15, UPF

Table 11. Results of the secondary tests for the species level identification of isolates belonging to the genus *Bacillus*

Biochemical tests	UPF				RPF			CH			
	2	7	11	24	2	4	8	1	3	20	23
Growth at 45^{0}C	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 5.7	+	+	+	+	+	+	+	+	+	+	+
Growth in 7 per cent NaCl	+	-	+	-	-	-	-	-	-	-	+
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth in glucose broth	+	+	+	+	+	+	+	+	+	+	+
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+
Arabinose fermentation	-	+	-	-	-	-	+	+	-	-	-
Mannitol fermentation	-	+	-	-	+	+	+	+	+	-	-
Xylose fermentation	-	-	-	+	-	-	+	-	-	-	-
VP	-	+	-	-	-	-	-	+	-	-	-
Hydrolysis of starch	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	+	+	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of gelatin	-	-	-	-	-	-	-		-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-

+ = Fermentation/hydrolysis/ production/utilization
- = No changes observed

Table 12. The results of the second stage biochemical tests for the identification of the isolates beonging to the genus *Staphylococcus*

Biochemical tests	UPF										RPF								CH										
	2	3	10	15	18	19	22	24	29	31	1	3	5	7	9	16	21	25	1	4	5	7	8	11	13	19	21	24	26	27
VP	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+	-	-	+
Nitrate reduction	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose fermentation	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol fermentation	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose fermentation	-	-	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
Xylose fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	+	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coagulase	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Fermentation/hydrolysis/ production/utilization

- = No changes observed

31, RPF 1, RPF 3, CH 1 and CH 24 and gelatin hydrolysis in UPF 24, UPF 31, RPF 1, CH 5, CH 8 and CH 21.

4.4.1.3. Species level identification of isolates belonging to genus *Pseudomonas*

The results of the second stage boiochemical test of the isolates are presented in Table 13. The test results of all the eight isolates were uniform and comparable with that of *Pseudomonas aeruginosa.*

4.4.1.4. Identification of isolates belonging to the family *Enterobacteriaceae*

The results of the second stage biochemical tests of the isolates are presented in Table 14. The test results of all the 45 isolates were uniform and comparable with that of *Escherichia coli.*

Numbers of isolates were significantly less from sample collected at the early stage of infection. As the infection advanced, the number of isolates per sample also showed an increase.

4.5. Isolation of fungus

Conjunctival swabs from 83 affected birds were utilized for the study. The swabs were inoculated on to SDA plates with and free of microbial inhibitors and kept both at 37^0C and room temperature for a period

Table 13. The results of second stage biochemical tests for the sp. level identification of isolates belonging to the genus *Pseudomonas*

Biochemical tests	UPF			RPF			CH	
	2	11	18	4	5	12	5	15
Citrate	+	+	+	+	+	+	+	+
Lactose fermentation	-	-	-	-	-	-	-	-
Maltose fermentation	-	-	-	-	-	-	-	-
Mannitol fermentation	+	+	+	+	+	+	+	+
Xylose fermentation	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+	+	+
Arginine hydrolysis	+	+	+	+	+	+	+	+
Tween 80 hydrolysis	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+
Lysine decarboxylation	-	-	-	-	-	-	-	-
Ornithine decarboxylation	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+

+ = Fermentation/hydrolysis/ production/utilization

- = No changes observed

Table 14. The results of second stage biochemical tests for the species level identification of isolates belonging to the family Enterobacteriaceae

Bio-chemical tests	UPF																			RPF		
	1	4	5	8	9	10	12	14	15	16	17	18	19	22	23	24	26	28	30	1	3	5
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
Metallic sheen on EMB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eijkman's test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Contd.

Table 14 (Contd.)

Bio-chemical tests	RPF								CH														
	6	7	8	10	11	14	22	24	3	4	5	7	8	10	11	13	14	15	16	17	20	21	27
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metallic sheen on EMB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eijkman's test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

of two weeks for the isolation of yeast and fungus respectively. Specific growth of different fungal colonies could be observed on SDA plates kept at room temperature (25^0C). Of the 83 samples examined, 18 fungal isolates were obtained from 15 samples (Table 15 and 16). None of the SDA plates incubated at 37^0C presented any yeast or yeast-like colony even after two weeks.

Primary characterizations of these fungal isolates were done based on the colony morphology and microscopical structures (Table 16). The isolates obtained included *Allescheria sp.*, *Penicillium sp.* and *Scopulariopsis sp.* Samples UPF 10 and RPF 12 were positive for both *Allescheria sp.* and *Penicillium sp.* and RPF 21 for *Allescheria* and *Scopulariopsis sp.*

The different type of organisms isolated from samples collected at different stages of infection are represented in Table 17. On an average the isolation rate of organisms per sample screened at beginning, mid and advanced stages of infection were 0.5, 1.3 and 2.8 respectively.

4.6. Experimental infection

Eight-week-old chicks, protected against Ranikhet disease and Infectious Bursal disease, procured from University Poultry Farm, Mannuthy, were inoculated with 96-hour-old. BHI broth culture of MG, 18-hour-old nutrient broth culture of eubacterial isolates and 18-hour-old Sabouraud's dextrose broth culture of fungal isolates (Table 4). None of the

Table 15. Details of the fungal organisms isolated

No. of samples utilized for the study	No. of samples positive for fungal growth	Total number of isolates		
		Allescheria sp.	*Penicillium sp.*	*Scopulariopsis sp.*
83	15	10	5	3

Table 16. Genus level classification of the fungal isolates based on colony morphology and microscopical structure

Samples found positive	Colony morphology of the isolate	Microscopical structure	Genus
UPF 10, UPF 16, RPF 4, RPF 5, RPF 12	50-60 mm in diameter in 12 days, flat with powdery bluish green surface surrounded by a narrow white border	Penicillus was seen consisted of chains or spores pinched off from flask- shaped sterigmata borne in whorls from the ends of metulae arising from branched or unbranched conidiophores	*Penicillium sp.*
UPF 3, UPF 10, UPF 31, RPF 8, RPF 9, RPF 12, RPF20, RPF 21, RPF 22, CH 23	Colony was white, abundant and with cottony aerial mycelium which later turned grey. The reverse of the colony was at first white and became grey later on	Mycelium was hyaline, septate, broad with many conidia in clusters on simple, straight, short conidiophores	*Allescheria sp.*
UPF24, RPF 21, CH 1	60-65 mm in diameter in 12 days, flat, white at first, later turned light brown and powdery with a light tan periphery. Reverse of the colony was brownish at centre, fading gradually to a light tan	Hyphae seen with many short, simple conidiophores which suggested 'penicillus' of *Penicillium*. The sterigmata produced chains of lemon-shaped conidia with a somewhat pointed apex and truncate base. Mature spores were echinucleate.	*Scopulariopsis sp.*

T able 17. Details of organisms isolated from samples taken at different stages of infection

Stages of infection	No. of samples collected	Conjunctivitis of chickens			No. of samples failed to give isolates
		Sl. No.	Organisms isolated	No. of isolates	
Beginning stage	24	1	MG	3	12
		2	Mycoplasma (Uncharacterized)	1	
		3	EC	8	
Mid stage	42	1	EC	12	3
		2	SA	6	
		3	BC	2	
		4	AS	1	
		5	SE	1	
		6	EC, BC	3	
		7	EC, AS	1	
		8	EC, PA	1	
		9	EC, SA	6	
		10	BC, AS	1	
		11	EC, PS	1	
		12	BC, PA	1	
		13	SA, AS	3	
Advanced stage	17	1	EC, SA	5	-
		2	SE, PA	1	
		3	EC, BC,AS	1	
		4	EC, SA, PA	3	
		5	EC, BC, PS	1	
		6	SA, AS, SS	1	
		7	SA, BC, SS	1	
		8	PA, PS, AS	1	
		9	EC, SA, PA, PS	1	
		10	EC, SA, BC, SS	1	
		11	EC, SA, AS, PS	1	

MG - *Mycoplasma galliseptium*
SE – *Staphylococcus epidermidis*
EC – *Escherichia coli*
SA – *Staphylococcus aureus*
BC – *Bacillus coagulans*
PA – *Pseudomonas aeruginosa*
AS – *Allescheria sp.*
PS – *Penicillium sp.*
SS – *Scopulariopsis sp.*

eighteen groups inoculated with the isolates alone or in combination by different routes were able to reproduce the eye disease except for group 3, the only group given intra-abdominal inoculation of MG isolate, which developed caseous exudates on air sacs when sacrificed and examined on day 36 post inoculation (Fig. 10). Reisolation of MG from lesions of air sacs was unsuccessful due to contamination.

4.7. Sensitivity to chemotherapeutic agents

The results of the antibiogram studies of the eubacterial isolates are presented in Table 18.

All the gram-positive isolates were generally sensitive to gentamicin, tetracycline, ciprofloxacin and cephalexin, while gram negative organisms except *Pseudomonas aeruginosa* (PA) were sensitive to gentamicin and ciprofloxacin. *Pseudomonas aruginosa* was resistant to all the antimicrobial agents. All the isolates in general showed uniform sensitivity to ciprofloxacin except for PA.

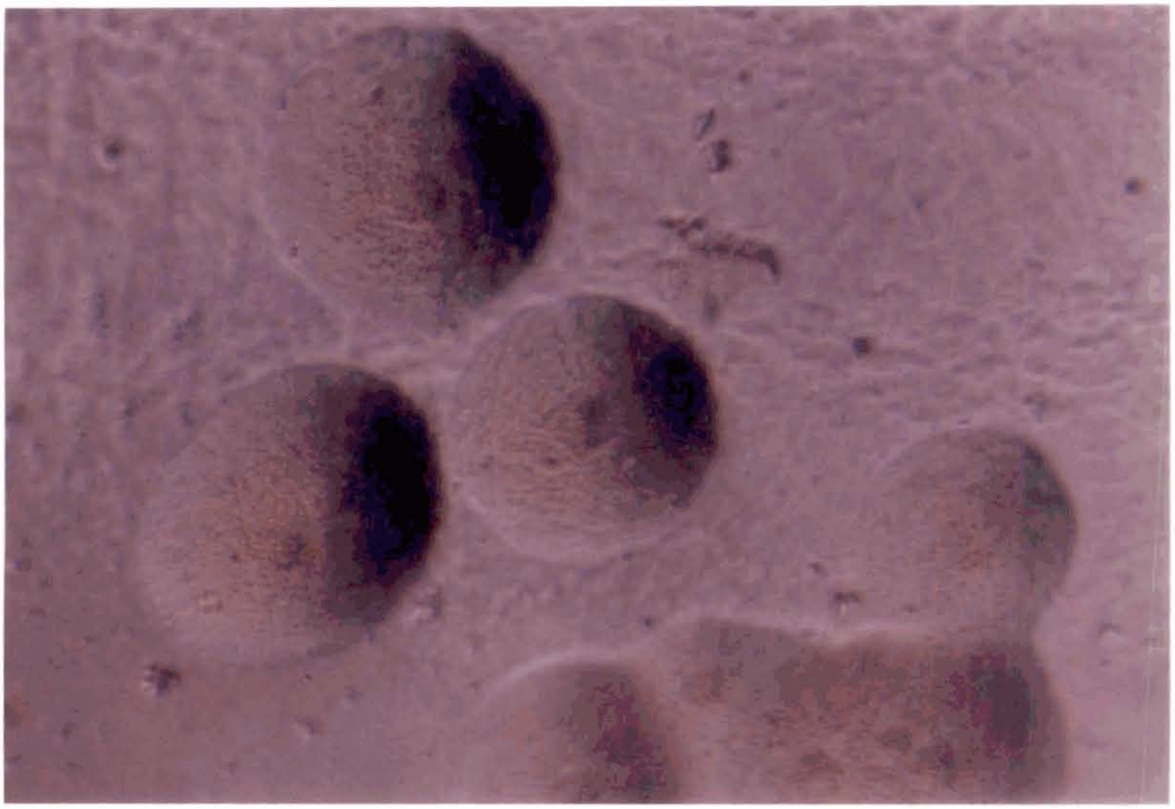

Fig.9 Unstained colony of *Mycoplasma gallisepticum* isolate CH 25 (10 x magnification)

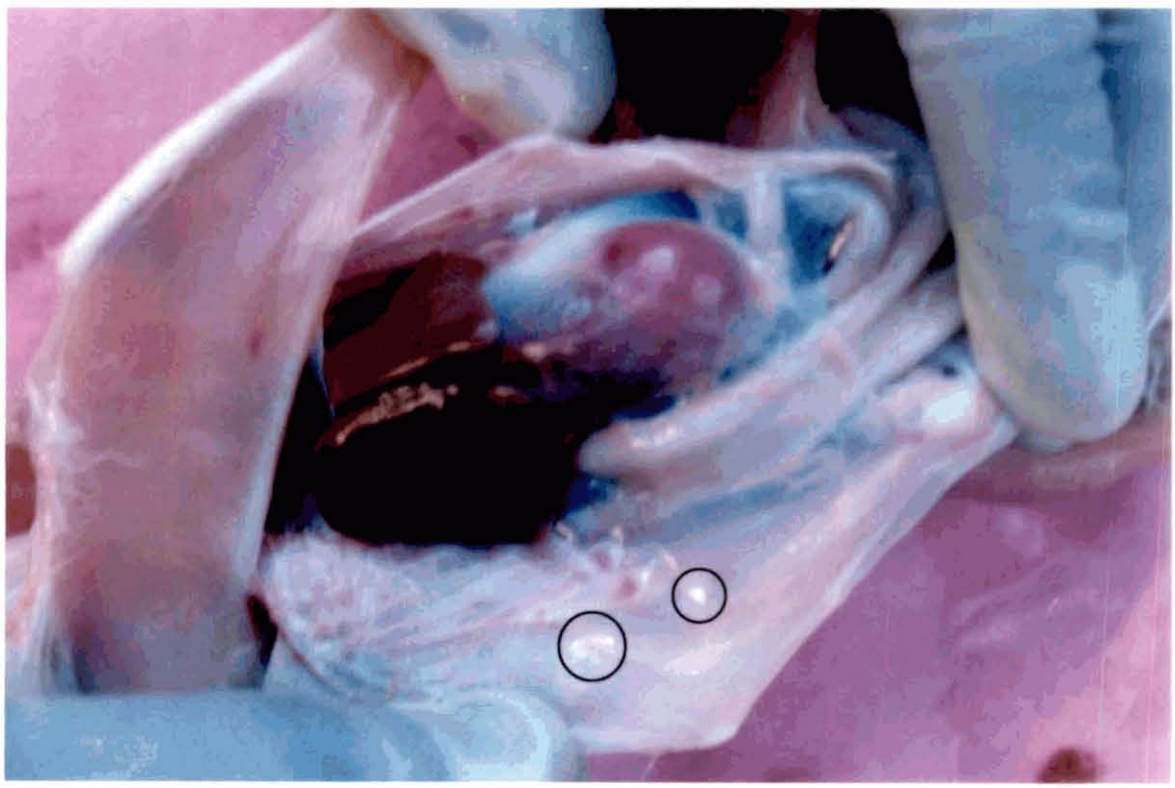

Fig.10 Air sac lesions of bird experimentally infected with *Mycoplasma gallisepticum* intra-abdominally

Table 18. Antibiogram of eubacterial isolates

Organism	Antibiotic discs							
	Erythromycin (15 mcg)	Ciproflaxacin (5 mcg)	Tetracyclie (30 mcg)	Chloramphenicol (30 mcg)	Streptomycin (10 mcg)	Penicillin (100 mcg)	Gentamicin (10 mcg)	Cephalexin (30 mcg)
Bacillus coagulans	+	+++	+++	++	-	++	+++	++
Staphylococcus epidermidis	-	+++	++	+	-	++	+++	++
Staphylococcus aureus	+	+++	++	++	+	++	+++	+++
E. coli	-	+++	-	++	-	-	+++	+
Pseudomonas aeruginosa	-	-	-	-	-	-	-	

- = resistant

+ = sensitive

Discussion

5. DISCUSSION

There are quite a few microorganisms that have been isolated from eye infection of chicken and other species of poultry. Among those causing unilateral conjunctivitis in birds, *Mycoplasma gallisepticum* is the most significant pathogen, followed by *Aspergillus fumigatus*. *Mycoplasma gallisepticum* produces conjunctivitis not only in chickens but also in other species of birds such as house finches, chukar partridges, mocking birds, blue jays and turkeys. Other microbes associated with eye infection include ILT virus, Marek's disease virus, New castle disease virus, *Salmonella typhimurium*, *Staphylococcus hycus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa,* but typical unilateral conjunctivitis is reported only in MG, *S.typhimurium* and *A. fumigatus* infections (Cantini and Rossi, 1972; Reis, 1940).

The incidence of this disease was more during the period from August to December, suggesting that high humidity and temperature were the predisposing factors. This is further augmented by the fact that change in the environment and proper treatment with antibiotics rapidly led to recovery. Shome *et al.* (2000) observed that high humidity, temperature, vitamin A deficiency and poor sanitation were the predisposing factors of conjunctivitis of chickens in Andamans.

5.1. Isolation of avian mycoplasma

The biomaterials collected for the isolation of mycoplasma were the conjunctival swabs and the air sac materials. Conjunctival swabs yielded good isolation rates of MG from house finches with conjunctivitis (Ley *et al.*, 1996). Air sacs 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). Air sacs and conjunctival swabs collected from mid and advanced stages of infection showed heavy contamination so that isolation of mycoplasma was unsuccessful from those samples. Heavy contamination with bacteria was reported by Majid (1986) when specimen from fowl was directly inoculated on to mycoplasma medium for MG isolation.

The critical requirement in isolation of avian mycoplasmas is to have a good media base, which could be used for isolation of all the pathogenic avian mycoplasma. In the present study, out of two media bases tested, buffalo heart infusion broth (Lecce and Sperling, 1954) was found to be ideal for isolation and propagation of avian mycoplasmas, compared to FM_4 broth (Frey *et al.*, 1968). This might be due to the fact 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 mycolasmas than other media compared. Alder *et al.* (1954) proposed a blood agar slope overlaid with PPLO broth containing 20 per cent horse serum, whereas Fabricant (1958) used chick embryo inoculation for the isolation of avian mycoplasmas. But these methods had their own limitations as it was very difficult to secure chick embryos free from mycoplasmas.

5.1.1. Characterization of mycoplasma cultures

In the present study, the mycoplasma isolates were identified based on the morphological, biochemical and serological tests. *Micoplasma gallisepticum* isolates produced typical fried egg appearance on the mycoplasma agar surface and were confirmed by the Diene's method of staining (Madoff, 1960). The biochemical tests such as fermentation of glucose, reduction of tetrazolium salt, production of film and spots and non utilization of urea as sole source of nitrogen and non decarboxylation of arginine confirmed the isolates 2, 3 and 4 as *M. gallisepticum* as reported earlier by Fabricant (1969) and Aluotto *et al.* (1970). The characterization of the isolate 1 was not was not done as it was lost during the study.

Katoch and Chandiramani (1984) reported that MG did not produce haemolysis of chicken erythrocytes, but only caused browning of the erythrocytes. In the present study all the three isolates tested showed only browning of erythrocytes, without haemolysis.

Growth inhibition and metabolic inhibition tests using known specific antiserum against MG also confirmed isolates 2, 3 and 4 as MG since the species specific antiserum inhibited both the metabolism and growth of the specific species. This finding is in accordance with the observation of Clyde (1964) and Woode and Mc Martin (1973). The antigens on the mycoplasma, associated with inhibition of the growth by antiserum, appeared to be essential physiologically active receptors (Woode and Mc Martin, 1973).

5.2. Isolation of chlamydia

The impression smears of conjunctival tissue were screened for elementary bodies (EBs) of chlamydia by various staining methods viz., Giemsa, Modified Ziehl Neelsen and Gimenez. None of the smears showed presence of EBs by these staining methods. Krishna (1990) and Aitken (1993) found the EBs of chlamydia on staining the smears of clinical specimens using the above mentioned methods.

The processed samples were inoculated into chicken embryo by YS route. Three blind passages were performed for each sample and none of the samples yielded chlamydial isolates. Epidemiological and laboratory evidence indicated that chickens appeared to be relatively resistant to disease caused by *C. psittaci* (Grimmes and Wyrick, 1991). Power and Jordan (1976) and Nunoya *et al.* (1995) could isolate only *M. gallisepticum* from cases of unilateral/bilateral conjunctivitis in chickens and did not find the involvement of chlamydia. Hence the findings of the present study suggest

that chlamydia are not involved in this disease process either as primary agent or secondary invader.

5.3. Isolation of virus

Isolation of virus was tried using embyonated eggs by three routes viz., YS, allantoic cavity and CAM. Three blind passages were carried out by each route and no haemagglutinating agents could be isolated. Usually in viral infections conjunctivitis will be associated with other clinical manifestations with a high morbidity. The birds subjected to the study were only showing unilateral conjunctivitis without any other systemic signs, with a low morbidity of five to ten per cent, except for the air sac lesions in nine birds. Power and Jordan (1976) and Nunoya *et al.* (1995) could isolate only MG from cases of unilateral/bilateral conjunctivitis in chickens and did not find the involvement of any virus. The failure to isolate any of the viral agents suggest that virus may not be the causative agent of recently reported eye disease of chickens.

5.4. Isolation of eubacteria

Cotton swabs soaked in peptone water were used to collect conjunctival swabs from the affected chickens. The swabs collected were directly inoculated on to two sets of plates with blood agar, chocolate agar, TSA and Mc Conkey agar and incubated at 37^0C up to 96 h. in both aerobic and microaerophilic condition and the negative plates were then discarded.

The organisms isolated were subjected to various primary and secondary tests for the identification (Barrow and Feltham, 1993) and found that *Escherichia coli* was the organism isolated in large numbers, either alone or in combination with other organisms, as it was isolated from 45 cases out of 83 studied, followed by *Staphylococcus aureus* from 28 cases, *Bacillus coagulans* from 11 cases and *Pseudomonas aeruginosa* from eight cases. *E. coli* was also seen to be associated with all the three stages of infection, while *S. aureus, B. coagulans* and *P. aeruginosa* were associated only with mid and advanced stages of infection.

Unilateral eye lesions in bacterial infection of birds were not common except in *Salmonella typhimurium* infection, as reported by Cantini and Rossi (1972) who found about five per cent of 4000 female chicks infected with *S. typhimurium* exhibited a lesion in one eye only. In all other cases of bacterial infection the lesions were seen bilaterally with other clinical manifestations (Evans *et al.*, 1955; Nillo, 1959; Kowalski and Stephens, 1968; Cheville *et al.*, 1988; Rhoades and Rimler, 1991; Garcia *et al.*, 1992; Pocknell *et al.*, 1996; Gustafson *et al.*, 1998). Of the 24 samples screened at the beginning stage of the infection only 12 isolates were obtained viz., four mycoplasma isolates and eight *E. coli* isolates and 12 samples did not yield any organism. The eubacterial isolates obtained from 42 samples screened at the second stage of infection and 17 screened at the advanced stage of infection were 49 and 37 respectively indicating increase

in number and type of organisms as the disease advanced. As Salmonella was not isolated from any of the cases and the numbers of eubacterial isolates in the initial phase of infection were considerably low, the eubacterial isolates obtained might be secondary invaders complicating the condition. Since *E. coli* was isolated from 45 samples, it could be possible that concurrent infection of *E. coli* and mycoplasma existed in these farms.

5.5. Isolation of fungus

Of the 83 samples examined, 18 fungal isolates were obtained from 15 samples (Table 15 and 16). Primary characterizations of these fungal isolates were done based on the colony morphology and microscopical structure (Table 16). The isolates obtained included *Allescheria sp.*, *Penicillium sp.* and *Scopulariopsis sp.* No fungal isolates were obtained from samples screened at the beginning stage of infection and the number of isolates obtained at the second and third stage of infection were five and 13 respectively, suggesting that the three fungal species obtained could be secondary invaders and moreover there are no published reports of these fungal species causing any eye lesion in chickens.

5.6. Experimental infection

Eight-week-old chicks, protected against Ranikhet disease and Infectious Bursal Disease, procured from University Poultry Farm, Mannuthy were inoculated with 96 h. BHI broth culture of MG, 18 h.

nutrient broth culture of eubacterial isolates and 18 h. Sabouraud's dextrose broth culture of fungal isolates (Table 4). Mc Martin *et al.* (1996) could reproduce natural eye infection in chukar partridges when 48 h culture of a field isolate of MG having a titre of $1x10^7$ CFU/ml was experimentally inoculated into the eye. The same isolate when inoculated intra-abdominally to six-week- old chicks produced air sacculitis at 21 days post-inoculation. In both the experiments the birds were sacrificed 36 days post-inoculation and they were successful in reisolating the organism from experimentally infected birds. In the present study none of the 18 groups, inoculated with the isolates alone or in combination, by different routes were able to reproduce the eye disease except for group 3, the only group given intra abdominal inoculation of MG isolate. This group developed caseous exudates on air sacs when sacrificed and examined on day 36 post inoculation (Fig. 10). Reisolation of MG from lesions of air sacs was unsuccessful, due to contamination. *Staphylococcus sp.*, *E. coli* and *Streptococcus sp.* isolated from cases of acute fibrinopurulent lesions of eyelids in chickens and turkeys failed to reproduce the natural disease when inoculated alone or in combination by intra-dermal scarification into the epithelium of the eyelid (Cheville *et al.*, 1988). Coagulase positive *Staphylococci* and *E. coli* isolated from acute ophthalmitis in chickens failed to reproduce the disease even when the isolates of *Escherichia coli* and *Staphylococus* were inoculated intra-conjunctivally (Shome *et al.*, 2000).

They observed that the infection was seen among the birds of different age groups and breeds in Andamans and the incidence was more during the rainy season. The failure to reproduce the disease in the present study might be due to the following reasons

1. the birds utilised for the study might not be free from subclinical mycoplasma infection
2. failure to simulate the conditions under which the natural disease progressed

The following findings of the present study suggest that MG could be the primary agent leading to recently reported unilateral/bilateral eye infection of chickens in Kerala

1. MG was isolated only from the beginning stage of infections and no other organisms viz., eubacteria/chlamydia/fungus/virus could be isolated from the samples screened at the beginning stage of infection. Twelve samples failed to give any organism, showing that the number of isolates were considerably low at the beginning stage of infection. Shome *et al.* (2000) isolated coagulase positive *Staphylococci* and *E. coli* from acute ophthalmitis in chickens in Andamans and were not successful in reproducing the disease when the isolates were inoculated intra-conjunctivally and suggested mycoplasma as the probable causative organism

2. No virus/chlamydia could be isolated from any of the samples

3. The isolation rate of organisms on an average from the initial, mid and advanced stages of infection were 0.5, 1.3 and 2.8 respectively showing that the number of eubacterial and fungal isolates per sample screened were on an increase as the disease advanced. The affected birds which were promptly treated with antibiotics showed rapid recovery without loss of vision. These findings suggest that the eubacterial and fungal isolates obtained could be secondary invaders complicating the condition.

In untreated cases of conjunctivitis loss of vision occurred. This might be due to the infection spreading into the eye and subsequent pressure atrophy of the eye ball by the caseated mass.

The chicks on the second and fourth day of hatching contracted the disease probably due to vertical transmission of MG. As *E. coli* was the organism isolated from 45 cases, it could be possible that concurrent infection of *E. coli* and MG existed in these farms. Jordan (1979) observed that both egg and air borne transmission may be influenced by concurrent infections.

The antibiogram of the eubacterial isolates suggest that if proper treatment with antibiotics like ciproflaxacin/gentamicin is given at the

beginning stage of infection, the secondary invaders can be prevented from complicating the condition.

The present case of eye infection is seen to be unusual because under natural conditions MG predominantly causes, respiratory disease in chickens. The pathogenesis of ocular lesions induced by mycoplasma infection is poorly understood due to lack of previous research reports clarifying the relationship between lesions and mycoplasma infection. Hence for the complete understanding of the source of infection and its pathogenesis, further research on this aspect is warranted.

Summary

6. SUMMARY

Poultry production in India has achieved a remarkable growth from an age-old backyard farming to a dynamic and most sophisticated agro-based industry in recent times. But the problems facing this industry are many and diverse, the major problem being the lack of adequate facilities for the diagnosis and prevention of emerging poultry diseases. Intensive breeding and managemental practices to increase the production have greatly contributed to higher incidence of several infectious diseases.

Recently from the year 1998 onwards, unilateral conjunctivitis of unknown etiology in chicken was reported extensively in many parts of Kerala, with low mortality and a morbidity ranging from five to ten per cent. The condition was unilateral in 99 per cent of cases and most of the affected birds recovered when timely treatment with antibiotics was given at the beginning stage of infection. The incidence of disease was more in seasons with higher temperature and humidity. The present study was undertaken to find out the microbial agents associated with the condition.

A total of 83 birds utilised for the study were obtained from University Poultry Farm, Kerala Agricultural University, Mannuthy; Regional Poultry Farm, Kodappanakunnu and Central Hatchery, Chengannoor. Biomaterials utilised for the study included the conjunctival swabs and air sac materials. In order to find out the primary etiological

agent and secondary invaders, samples were collected from the beginning, mid and advanced stages of infection.

Out of the 24 samples screened at the beginning stage of infection, 12 isolates were obtained, of which four were mycoplasmas and eight were *Escherichia coli* and 12 samples did not yield any microorganisms. On further characterization of mycoplasma isolates using biochemical and serological methods three were found to be *Mycoplasma gallisepticum* while one isolate was lost during the study. Twenty four isolates of *E. coli*, 15 isolates of *Staphylococcus aureus*, seven isolates of *Bacillus coagulans*, two isolates of *Pseudomonas aeruginosa*, one isolate of *Staphylococcus epidermidis*, six isolates of *Attescheria sp.* and one isolate of *Penicillium sp.* were obtained from 42 samples screened at the mid stage of infection. The samples screened at the advanced stage of infection yielded 13 isolates of *E. coli*, 13 isolates of *S. aureus*, six isolates of *P. aeruginosa*, four isolates of *B. coagulans*, one isolate of *S. epidermidis*, four isolates of *Allescheria* sp., four isolates of *Penicillum* sp. and three isolates of *Scopulariopsis* sp.

Attempts to isolate viral agents through embryonated egg inoculation by yolk sac, allantoic and chorio-allantoic membrane routes and chlamydia by YS route did not yield any organism. Eighty three samples screened in the present study yielded a total of 116 isolates of different species of microorganisms. On an average the isolation rate of microbial agents per sample at the beginning, mid and advanced stages of infection were 0.5, 1.3

and 2.8 respectively showing increase in number and type of microorganism as the disease advanced except for MG which was isolated only from the beginning stage of infection.

The efforts to reproduce the disease experimentally was not successful, probably due to the failures in obtaining experimental birds free of subclinical mycoplasma infection and in simulating the condition under which the natural disease progressed.

The findings of the present study in correlation with the management practices suggest that the primary etiological agent leading to the eye infection in chicken in Kerala could be MG, and the physical parameters such as high temperature and humidity, overcrowding and ammonia fumes could act as the predisposing factors. Since *E. coli* was the organism isolated in large numbers ie., from 45 samples out of 83 screened, it could be possible that intercurrent infection of *E. coli* and mycoplasmas existed in these poultry farms. Other eubacterial and fungal agents isolated might be secondary invaders complicating the conditions.

The antibiogram of the eubacterial isolates suggest that if proper treatment with antibiotics like ciprofloxacin/gentamicin is given at the beginning stage of infection, the secondary eubacterial invaders can be prevented from complicating the condition.

The present case of eye infection seems to be unusual because under natural conditions MG predominantly causes respiratory disease in chickens. The pathogenesis of ocular lesions induced by mycoplasma infection is poorly understood due to lack of clarification of the relationship between lesions and mycoplasma infection in previous research reports. Hence for the complete understanding of the source of infection and pathogenesis, further research on this aspect is warranted.

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MICROBIAL AGENTS ASSOCIATED WITH EYE INFECTION IN CHICKEN

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ABSTRACT OF THE THESIS

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ABSTRACT

A study was undertaken to find out the microbial agents associated with the recently reported eye infection of chickens in many parts of Kerala. Samples were collected from 83 birds from different parts of Kerala. The bio-materials employed for the study were the conjunctival swabs and air sac materials. Organisms isolated from different stages of infection include 45 isolates of *E. coli*, 28 isolates of *S. aureus*, 11 isolates of *B. coagulans*, eight isolates of *Pseudomonas aeruginosa*, two isolates of *S. epidermidis*, ten isolates of *Alloscheria sp.*, five isolates of *Penicillin sp.* and three isolates of *Scopulariopsis sp.* and three isolates of *M. gallisepticum*. On an average, the rate of isolation of microbial agents per sample at the beginning, mid and advanced stage of infection were 0.5, 1.3 and 2.8 respectively, showing that the number and type of organism isolated increased as the disease advanced except in the case of MG which was isolated only from the beginning stage of infection. No viral agents or chlamydia could be isolated through embryonated egg inoculation. The findings of the present study suggest that MG could be the primary agent leading to conjunctivitis while other eubacterial and fungal isolates could be secondary invaders complicating the condition. The antibiogram of the eubacterial isolates suggest that proper treatment with antibiotics like ciproflaxacin/gentamycin at the beginning stage of infection could prevent secondary invaders from complicating the condition.