BACTERIA ASSOCIATED WITH RESPIRATORY INFECTIONS IN POULTRY

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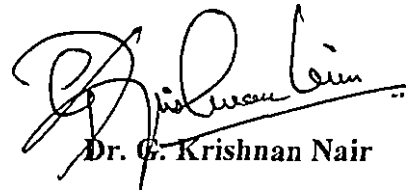MANNUTHY, THRISSUR-680651
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I hereby declare that the thesis entitled " BACTERIA ASSOCIATED WITH RESPIRATORY INFECTIONS IN POULTRY " is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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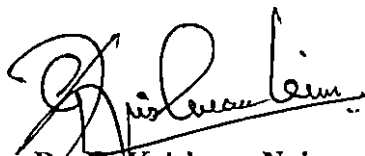
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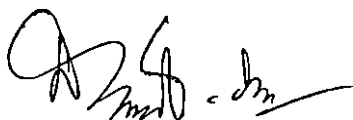College of Veterinary and
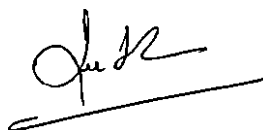Animal Sciences, Mannuthy.

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Dedicated to Saint Mary

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Introduction

1. INTRODUCTION

Birds have fascinated man from pre-historic times, probably due to their capability to fly. Archaeological evidence for domestication of fowl dates to approximately 2500 BC in the Indus valley, India (Crawford, 1990).

By 2000 AD, almost 4000 years after the domestication of jungle fowl, the total world poultry meat production reached 66 million tons which contribute to 28.5 per cent of total meat production in world. No other proof is required to show the success of this industry. The reasons for this success can be attributed to the population explosion, which led to an increased demand for nutritious food, especially for animal protein, at reasonable price. Under these circumstances, poultry products got a golden opportunity to capture the animal product market. Poultry industry succeeded through rapid development and poultry products got global acceptance.

Presently, poultry industry occupies a prominent position in agricultural sector all over the world. The worldwide success of this industry found its reflections in India also. Our poultry sector has shown a much accelerated growth in the last three decades, evolving from backyard ventures to a full-fledged commercial agro industrial business. This is mainly due to comprehensive research and development initiated by the Government and subsequently taken up by the organized private sector. India is now the world's fifth largest egg producer and the eighteenth large producer of broilers (FAO, 2003).

Advances in breeding, nutrition and management improved poultry production. But on the other side, spates of disease outbreaks have repeatedly disrupted poultry production systems and created instability in the industry. The recent disease outbreaks, especially of avian influenza, indicate that poultry diseases also possess serious zoonotic risk. Therefore, research needs to be redirected to

diagnose and control avian diseases in order to maintain the harmony and stability of the poultry industry and for security of the human population.

Disease is a condition which results from the failure of the body to perform its normal functions, the severity of which is directly proportional to the degree of impairment of the body / system(s). The impairment which leads to the disease condition may be due to infectious or parasitic agents, injury, physical stress, metabolic disorders or nutrient deficiencies.

The immune system that protects the body from diseases is complex in birds as in mammals. The proper functioning of this system is of special importance in preventing disease among poultry, because commercial poultry production is based on intensive rearing, and under such conditions the flocks are vulnerable to rapid spread of infectious agents and disease outbreaks (Sharma, 2003).

Respiratory infections in chicken and turkeys are seen worldwide and numerous factors including infectious agents, non-infectious agents and environmental factors may contribute to the disease complex. The infectious causes may be viruses like Infectious Bronchitis, Avian pneumovirus, Lentogenic Newcastle disease virus, Avian influenza, fungi like *Aspergillus* sp. and bacteria like *Escherichia coli(E. coli)*, *Haemophilus* sp., *Klebsiella* sp., *Mycoplasma* sp., *Pasteurella* sp., *Staphylococcus* sp. *and Streptococcus* sp. Dust, ammonia, other gases and factors associated with poor ventilation, may act as predisposing factors for infectious diseases.

Bacterial respiratory disease outbreaks can occur either as primary disease or as secondary complication to viral diseases, stress and other managerial faults. In all cases, the involvement of bacterial pathogens will increase mortality, decrease production and thereby cause economic losses. Therefore, the management of a sustainable poultry production system entails control of bacterial respiratory

pathogens and the isolation and identification of the causative agent if present, and preventing multiplication and spread of the same.

Considering the importance of these problems, this study was undertaken with the following objectives.

1) Isolation and identification of bacteria from the respiratory tract of poultry.

2) Biochemical identification of isolated bacteria.

3) To study the antibiogram of the isolates.

Review of Literature

2. REVIEW OF LITERATURE

2.1 PECULIARITIES OF AVIAN RESPIRATORY SYSTEM

2.1.1 Bacteria

Many bacteria are essential for the animal's well-being since they maintain an environment on the body surface that is hostile to other potential invaders and assist in digestion of food such as celluloses. Thus, some bacteria act as commensals. Nevertheless many commensal bacteria are also potential pathogens and so it could be concluded that bacterial disease is not an inevitable consequence of the presence of pathogenic organisms on body surfaces and the development of disease depends on many other factors such as host response, location of bacteria and virulence of bacteria. The disease occurs when there is an upset in the balance between host immunity and bacterial virulence (Tizard, 2004).

2.1.2 Immune System of Birds

The avian immune system differs from that of mammals in that birds possess bursa of Fabricius. Lymph nodes are absent in chickens and turkeys, but present in water birds (King, 1975). However, the basic mechanisms involved in immune system are the same for both birds and mammals (Sulochana, 1998).

The proper functioning of the immune system is of special importance to birds because the intensive rearing conditions of the commercial poultry flock make them vulnerable to rapid spread of infectious agents and disease outbreaks (Sharma, 2003). Protection against invasive bacteria is usually mediated by antibodies which

make them prone to phagocytosis by opsonising them. The antibodies also activate the classical complement pathway (Tizard, 2004).

2.1.3 Respiratory Disease in Poultry

Maina and Cowley (1998) observed that, the respiratory system of birds has developed several cellular defense lines that include surface macrophages, epithelial, sub epithelial and interstitial phagocytes, and pulmonary intra-vascular macrophages (PIMs).

Nganpiep and Maina (2002) observe that, birds have significantly fewer free (surface) avian respiratory macrophages (FRMs) compared to the rat. In the birds, the FRMs have been found both in the lungs and in the air sacs. Under similar experimental conditions, the most robust FRMs were those of the domestic fowl followed by those of the rat and the duck. Flux of macrophages into the respiratory surface from the sub epithelial compartment and probably also from the pulmonary vasculature was observed in the birds but not in the rat. Considering the above described facts, they explain that rather than paucity of FRMs, factors such as inadequate management and husbandry practices and severe genetic manipulation for fast growth and high productivity may have weakened cellular and immunological defenses. This may be the reason for the purported high susceptibility of poultry to respiratory ailments and afflictions.

2.2 BACTERIA ASSOCIATED WITH RESPIRATORY TRACT INFECTIONS.

According to Walker (2004) the important bacterial infectious agents affecting the respiratory system of poultry are *Pasteurella multocida (P. multocida)* causing air sacculitis and pneumonia; *E. coli* causing colisepticaemia and secondary pneumonia; *Haemophilus paragallinarum* causing rhinitis and sinusitis; *Mycoplasma* sp. causing air sacculitis and sinusitis ; *Ornithobacterium rhinotracheale* causing air

sacculitis , bronchopneumonia and sinusitis and *Bordetella avium* causing tracheitis and sinusitis.

Although there are so many bacteria involved in respiratory diseases in poultry, only those organisms that are obtained in this study have been included in this review of literature.

2.2.1 *Escherichia coli* Infections
2.2.1.1 *Incidence and Prevalence*

Avian coli septicaemia occurred with the invasion of the trachea by virulent strains of *E. coli*, with strains of serotype O78 responsible for about 50 per cent of the cases. (Yerushalmi *et al.*, 1990)

Escherichia coli caused a variety of diseases in poultry including respiratory tract infection, omphalitis, swollen-head syndrome, enteritis, septicaemia, and cellulitis. These diseases were responsible for major economic losses in the chicken industry (Norton, 1997).

Escherichia coli is a part of normal intestinal flora of mammals and birds and in the organs outside the intestinal tract, it could cause a variety of diseases depending on presence of specific virulence factors   (Altwegg and Bockemuhl, 1998).

Although *E. coli* is present in the normal intestinal flora of birds , only some strains with specific virulence attributes, designated as avian pathogenic *E. coli* (APEC), are able to cause diseases such as acute coli septicaemia, fibrinopurulent polyserositis, airsacculitis, pericarditis, salpingitis, synovitis, yolk sac infection, swollen head syndrome, coligranuloma, and cellulitis (Vidotto *et al* ., 1990; Dozois

et al ., 1994; Gomis *et al* ., 1997; Pourbakhsh *et al* ., 1997; Dho-Moulin and Fairbrother, 1999).

Serotypes O1, O2 and O78 are present in 15 per cent to 61 per cent of colibacillosis clinical cases (Dho-Moulin & Fairbrother, 1999).

An investigation on the occurrence of poultry diseases in Sylhet region of Bangladesh conducted by Islam *et al.* (2003) revealed that colibacillosis contributed to 5.17 per cent out of the total deaths occurring in poultry.

Manohar and Moorthy (2004) obtained 32 *E. coli* isolates from 82 dead birds suspected for chronic respiratory disease (CRD) from commercial farms in and around Bangalore.

Gomes *et al.* (2005) describe *E. coli* infections as of significant concern to the poultry industry and as one of the most frequently encountered important bacterial avian pathogens causing a wide variety of disease syndrome in farmed birds. These organisms are responsible for 5-50 per cent of mortality in the poultry industry.

2.2.1.2 Clinical Signs and Lesions

According to Jordan and Pattison (1996), clinical signs of colisepticaemia include decreased feed intake, listlessness, ruffled feathers, laboured breathing, occasional gasping and characteristic "snicking". The gross lesions are airsacculitis, pericarditis, peritonitis and perihepatitis.

Younus (1996) describe that *E. coli* often affects respiratory system of birds, causing respiratory distress and in such cases the air sacs are thickened and often have caseous exudates.

2.2.1.3 *Pathogenesis and Virulence*

2.2.1.3.1 *Pathogenesis*

Studies conducted by Tufft *et al.* (1988), showed that in early infection due to *E. coli*, some of the disease responses might be due to endotoxins, whereas the later responses might be due to other aspects of infection such as stress.

Jordan and Pattison (1996) describe that the pathogenic *E. coli* serotypes that most frequently cause colisepticaemia were likely to be found in throat and upper trachea and they invade the bird's body to produce the characteristic condition.

Barnes and Gross (1997) suggest that yolk sac infection could cause the spread of *E. coli* into the bloodstream, which may lead to septicaemia. In this case, the pathogenesis will be different from the more studied colibacillosis of respiratory origin.

Pourbakhsh *et al.* (1997) observe that both air sac and lung could act as the portal of entry for *E. coli* into systemic circulation, probably via damaged epithelium. In experimental studies, they observed a gradual increase in bacterial counts in the trachea, lungs, air sac and liver following inoculation of pathogenic *E. coli* into air sac.

2.2.1.3.2 Virulence

α-Hemolysin of *E. coli* is an extra cellular toxin frequently produced by pathogenic isolates from human and animals (Cruz and Muller 1980).

The ability of bacteria to adhere to host epithelial cells is considered to be a prerequisite for the establishment of infectious diseases, mainly through expression of fimbriae (Naveh *et al.*, 1984).

According to Ike *et al.* (1990), APEC generally possess type 1 and P fimbria.

Several O78 strains isolated from poultry with colisepticaemia produced pili that mediated adherence to avian epithelial tissues *in vitro* and *in vivo* (Yerushalmi *et al.*, 1990).

Orndorff and Falkow (1994) describe that type 1 fimbriae are characterized by the ability to agglutinate chicken and guinea pig erythrocytes in the absence of D-mannose.

Dho-Moulin and Fairbrother (1999) report that P fimbriae is an important factor in the later stages of infection by *E. coli* as it helps in adhesion to internal organs.

Temperature-sensitive haemagglutinin (Tsh) of *E. Coli* play a role in the colonization of air sacs (Dozois *et al.*, 2000)

Haemolysin E (HlyE) is a novel pore-forming toxin that has been identified in K 12 strain and is believed to have the ability to lyse red blood cells (Wyborn *et al.*, 2004).

2.2.1.3.3 Detection of Virulence

2.2.1.3.3.1 Congo Red (CR) Dye Binding Test

Berkhoff and Vinal (1985) found a direct correlation between the ability of clinical isolates of *E. coli* to bind CR dye and their ability to cause septicaemic infection in chickens. They suggested that CR dye binding could be used as a phenotypic marker to distinguish between invasive and non-invasive *E. coli* isolates.

Panigrahy and Yusen (1990) opined that, in contrast to pathogenic *E. coli,* however, nonpathogenic isolates absorbed CR dye early, between 18 to 24 hours of incubation. Although CR dye binding does not correlate well with pathogenicity, it may be an identifiable property of some serotypes of *E. coli.*

2.2.1.3.3.2 *Iron Uptake Abilities*

According to Dho and Lafont (1984), the ability to grow in limited iron conditions is strongly correlated with lethality. In a study conducted by them 52 per cent of the lethal *E. coli* strains but none of the non lethal strains , possessed both adhesive and iron-uptake abilities and hence, it was suggested that these two played a major role in the virulence of *E.coli* isolated from poultry.

2.2.1.3.4 *Plasmids*

Meyers *et al.* (1976) describe a simple agarose gel electrophoretic method for the identification and characterization of plasmid DNA.

Mini *et al.* (2005) reported plasmid isolation from 10 *E. coli* isolates and plasmids varied from one to three in each isolate and the size of the plasmids ranged from 1.86 to 48.06 kb and one isolate did not show any plasmid.

Rodriguez-Siek *et al.* (2005) opined that, the presence of large plasmids appeared to be one of the most important characteristics of the APEC pathotype.

The findings of Skyberg *et al.* (2006) suggest that acquisition of avian pathogenic *Escherichia coli* plasmids like pAPEC-O2-ColV, by a commensal *E. coli* isolate enhance its abilities to kill chicken embryos, thereby proving their ability to contribute to the pathogenesis of avian colibacillosis. Hence, a better understanding of the virulence mechanisms of the causative APEC strains is needed to guide the development of preventive measures.

2.2.1.4 Isolation and Identification

2.2.1.4.1 Isolation

Jordan and Pattison (1996) state that confirmatory diagnosis of *E. coli* infection could be made by isolating a profuse pure growth on direct culture from the organ in Mac Conkey agar.

Serotyping of the *E. coli* isolates revealed that the predominant serotype among 32 isolates from 82 dead birds suspected for chronic respiratory disease (CRD) obtained from commercial farms in and around Bangalore was O8 followed by rough strains. The other serotypes obtained were O173, O15 and untypable (Manohar and Moorthy, 2004).

2.2.1.4.2 Identification

2.2.1.4.2.1 Cultural characters

E. coli strains are usually strong lactose fermenters and the colonies on Mac Conkey agar were bright pink. On Eosin methylene blue agar (EMB) they had a unique and characteristic metallic sheen. (Quinn *et al.*, 1994)

2.2.1.4.2.2 Biochemical characterization

Rosenberger *et al.* (1985) stated that although 197 *E. coli* isolates obtained from clinically affected broilers had similar metabolic activity, as determined by amino acid decarboxylation and carbohydrate fermentation, the fermentation of adonitol was an exception as it was seen more frequently with higher pathogenic strains.

The IMViC test (indole + / MR+ / VP- / Citrate-) could be used as a quick presumptive method of identifying *E. coli* as almost no other lactose positive member of *Enterobacteriaceae* gives this combination of results (Quinn *et al.*,1994).

On triple sugar iron agar (TSI) *E. coli* gives yellow slant and yellow butt with out hydrogen sulphide production (Quinn *et al.*, 2002).

2.2.1.4 Antibiotic Sensitivity

There is strong evidence that the use of antimicrobial agents can lead to the emergence and dissemination of resistant *E. coli* (Linton *et al.*, 1977).

In intensively reared food animals, antibiotics might be administered to whole flocks rather than individual animals, and antimicrobial agents might be continuously fed to food animals such as broilers and turkeys as antimicrobial growth promoters. Therefore, the antibiotic selection pressure for resistance in bacteria in poultry is high and consequently their faecal flora contained a relatively high proportion of resistant bacteria (Caudry and Stanisch, 1979).

According to Panigrahy *et al.* (1983) greater intensification of poultry husbandry and ubiquity of pathogenic *E. coli* in litter dust and the concomitant increased risk of infection makes colibacillosis difficult to control and protection obtained by antibiotics is transient and unreliable.

A resistance of 98 per cent was observed against erythromycin in the study conducted by Allan *et al.* (1993). They also observed a high frequency of resistance to tetracycline, kanamycin, neomycin cephalothin, streptomycin.

Jordan and Pattison (1996) stated that the best method to control colisepticaemia is by maintaining higher standards of flock management. Although *E. coli* infection could be treated with a number of antibacterial agents, the logical

approach to treatment is to isolate the causal serotypes and carry out an antibiotic sensitivity test to choose the most effective antibiotic.

In vitro antibiotic susceptibility test was found to be the most ideal technique for the selection of effective antimicrobial agents in the treatment of respiratory diseases (Martel, 1996).

The resistance of avian *E. coli* isolates to tetracycline has been attributed in part to widespread and lengthy use of tetracycline in the poultry industry (Piddock, 1996).

Cormican *et al.* (2001) obtained 100 per cent sensitivity to ciprofloxacin among *E. coli* isolated from hens.

Miles *et al.* (2006) obtained a resistance of 82.4 per cent to tetracyclines among *E. coli* isolates from poultry.

Giovanardi *et al.* (2005) reported that *E. coli* O78 strains showed a high level of resistance to amoxycillin, enrofloxacin, tetracycline and trimethoprim-sulfamethoxazole. Eighteen *E. coli* O139 isolates showed resistance to amoxycillin, enrofloxacin and tetracycline.

2.2.2 *Pasteurella multocida*

Fermentation patterns of dulcitol and sorbitol by *P. multocida* were of taxonomic significance (Mutters *et al.*, 1985). Based on these criteria the taxon *P. multocida* could be divided into three subspecies. The sorbitol and dulcitol positive variety became *Pasteurella multocida* subsp. *gallicida*; those strains negative for both became *Pasteurella multocida* subsp. *septica* and those positive for sorbitol but negative for dulcitol were *Pasteurella multocida* subsp. *multocida*.

Fowl cholera (pasteurellosis), caused by *P. multocida* sp. occurrs sporadically or enzootically in most countries of the world wherever intensive poultry production is prevalent. It is known as a bacterial disease of major economic importance due to its high mortality (Rimler and Glisson, 1997).

The genus *Pasteurella* was named in honour of Louis Pasteur in commemoration of his work on these bacteria. *Pasteurella* comes under the family *Pasteurellaceae* along with other genera *Actinobacillus and Haemophilus*. *Pasteurella multocida* is the most common pathogen of the genus causing severe disease in poultry leading to heavy economic loss (Bottone, 1998).

Fowl cholera is a contagious disease affecting both domesticated and wild birds. The disease usually appears to be septicaemic with high morbidity and mortality although chronic or benign conditions also occur (Glisson *et al.*, 2003).

2.2.2.1. *Incidence and Prevalence*

2.2.2.1.1 *Incidence*

Although all species of birds are susceptible to pasteurellosis, turkey, ducks and geese are more susceptible than fowl and adult birds seem to be more susceptible than younger stock. The immune status of the bird gives protection against the strain of organism with which they have previous contact, but birds are often susceptible to other strains. The carrier birds, clinically diseased birds and their excretions, carcasses of birds which have died of infection and rats act as source of infection (Jordan and Pattison, 1996).

Petersen *et al.* (2001) proved that *P. multocida* subsp. *multocida* strain isolated from fowl cholera outbreak in wild birds was highly virulent for turkeys, partridges and pheasants, while chickens were more resistant when given

experimentally as intra tracheal challenge, thereby underlining the importance of wild birds as a reservoir for *P. multocida*.

Chronically infected birds appeared to be the major source of infection. Transmission of organism through eggs occur seldom (Glisson *et al.*, 2003).

Molecular epidemiological investigation of outbreaks of fowl cholera in geographically related poultry flocks conducted by Kardos and Kiss (2005) had shown that most strains isolated from epidemiologically related outbreaks showed genetic relatedness, as revealed by enterobacterial repetitive intergenic consensus-PCR and pulsed-field gel electrophoresis (PFGE), suggesting that the consecutive outbreaks were due to recurrences rather than reinfections.

2.2.2.1.2 *Prevalence of the Disease Outside India*

Fowl cholera outbreak in a flock of turkeys in USA produced a loss of over 68 per cent within six days (Alberts and Graham, 1948)

Carpenter *et al.* (1989) recovered 49 isolates of *P. multocida* from 11 cases of fowl cholera out breaks in turkeys and failed to isolate *P. multocida* from non outbreak cases even though isolation trials were conducted. Their results demonstrated that for purposes of disease control, meat birds in fowl cholera outbreak flocks should be considered carriers of potentially virulent *P. multocida* for the life of the flock.

Investigations on poultry diseases occurring in Bangladesh revealed that fowl cholera was implicated in 3.15 per cent of the cases (Talha *et al.*, 2001).

2.2.2.1.3 Occurrence in India

Mulbagal *et al.* (1972) recorded the incidence of fowl cholera in ducks in India as early as 1947.

Sambyal *et al.* (1988) reported isolation and characterization of *P. multocida* from an outbreak of fowl cholera in ducks in Srinagar, Jammu and Kashmir.

Antony (2004) reported isolation of 25 different isolates out of 157 samples originating from ducks and fowl in Kerala. Two isolates were from fowl and rest from ducks. Two biotypes were observed among the 27 isolates and they were *P. multocida* subsp. *Septica* and *P. multocida* subsp. *multocida.*

2.2.2.2 Clinical signs and lesions

According to Rhoades and Rimler (1989), pneumonia was common in turkeys infected with *P. multocida.* The other pathological changes observed were swollen liver with multiple small areas of coagulative necrosis, heterophilic infiltration of lungs and necrotic foci of spleen. Localized lesions were observed in chronic cases and tissues involved were sinuses, pneumatic bones, lungs, air sac, wattles, sternal bursa, foot pad, hock joints, peritoneal cavities, oviduct, meninges and conjunctivae.

Clinical signs varied in different forms of disease. In per acute form, birds were found to be dead in good bodily condition without any premonitory sign. In acute forms signs included depression, anorexia, mucous discharge from orifices, cyanosis and fetid diarrhea. In chronic form, the signs were depression, conjunctivitis and dyspnoea with more survivability (Jordan and Pattison, 1996).

2.2.2.3   Pathogenesis and virulence

Heddleston *et al.* (1964) stated that loss of ability of a virulent strain to produce the capsule resulted in loss of virulence.

Heddleston and Rebers (1975) extracted loosely bound endotoxin from *P. multocida* using cold formalinized saline solution and it was found to be nitrogen containing lipopolysaccharides. This endotoxin was found to be capable of inducing signs of acute fowl cholera when injected at functional amounts to fowl. The serologic specificity of the endotoxin was associated which lipopolysaccharide and free endotoxin was able to induce active immunity.

Roberts (1996) described that polysaccharide capsules were found on the surface of a wide range of bacteria. With Gram negative bacteria, the capsule lies outside the outer membrane and is composed of highly hydrated polyanionic polysaccharides. Polysaccharide capsules might mediate a number of biological processes, including invasive infections.

Boyce and Adler (2000) demonstrated that acapsular *P. multocida* were readily taken up by murine peritoneal macrophages and removed from blood spleen and liver, while wild-type capsulated bacteria were significantly resistant to phagocytosis and multiplied in body following intra peritoneal challenge in mice. Thereby they had unequivocally shown the presence of the capsule to be a crucial virulence determinant for *P. multocida.*

2.2.2.4   Isolation and Identification

2.2.2.4.1   Isolation

Pasteurella multocida was easily isolated from tissues such as liver, spleen, lungs and from the heart blood of birds which succumbed to the acute form of the

disease and from localized lesions in chronically affected birds. *Pasteurella multocida* is somewhat fastidious and isolation from clinical specimen is usually made on media containing five per cent sterile serum or blood. Usually bovine, ovine or equine blood is preferred (Rhoades and Rimler, 1989).

Rajini *et al.* (1995) isolated *P. multocida* from long bones, heart blood swabs, liver and lungs from dead birds on ovine blood agar.

Jayakumar (1998) isolated *P. multocida* from ducks from Kuttanad area of kerala on bovine blood agar.

Tissue or blood smear from septicaemic cases, stained by Giemsa or Leishman methods, may reveal large number of bipolar organisms (Quinn *et al.*, 2002).

Antony (2004) conducted isolation of *P. multocida*. Biomaterials collected from the birds were streaked on ovine/bovine blood agar. The petri plates were incubated at 37°C for 18-24 h in a candle jar. Following incubation, mucoid, convex, greyish-white and non-haemolytic colonies were obtained.

2.2.2.4.2   *Identification*
2.2.2.4.2.1 *Cultural Characters*

P. multocida was found to grow well in the temperature range of 12 to 43°C with an optimal temperature of 37°C (Smith and Philips, 1990).

Jayakumar (1998) isolated *P. multocida* from ducks by inoculating the clinical material into blood agar and incubating at 37^0 C for 24 hours under increased carbon dioxide tension. The colonies obtained were found to be smooth convex and non haemolytic.

Chung *et al*. (2001) used dextrose starch agar, supplemented with six per cent chicken serum, for determination of colony morphology of *P. multocida.*

Colonies of *P. multocida* were found, greyish shiny and non hemolytic on blood agar. Colonies of some pathogenic strains were mucoid due to production of thick capsules. The colonies had a subtle but characteristic sweetish odour. Most pathogenic *Pasteurella* sp. did not grow on Mac Conkey agar (Quinn *et al.,* 2002).

On primary isolation from birds with fowl cholera, *Pasteurella* colonies might be iridescent, sectored with various intensities of iridescence, or blue with little or no iridescence when observed with obliquely transmitted light. Iridescence is related to presence of a capsule (Glisson *et al.*, 2003).

2.2.2.4.2.2   *Biochemical Characterization*

Wijewardana *et al*. (1986) observed that out of a wide range of *Pasteurella multocida* isolates none fermented lactose, trehalose, or salicin while sugar fermentation tests were conducted for various sugars.

Bisgaard *et al*. (1991) used de-carboxylation of ornithine, production of acid from mannitol and indole production for the sub species classification of *Pasteurella multocida*

Kumar *et al*. (1996) conducted biochemical studies of 43 *Pasteurella multocida* isolates of animal and avian origin from India. All the isolates fermented dextrose, mannose and fructose and all were negative for inulin, lactose, salicin, maltose, rhamnose, inositol and dextrin.

2.2.2.4.2.3 *Serotyping and Serogrouping*

A typing system based on passive protection of mice by serum against live organisms was developed by Roberts (1947). Four types designated I, II, III and IV were recognized among 37 cultures. Type V was distinguished later by Hudson (1954). Isolates of *P. multocida* causing haemorrhagic septicaemia in cattle and buffalo were grouped in Type I.

Carter (1955) described passive haemagglutination of erythrocytes by capsular antigen as a method for specific capsular serogrouping. Five serogroups A, B, D, E and F had been reported in the Carter system for *Pasteurella multocida.*

Heddleston system of serotyping of *Pasteurella multocida* based upon gel diffusion precipitin tests employing heat extracted antigen and anti *Pasteurella* sera prepared in chicken was described by Heddleston *et al.* (1972) and sixteen serotypes (named 1 to 16) were recognized under this system.

According to Carter and Chengappa (1981) the Carter and Heddleston systems may be combined and used to designate serotype, so that a serotype would be designated by its capsular type, followed by its somatic type as determined by the agar gel precipitin test.

2.2.2.4.2.4 *Polymerase Chain Reaction (PCR)*

Lee *et al.* (1999) modified *Pasteurella multocida* specific PCR for detection of *P. multocida* from chicken alimentary tract even when only ten organisms were present in the sample.

Townsend *et al.* (2001) developed a *Pasteurella multocida* specific PCR (PM-PCR) that identified all subspecies of *P. multocida* viz., subsp. *multocida*, subsp. *gallicida* and subsp. *septica*, through specific amplification of an

approximately 460 bp DNA fragment within the KMT1 gene. Genomic subtractive hybridization of closely related *P. multocida* isolates had generated clones useful in distinguishing HS causing type B strains from other *P. multocida* serotypes. Oligonucleotide primer pair KTT72 and KTSP61 designed from the sequence of the clone 6b specifically amplified a DNA fragment from types B:2, B:5 and B: 2, 5 *P. multocida.*

Gautam *et al.* (2004) developed a PCR assay targeting the hyaC-hyaD gene, to identify strains of *P. multocida* belonging to serogroup A. A set of serogroup specific PCR primers amplified a 564 bp product from genomic DNA. This method detected as low as 10 ng of bacterial DNA and had a specificity of 100 per cent for *P. multocida* serogroup A.

Shivachandra *et al.* (2005) detected multiple strains of *P. multocida* in fowl cholera outbreaks by PCR-based typing. Their investigations of fowl cholera outbreaks in a poultry farm indicated that molecular methods of detection and typing were rapid in comparison with conventional methods for epidemiological investigations, although both methods were efficient in the identification and characterization of *P. multocida* strains.

2.2.2.5   Antibiotic sensitivity

Bauer *et al.* (1966) developed a single disc method based on the measurement of zones in the antibiotic susceptibility testing of bacterial isolates.

Morris *et al.* (1989) reported that pencillin had the greatest activity against *P. multocida* isolated from turkeys.

In a study conducted by Rammanath and Gopal (1993) *P. multocida* isolates of duck origin were found to be sensitive to chloramphenicol, chlortetracycline,

oxytetracycline, co-trimoxazole, nalidixic acid, gentamicin, nitrofurantoin, streptomycin, kanamycin and neomycin and to a lesser extent against polymyxin-B, penicillin G, amoxycillin, cloxacillin, lincomycin and vancomycin.

Williams and Horne (1993) reported a high pencillin sensitivity of 99.4 per cent among 176 isolates of *P. multocida.*

Antibiogram studies on forty-five avian strains of *P. multocida* showed that all the strains were resistant to streptomycin, lincomycin and trimethoprim and susceptible to ampicillin, penicillin, gentamicin, erythromycin, trimethoprim, nitrofurantoin and sulfanilamide (Diallo *et al.*, 1995).

Per acute fowl cholera is rapid and hence antibiotic treatment is rarely of value. In less acute forms a number of drugs have proved to be effective. In order to eradicate infection from premises, it is necessary to depopulate, to cleanse and disinfect buildings and equipments and to eradicate pathogen (Jordan and Pattison, 1996).

Antony (2004) found that out of 27 *P. multocida* isolates of avian origin, all the isolates were uniformly sensitive to enrofloxacin, chloramphenicol and pefloxacin. Seven isolates representing 25.92 per cent of total number of isolates were resistant to co-trimoxazole.

2.2.3 *Staphylococcus* sp.

Members of genus *Staphylococcus* are Gram positive cocci (0.5-1.5 μm) that occur singly, in pairs, tetrads, short chains and irregular grape like clusters. They were non motile and non spore forming and most species demonstrate catalase activity and are facultative anaerobes, growing better under aerobic conditions than anaerobic conditions (Kloos, 1998).

2.2.3.1   Incidence and Prevalence.

Derriese *et al.* (1994) reported isolation of *Staphylococcus intermedius* from the lungs of canaries. They described that the species is rarely isolated from lesions in canaries.

Devriese *et al.* (1994) isolated *Staphylococcus aureus* and *Staphylococcus hyicus* from the lungs of birds showing dyspnoea and sudden death.

In a study conducted by Dias and Montali (1994) mortalities of 1.7% was reported due to staphylococcosis in wild waterfowl kept in captivity.

Kawano *et al.* (1996) isolated methicillin-resistant coagulase-negative *Staphylococcus sciuri*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* from the nares and skin of one to eight week old healthy chickens in three flocks from a farm. According to them, although coagulase-negative staphylococci (CoNS) in chickens had generally been accepted as harmless inhabitants, they manifested pathogenicity under suitable conditions, thereby producing opportunistic infections.

There are 32 species currently recognized in the genus *Staphylococcus* and members of same species demonstrate relative DNA binding values of generally 70 per cent or greater when reactions performed at optimal criterion and much lower relative binding percentage at stringent criterion (Kloos, 1998).

2.2.3.2   Clinical Signs and Lesions

Sandhu (1988) observed septicaemic infection among white peckin duckling caused by *Staphylococcus faecium*, with mortalities of 0.5-5 per cent. The gross lesions observed were primarily hepatomegaly and enlarged necrotic spleens.

Younus (1996) observed that necrosis and vascular congestion of lungs could occur in staphylococcal infections.

2.2.3.3 Pathogenesis and Virulence

2.2.3.3.1 Pathogenesis

Septicaemia has been the most common sequel to a primary load staphylococcal focus. It could result in sudden death with marked congestion of the carcass and appearance of hemorrhages and necrotic foci in the liver, lungs, spleen and myocardium. Endocarditis may also occur (Jordan and Pattison, 1996).

Staphylococcus sp. could readily and reversibly convert lactic acid and lactate to pyruvic acid, thereby allowing them to enter pathways of pyruvate metabolism and assimilation which in turn enabled them to survive and multiply on skin, where lactic acid and lactate are the major available carbon sources (Kloos, 1998).

2.2.3.3.2 Virulence

Many studies have been initiated on CoNS since 1958 as a result of the growing recognition that CoNS are clinically important (Smith *et al.*, 1958).

Quinn *et al.* (2002) has described that coagulase promoted conversion of fibrinogen to fibrin and fibrin production might shield staphylococci from phagocytic cells. So coagulase production by *Staphylococcus* acts as an important indicator of pathogenicity. According to them the additional markers of pathogenicity are DNAse activity and protein A production.

Pak *et al.* (1999) reported pathogenicity testing of *Staphylococcus aureus* isolates by intra peritoneal inoculation of 0.5ml of 10^8 -10^9 organisms suspended in

phosphate buffered saline to mice and observing them for a period of 15 days for death

The results of the study conducted by Turkyilmaz and Kaya (2005) revealed that CoNS isolated from animals had virulence factors and might have an important role in the pathogenesis of infections.

2.2.3.4   Isolation and Identification.

2.2.3.4.1   Isolation

Vanenk and Thompson (1992) used Oxacillin-containing mannitol-salt-based selective and differential medium for isolation of Methicillin resistant *Staphylococcus aureus* (MRSA) isolates from respiratory specimens. They recovered 45 per cent more isolates than on non selective media alone.

Among 79 isolates of *Staphylococcus* sp. isolated by   Awan and Matsumoto (1998) from poultry 77 were coagulase-negative. Major species of CoNS isolated by them were *Staphylococcus lentus* (19 per cent), *Staphylococcus simulans* (18 per cent), *Staphylococcus cohnii* (13 per cent), *Staphylococcus gallinarum* (10 per cent), and *Staphylococcus captis* (7 per cent).

Staphylococci from a variety of clinical specimens could be isolated in primary culture on 5 per cent blood agar, following an incubation period of 18-24 h at $35^\circ - 37^\circ$ C. By that time most species produced circular, smooth, raised well isolated colonies of one to three millimeter in diameter, with butyrous consistency (Kloos, 1998).

2.2.3.4.2 Cultural and Biochemical Characterization

Gemmell and Dawson (1982) used API Staph System for identification of 100 strains of CoNS isolated from various body sites as the primary etiological agent of clinical infection. The increasing importance of staphylococci and their resistance to antibiotics provided the rationale for such an investigation. Over 90 per cent of the *Staphylococcus* sp. isolates were easily identified to species level on the basis of their reaction profile to 19 biochemical tests included in the kit. The isolates identified were *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus cohnii, Staphylococcus warneri, Staphylococcus xylosus and Staphylococcus saprophyticus.*

Staphylococcus aureus were usually hemolytic and often produced both the alpha and beta hemolysin and so exhibited double haemolysis. Also coagulase test, DNAse test, haemolysis, pigment production, alkaline phosphatase test, urease test, fermentation of mannitol, maltose and esculin hydrolysis, could be used for characterization of *Staphylococcus* sp. (Quinn *et al.*, 1994).

Most staphylococci are non motile, non sporulating, oxidase negative facultative anaerobes which are catalase positive. Coagulase production correlates with pathogenicity. Although CoNS were usually of low virulence, some occasionally caused disease in animals and man. (Quinn *et al.*, 2002)

2.2.3.4.3 Molecular Characterization

Brakstad *et al.* (1992) had reported the rapid detection of *Staphylococcus aureus* by PCR of the *nuc* gene.

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The genetic heterogeneity among different *Staphylococcus aureus* isolates using random amplified polymorphic DNA (RAPD) technique and PFGE was demonstrated by Ahmed *et al.* (1998).

Palomares *et al.* (2003) reported the rapid detection and identification of *Staphylococcus aureus* from culture specimens using real time fluorescence PCR.

2.2.3.4 *Antibiotic Sensitivity*

Gemmell and Dawson (1982) isolated 100 strains of coagulase-negative staphylococci from various body sites as the primary etiological agent of clinical infection. Antibiogram studies showed general resistance to sulfamethoxazole and nalidixic acid among all strains and resistance to penicillin was widespread among strains of several species.

The major cause of the drug resistance is ascribed to the production of penicillin-binding protein (PBP), which is encoded by the *mecA* gene which resulted in a low affinity for β-lactam antibiotics. Other than MRSA isolates, isolates of CoNS from patients, such as *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis* had been found to be methicillin resistant and to possess *mecA* and PBP 29. The methicillin resistance in those CoNS had been considered to be controlled by the same mechanism as that which controls methicillin resistance in MRSA (Pierre *et al.*, 1990).

Control of staphylococcosis was based on avoiding predisposing and debilitating factors and use of drugs such as the fluroquinolones, tetracyclines, streptomycin or pencillin. Antibiotic sensitivity tests should always be done to support flock medication (Jordan and Pattison, 1996).

Methicillin-resistant *Staphylococcus aureus* (MRSA) showing resistance to many β-lactam and other antibiotics isolates have been known to be a cause of serious nosocomial disease in man. The major cause of the drug resistance is ascribed to the production of penicillin-binding many β-lactam and other antibiotics (Kawano *et al.*, 1996).

Khan *et al.* (2000) demonstrated that the poultry *S. aureus* strains could transfer erythromycin resistance to human *S .aureus* strains by transposition and transformation. This indicated that the strains belonging to different ecosystems could contribute to the spread of antibiotic resistant genes.

According to Quinn *et al.* (2002), currently available vaccines are ineffective in preventing staphylococcal infections and hence, antibiotic susceptibility testing should precede treatments.

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and plastic ware of Tarsons were used in this study. All Sterile swabs, ready-made media, antibiotics and antibiotic discs were procured from M/S Hi-media Laboratories Private Limited, Mumbai, unless otherwise mentioned.

3.1 ISOLATION OF BACTERIA FROM RESPIRATORY TRACT

3.1.1 Collection of Samples

Samples were collected from birds maintained in farms attached to Kerala Agricultural University and cases of respiratory infections in poultry brought to Department of Veterinary Microbiology and Centre of Excellence in Pathology, College of Veterinary and Animal Sciences, Mannuthy for disease diagnosis. Birds showing respiratory signs were sacrificed, postmortem examination was conducted and samples such as nasal, tracheal and air sac swabs and lungs were collected after taking all sterile precautions. A total of 105 samples were collected by sacrificing birds showing clinical signs and 31 bacterial isolates were obtained from them.

3.1.2 Method of Collection of Sample

3.1.2.1 Nasal, Tracheal and Air sac Swabs

Sterile cotton swabs, supplied by Hi-media Laboratories Private Limited, Mumbai, were used for the collection.

Birds were sacrificed and postmortem examination was conducted. Nasal, tracheal and air sac swabs were collected by swabbing the area with sterile swabs under aseptic conditions in the vicinity of a gas burner. Immediately after collection, before drying, the swabs were used for streaking the media.

3.1.2.2 Lung Samples

Lungs showing congestion or pneumonic lesions were collected and placed in a sterile Petri dish. The dorsal surface of the lungs was cleaned using sterile cotton dipped in alcohol. The surface was then seered using a hot spatula and a cut was made superficially on the surface using sterile scissors. The sterile loop was inserted through the cut, rotated inside lung tissue, taken back and streaked the media.

3.2 MEDIA USED FOR ISOLATION

3.2.1 Materials

Isolation of causative bacteria was made by culturing on brain heart infusion agar (BHIA), Mac Conkey agar and blood agar.

3.2.1.1 Blood Agar

Defibrinated ovine blood was collected under aseptic conditions and was stored under refrigeration up to a maximum period of one week. After autoclaving, the melted BHIA was cooled to a temperature just above the temperature at which solidification of agar takes place (around 45^0 C). Sterile ovine blood was then added at a concentration of five to ten per cent. Melted agar was mixed well and transferred to petri dish up to a thickness of four millimeter under sterile conditions. The agar plates were incubated for 24 hours to check for any contamination, packed in polythene bags and stored under refrigeration.

3.2.1.2 Brain Heart Infusion Agar

Brain heart infusion agar base (Hi-media) was prepared as per the manufacturer's instructions.

3.2.1.3 Mac Conkey Agar.

Mac Conkey Agar base (Hi-media) was used and agar plates were prepared as per the instructions of the manufacturer.

3.3 IDENTIFICATION OF BACTERIA

3.3.1 Morphology , Staining and Colony Characters

All the procedures were followed as described by Barrow and Feltham (1993).

3.3.2 Biochemical Identification of Isolates

Unless otherwise indicated, cultures were incubated at 37°C and the method followed was as described by Barrow and Feltham (1993).

3.3.2.1 Acid Production from Carbohydrates

The disc of the sugar to be tested was added to five milliliters of Andrade's peptone water taken in a test tube and it was inoculated with the test organism. Incubated the test tube at 37°C for five days. Acid production is indicated by the change in the colour of the medium to red.

3.3.2.2 Citrate Utilization Test

The test organism was inoculated as a single streak over the surface of the slope of Simmons' citrate medium slant, incubated at 37°C and examined daily for up to seven days for growth and colour change.

Blue colour and streak of growth   -   citrate utilized

Original green colour   -   citrate not utilized

3.3.2.3   Coagulase Test

0.5 ml. of undiluted rabbit plasma was mixed with an equal volume of an 18-24 hour broth culture of organism and incubated at 37°C for four hours. It was examined after one hour and four hours for coagulation. If negative, the tubes were left at room temperature overnight and then re-examined. A positive result is indicated by definite clot formation; granular or ropy growth is regarded as doubtful and the organism should be retested.

3.3.2.4   Decarboxylase Reactions

The organism taken from a plate culture with a straight wire was heavily inoculated, into tubes of the four media (arginine, lysine, ornithine and control) through the paraffin layer. It was then incubated at 37°C and examined daily for upto five days for any colour change. The media first becomes yellow due to acid production from the glucose; later, if decarboxylation occurs, the medium becomes purple. The control should remain yellow. With non-fermentative organisms, no acid (or insufficient acid) is produced from glucose and there is no change in colour of the media to yellow.

3.3.2.5   Growth on Mac Conkey Agar

The test organism was inoculated on Mac Conkey agar and incubated at 37^{0}C for 24 hours and observed for any growth. Lactose fermenters will produce pink colonies and non lactose fermenters will produce pale colonies on Mac Conkey agar. The indicator used was neutral red which turns pink in acidic pH produced by organic acids generated by fermentation of lactose incorporated in agar.

3.3.2.6   Indole Production

Peptone water was inoculated and incubated at 37°C for 48 hours. 0.5ml of

Kovacs reagent was added, shaken well and examined after about one minute. A red colour in the reagent layer indicates indole production.

3.3.2.7 Methyl red (MR) reaction

Glucose Phosphate peptone water was inoculated and incubated at 37°C for two days. Two drops of methyl red solution was added, shaken well and examined. A positive MR reaction is shown by the appearance of a red colour. An orange or yellow colour should be regarded as negative.

3.3.2.8 Motility

Young broth cultures of the organism were incubated at 37°C and examined in hanging drop preparations using a high-power dry objective with reduced illumination and observed for motility.

3.3.2.9 Nitrate Reduction

Inoculated Nitrate Broth lightly and incubated at 37°C up to five days. Added one ml of 'Nitrate reagent A' followed by one ml of 'Nitrate reagent B'. A deep red colour shows the reduction of nitrate to nitrite thereby indicating a positive reaction.

To tubes not showing a red colour within five minutes added powdered zinc (upto five milligram per milliliter of culture) and allowed to stand. Formation of red colour indicates that nitrate is present in the medium (i.e., not reduced by the organism). Absence of red colour indicates absence of nitrate in the medium (i.e. reduced by the organisms to nitrite, which in turn is itself reduced).

3.3.2.10 Oxidation or Fermentation of Glucose

The OF medium was steamed to remove dissolved air, and quickly cooled just before use and then stab-inoculated duplicate tubes with a straight wire. To one of the tubes added a layer of melted soft paraffin (petrolatum) to a depth of about three centimeters above the medium to seal it from air. The test tubes were incubated at 37 ^{0}C and examined daily for up to 14 days and read the results according to the following table.

Results	Open tube	Sealed tube
Oxidation	yellow	green
Fermentation	yellow	yellow
No action on Carbohydrate	blue or green	green

3.3.2.11 Phosphatase Test

Phenolphthalein phosphate agar was lightly inoculated to obtain discrete colonies, incubated at 37°C for 18 hours. 0.1 ml. ammonia solution (sp. gr. 0.880) was placed in the lid of the petri dish and inverted the medium above it. Free phenolphthalein liberated by phosphatase reacts with the ammonia and phosphatase positive colonies become bright pink.

3.3.2.12 Urease Activity

The slope of Christensen's urea agar slant was inoculated heavily with the test organism. Incubated at 37°C and examined after four hours and daily for five days. Red colour indicates positive reaction.

3.3.2.13 Voges – Proskauer Reaction; The Acetyl Methyl Carbinol Production (VP)

After completion of the MR test added 0.6 milliliter of five per cent α-naphthol solution and 0.2 ml 40 per cent KOH aqueous solution; shaken well, kept the tube in slanting position to increase the area of the air-liquid interface and examined after 15 minute and one hour. A positive reaction is indicated by a cherry red colour.

3.4   ANTIBIOGRAM

3.4. 1   Materials

Mueller–Hinton agar was used to study the antibiotic sensitivity pattern of the isolates. The G-1-plus OctoDiscs (OD-001) supplied by Hi media laboratories with known concentrations of antibiotics as noted in micrograms (µg) or international unit (IU) were used.

The antibiotics and its concentration per disc used in OctoDiscs were given below

1. Amoxycillin- clavulanic acid (Ac)-10µg
2. Cephalexin (Cp)-10µg
3. Ciprofloxacin (Cf)-10µg
4. Clindamycin (Cd)-2 µg
5. Cloxacillin (Cx)-1 µg
6. Co-Trimoxazole (Co)-25 µg
7. Erythromycin (E)-15 µg
8. Tetracycline-30 µg

The antibiotic discs used other than OctoDiscs are given below

1.   Ampicillin(A) - 10 μg

2.   Penicillin G (P) - 10 IU

3.   Enrofloxacin (Ex) - 10 μg

4.   Pefloxacin (Pf) - 5 μg

5.   Gentamicin (G) - 30 μg

6.   Streptomycin (S) - 10 μg

7.   Metronidazole (Mt) - 5 μg

8.   Nitrofurantoin (Nf) - 300 μg

9.   Chloramphenicol (C) - 30 μg

10.   Furazolidone (Fr) - 100 μg

11.   Bacitracin (B) - 10 IU

3.4.2   Method

Antibiotic sensitivity test was done as per the standard single disc diffusion method of Bauer *et al.* (1966) as described below.

1.   Sterile Mueller Hinton agar plates were prepared with a medium thickness of about four mm for rapidly growing aerobic organisms.

2.   Pure culture was used as inoculum. Three to four similar colonies were selected and transferred them into about five milliliters of tryptone soya broth. Incubated at 35°C for two to eight hours till light to moderate turbidity developed. The turbidity was adjusted to yield a uniform suspension containing $10^5 - 10^6$ cells/ml.

3.   A sterile non-toxic cotton swab was dipped on a wooden applicator into the standardized inoculum and rotated the soaked swab firmly against the upper inside wall of the tube to express excess fluid. Streaked the entire agar surface of the plate with the swab three times, turning the plate at 60°

angle between each streaking. The inoculum was allowed to dry for 5-15 minutes with lid in place.

4. The discs were applied using aseptic technique. Deposited the discs with centre at least 24 mm apart. Deposited OctoDiscs in the centre of the plate.

5. The plates were incubated immediately at 37°C and examined after 14-19 hours or later if necessary. The zones were measured showing complete inhibition and recorded the diameters of the zones to the nearest millimeter.

3.5 PLASMID PROFILE OF *E. coli* ISOLATES

3.5.1 Isolation of Plasmid DNA

3.5.1.1 *Materials*

3.5.1.1.1 *Luria Bertani Broth*

Yeast extract   5 g

NaCl   10 g

Tryptone   10 g

Distilled water to   1000 ml

Autoclaved at 121° C and 15 lb pressure for 15 min.

3.5.1.1.2 *1M Tris – HCl (pH 8.0)*

Tris base   12.11 g

Conc. HCl   0.2 ml

The above ingredients were dissolved in 90 ml triple distilled water. The volume was made up to 100 ml with distilled water and sterilized by autoclaving at 121^0 C at 15 lb pressure for 15 min.

3.5.1.1.3 3M Sodium Acetate Solution (pH 4.8)

Sodium acetate   40.81 g

Triple distilled water   70 ml

The pH was adjusted with glacial acetic acid and stored at 4^0 C.

3.5.1.1.4   1N NaOH

NaOH   4 g

Triple distilled water to   100 ml

Stored at room temperature.

3.5.1.1.5   Tris EDTA Glucose (TEG) Buffer pH (8.0)

1M Tris   2.5 ml

Glucose (50mM)   9.008 g

EDTA (10mM)   2ml of 0.5 M EDTA

Distilled water was added to make it to 100 ml, autoclaved at 121^0 C at 15 lb pressure for 15 min and stored at room temperature.

3.5.1.1.6 Lyzozyme

Lyzozyme- 10mg

TEG buffer-1 ml

Distributed into aliquots and stored at $-20\ ^{0}C$.

3.5.1.1.7 Sodium Dodecyl Sulphate –NaOH (SDS- NaOH)

10 percent SDS   0.5 ml

1N NaOH   1.0 ml

Triple distilled water 3.5 ml

The solution was prepared fresh each time .

3.5.1.1.8   Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

Procured from M/s Sigma Fine Chemicals and was used as such.

3.5.1.1.9 Chloroform: Isoamyl Alcohol (24: 1)

Chloroform   24 ml

Isoamyl alcohol   1 ml

Mixed and stored in amber coloured bottles

3.5.1.1.10 Ethanol (Absolute)

Molecular grade absolute ethanol procured from Changashu Chemicals Company, China was used

3.5.1.1. 11 Ethanol 70 Percent

Ethanol (absolute)   70 ml

Distilled water   30 ml

Mixed and stored in amber coloured bottles

3.5.1.1.12   Ribonuclease A

Ribonuclease A   10 mg

Distilled water   2 ml

Distributed into aliquots and stored at ⁻20 ^{0}C

3.5.1.1.13 10 x Tris Borate Electrophoresis buffer (TBE)

Tris base-108.0 g

Boric acid-55.0g

EDTA-   9.3g

The chemicals were dissolved in 700 ml of distilled water. The pH was adjusted to 8.3 and made up to 1000 ml, autoclaved and stored at room temperature.

3.5.1.1.14   Tris EDTA Buffer (TE) pH (7.8)

Tris   1 ml of 1M Tris

EDTA   0.2 ml of 0.5 M EDTA

3.5.1.1.15   Gel Loading Buffer (6x)

Bromophenol blue 0.25 % - 50 mg.

Xylene cyanol FF   0.25 % - 50 mg.

Sucrose   40.0% - 08g.

Stirred well in 20 ml of distilled water and stored at 4 ^{0} C.

Distilled water was added to 100 ml, autoclaved at 121^0 C at 15 lb pressure for 15 min and stored at room temperature.

3.5.1.2   Method

Pure culture of *E coli* was inoculated in 10 ml of Luria Bertani broth and incubated at 37 °C for eight hours.

The broth was centrifuged at 8000 x g for 15 min. Resulting pellet was resuspended in 100 µl of TEG buffer containing lysozyme to a final concentration of 10 mg/ml. The mixture was kept on ice for 15 min. This was followed by addition of 200 µl of SDS-NaOH and mixed gently until solution became translucent. The tube was further incubated on ice for 15 min. To this mixture added 150 µl of 3 M sodium acetate, mixed gently and incubated on ice for another 15 min. The mixture was

centrifuged at 12000 x g for 30 min at 4°C. Transferred the supernatant carefully to a fresh Eppendorf tube and added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mixed by gentle inversion for 10 min and centrifuged at 10,000 X g for 5 min. The aqueous phase was transferred to a fresh tube, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed gently by inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred into a fresh Eppendorf tube, added double the volume of ice-cold ethanol, mixed by inverting the tube several times and allowed the plasmid DNA to precipitate at -70°C overnight. The tube was then thawed and pelleted DNA by centrifugation at 10,000 x g for 15 min. The DNA pellet was washed twice with 70 per cent ethanol, dried and resuspended in 20 µl of TE Buffer. 2 µl of ribonuclease A (10mg/ml) was added and incubated at 37 °C for half an hour and then stored at ⁻ 20°C. Plasmid DNA from *E. coli* V517 maintained in the Department of Microbiology was prepared in a similar manner.

3.5.1.3   Electrophoresis

The isolated plasmid DNA was analysed by submarine gel electrophoresis using 0.8 per cent agarose gel in 1 x TBE buffer. Approximately 20 µl of plasmid DNA was mixed with 6 x gel loading dye and loaded into the wells. *Escherichia coli* V517 plasmid DNA was used to ascertain the size of the plasmids. Electrophoresis was carried out at 40 V till the dye reached near the end of the gel. DNA fragments were viewed on a transilluminator and photographed using a gel documentation system (Bio-Rad, USA).

3.5.1.4 Determination of size of plasmids

The bands obtained for the plasmid DNA of *E. coli* V517 was used as standard to ascertain the size of the plasmids. Size of the plasmids was determined using the software Bio-Rad Quantityone (Version 4.2) provided by Bio-Rad, USA.

3.5.1.4 Dendrogram

A dendrogram was prepared based on plasmid profile using the software NTSYS.

Results

4. RESULTS

4.1. ISOLATION OF ORGANISM

A total of 105 samples were collected by sacrificing birds showing clinical signs. Samples comprised of nasal, tracheal and air sac swabs and lungs. All the samples were collected under sterile precautions and 31 bacterial isolates were obtained from them. Details are given in Table 1.

4.2. IDENTIFICATION OF ORGANISM

The identification of the isolates was done as described by Barrow and Feltham (1993) unless otherwise mentioned.

4.2.1 Identification of Gram Negative Bacteria

First stage identification of gram negative bacteria was done based on the following characters of the organism like shape, motility, growth in air, growth anaerobically, catalase, oxidase, O/F reaction. The isolates were identified up to the family level, the details of which were given in table 2 and 3.

Table 1. Details of sample collection

Isolate number	Species	Clinical signs or Symptoms reported	Isolated from
Ec-1	Fowl	Respiratory distress, droopiness	Lungs, Trachea
Ec-2	Quail	Sudden death	Lungs
Ec-3	Quail	Respiratory distress, droopiness , Sudden death	Lungs, Trachea
Ec-4	Duck	Respiratory distress, droopiness, Sudden death (50 % mortality)	Lungs
Ec-5	Fowl	Respiratory distress, droopiness	Lungs
Ec-6	Fowl	Respiratory distress, droopiness	Lungs
Ec 7	Fowl	Respiratory distress, droopiness	Lungs, Trachea
Ec 8	Quail	Respiratory distress, droopiness , Sudden death	Lungs
Es 9	Quail	Respiratory distress, droopiness	Lungs, Trachea
Ec 10	Duck	Respiratory distress, droopiness	Lungs
Ec 11	Fowl	Respiratory distress, droopiness	Lungs
Ec 12	Fowl	Droopiness , Sudden death	Lungs, heart blood
Pm 1	Duck	Respiratory distress , Sudden death	Lungs, heart blood
Pm2	Duck	Sudden death	Lungs, heart blood

Table 1. Continued

Isolate number	Species	Clinical signs or Symptoms reported	Isolated from
Pm3	Duck	Sudden death	Lungs, heart blood
Pm 4	Turkey	Sudden death without any clinical signs	Lungs, heart blood
S1	Fowl	Sudden death Respiratory distress, droopiness	Trachea, Air sac
S2	Fowl	Death, droopiness, Greenish diarrhea.	Lungs
S3	Fowl	Respiratory distress, death	Heart blood, Trachea,Air sac
S4	Fowl	Respiratory distress, sneezing	Lungs,Trachea Air sac
S5	Fowl	Respiratory distress,	Lungs, Air sac
S6	Fowl	Respiratory distress,	Lungs, Air sac
S7	Fowl	Respiratory distress, droopiness	Lungs Air sac
S8	Fowl	Respiratory distress, droopiness	Lungs, Air sac
S9	Fowl	Respiratory distress, droopiness	Lungs, Air sac
S10	Fowl	Respiratory distress, droopiness	Lungs, Air sac

Table 1. Continued

Isolate number	Species	Clinical signs or Symptoms reported	Isolated from
S11	Fowl	Respiratory distress, droopiness	Lungs, Air sac
S12	Fowl	Respiratory distress, droopiness	Lungs, Trachea, Air sac
S13	Fowl	Respiratory distress, droopiness	Lungs, Trachea Air sac
S14	Fowl	Respiratory distress, droopiness	Lungs, Trachea Air sac
S 15	Fowl	Respiratory distress, droopiness	Lungs, Trachea Air sac

Table 2. First stage identification of Gram negative bacteria

Test done	Shape	Motility	Growth in air	Growth anaerobically	Catalase	Oxidase	Acid from glucose	O/F	Family
Ec-1	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec-2	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec-3	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec-4	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec-5	R	'	+	+	+	'	+	F	Enterobacteriaceae
Ec-6	R	'	+	+	+	'	+	F	Enterobacteriaceae
Ec 7	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec 8	R	+	+	+	+	'	+	F	Enterobacteriaceae
Es 9	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec 10	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec 11	R	+	+	+	+	'	+	F	Enterobacteriaceae
Ec 12	R	+	+	+	+	'	+	F	Enterobacteriaceae

Isolates

R – Rod
F - Fermentative

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Table 2.   Continued.

Test done	Isolates			
	Pm 1	Pm 2	Pm 3	Pm 4
Shape	Co	Co	Co	Co
Motility	-	-	-	-
Growth in air	+	+	+	+
Growth anaerobically	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Acid from glucose	+	+	+	+
O/F	F	F	F	F
Family	Pasteurellaceae	Pasteurellaceae	Pasteurellaceae	Pasteurellaceae

Co - coccobaccillary or short rod
F - Fermentative

4.2.1.1 *Identification of Enterobacteria*

Identification of Enterobacteria up to species level was done by conducting a set of biochemical reactions, the details of which are given in table 3.

4.2.1.1.1 *Escherichia* **sp.**

A total of 12 *E. coli* isolates were obtained. All the isolates gave lactose fermenting rosy pink colonies on Mac Conkey agar (Fig.1). They gave a characteristic metallic sheen on EMB agar (Fig.2). The isolates were identified mainly based on the IMViC test for which they gave a result of Indole (+), Methyl red (+), VP (-) and Citrate (-). The other characters used for confirmatory identification were motility at 37^0C, Mac Conkey growth and biochemical tests urease production, H_2S production from TSI, ONPG, arginine dihydrolase (ADH), lysine decarboxylase, ornithine decarboxylase, nitrate reduction and haemolysis. Carbohydrate fermentation reactions were carried out for 21 different sugars. Details of characterization of *E. coli* isolates are given in tables 4. Five *E. coli* isolates *i.e.* Ec 3, Ec 4, Ec 5, Ec 10 and Ec 11 produced α- haemolysis on blood agar.

Table 3. Identification of *Enterobacteria*

Test done	Isolates											
	Ec-1	Ec-2	Ec-3	Ec-4	Ec-5	Ec-6	Ec 7	Ec 8	Ec 9	Ec 10	Ec 11	Ec 12
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Colony colour in Mac Conkey agar	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink
urease	-	-	-	-	-	-	-	-	-	-	-	-
H₂S from TSI	-	-	-	-	-	-	-	-	-	-	-	-
ONPG	+	+	-	+	+	+	+	+	-	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+
LDB	+	+	+	+	+	+	+	+	+	+	+	+
ODB	+	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+
Haemolysis (α, β or -)	-	-	α	α	α	-	-	-	-	α	α	-
Organism	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*

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Table 4. Carbohydrate fermentation reactions of *E. coli*

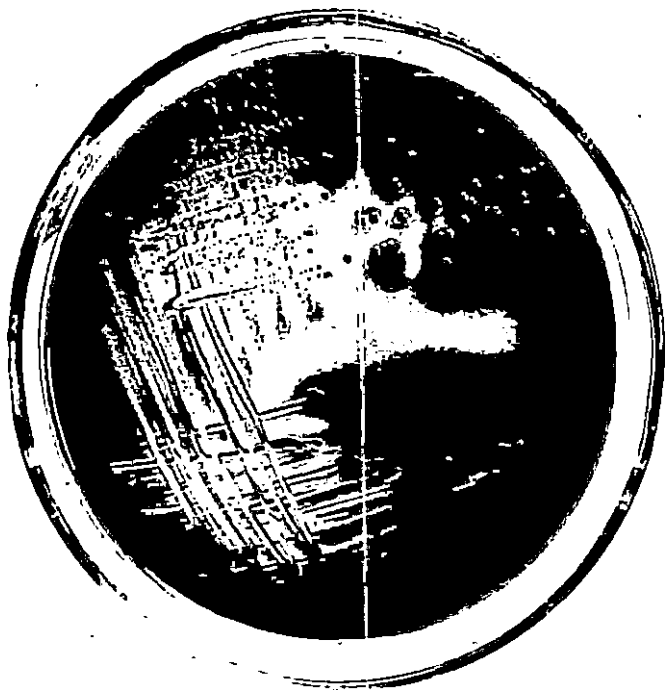| Carbohydrate utilized	Isolates											
	Ec-1	Ec-2	Ec-3	Ec-4	Ec-5	Ec-6	Ec 7	Ec 8	Ec 9	Ec 10	Ec 11	Ec 12
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+	+	-	+	+	+	+	+	+	+	+	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	+	+	-	-	-	+	-
Fructose	+	+	+	-	+	+	+	+	+	-	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	+	-	-	-	-	-	+	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	-	+	+	-	-	+	-	+	-
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	-	+	+	-	+	+	-	-	+	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+

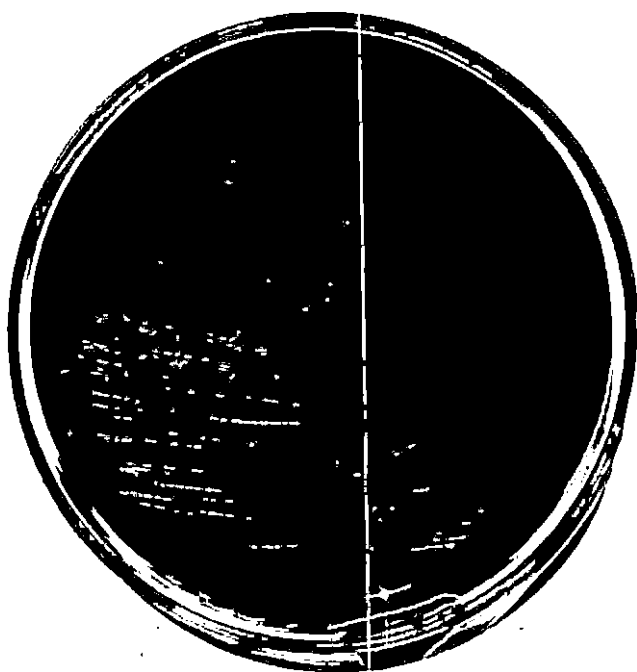Fig.1 *E. coli* on Mac Conkey agar

Fig.2 *E. coli* on EMB agar

4.2.1.2   Identification of Pasteurella sp.

The specimens collected for the isolation trials were tracheal swabs, heart blood and lung tissue.

The gross lesions observed during post mortem examination were epicardial petechiae; enlargement, congestion and pinpoint white multiple necrotic foci on the liver; congested spleen; hemorrhagic tracheitis and diffuse hemorrhages in internal organs like proventriculus, lungs and intestine.

Examination of the heart blood smears and impression smears from liver and spleen of the birds, stained with Leishman's stain revealed the presence of characteristic bipolar organisms.

Bio-materials collected from the birds were streaked on ovine blood agar. The petri plates were incubated at 37°C for 18-24 h in a candle jar. Following incubation, mucoid, convex, grayish-white ' and non-hemolytic colonies were obtained. A characteristic odour was observed when the petri plates were opened following overnight incubation at 37°C.

A total of four *Pasteurella* sp. isolates were obtained viz, Pm1, Pm 2, Pm 3 Pm 4. The isolates Pm1, Pm 2 and Pm 3 were isolated from cases of sudden death reported from outbreaks in ducks with a mortality percentage of 30. *Pasteurella multocida* 4 was isolated from turkey following a pasteurellosis outbreak that produced 40 per cent mortality in a turkey farm. Identification of *Pasteurella* sp. up to species level was conducted based on motility at 37^0C, Mac Conkey growth and biochemical tests like production of urease, H_2S production from TSI, ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, indole, MR, VP, Simmon's citrate, nitrate reduction, haemolysis on sheep blood agar and

carbohydrate fermentation reactions. Details of characterization are given in tables 5 and 6. All the isolates were found to be *P. multocida.*

The results of carbohydrate fermentation reactions show that Pm1, Pm 3 and Pm 4 isolates gave positive for sorbitol but negative for dulcitol fermentation and hence was biotyped as *Pasteurella multocida* subsp. *multocida.* The Pm 2 isolate gave negative reactions for both sorbitol and dulcitol and hence was biotyped as *Pasteurella multocida* subsp. *septica.*

When observed through a stereomicroscope, the colonies of all the four *P. multocida* isolates appeared to be translucent under directly transmitted light (Fig.3) and iridescent under obliquely transmitted light (Fig.4).

Table 5. Second stage identification of *Pasteurella* sp.

Test done	Isolates			
	Pm 1	Pm 2	Pm 3	Pm4
Motility	-	-	-	-
Maconkey growth	-	-	-	-
Urease	-	-	-	-
H₂S from TSI	-	-	-	-
ONPG	-	-	-	-
ADH	+	+	+	+
Lysine decarboxylase	+	+	+	+
Ornithine decarboxylase	+	+	+	+
Indole production	+	+	+	+
MR	-	-	-	-
VP	-	-	-	-
Simmons citrate	-	-	-	-
Nitrate reduction	+	+	+	+
Haemolysis	-	-	-	-
Beta-galactosidase activity	-	-	-	-
Organism identified	*Pasteurella multocida*	*Pasteurella multocida*	*Pasteurella multocida*	*Pasteurella multocida*

Table 6.   Carbohydrate fermentation reactions of *Pasteurella multocida*

Carbohydrate	Isolates			
	Pm 1	Pm 2	Pm 3	Pm 4
Glucose	+	+	+	+
Galactose	+	+	+	+
Inositol	-	-	-	-
Lactose	-	-	-	-
Maltose	-	-	-	+
Mannitol	+	+	+	+
Mannose	+	+	+	+
Salicin	-	-	-	-
Sucrose	+	+	+	+
Dulcitol	-	-	-	-
Sorbitol	+	-	+	+
Trehalose	-	-	-	+
Xylose	+	+	+	+
Arabinose	+	+	+	+

Fig. 3 *Pasteurella multocida* colonies under directly transmitted light in stereomicroscopic view (20 X)
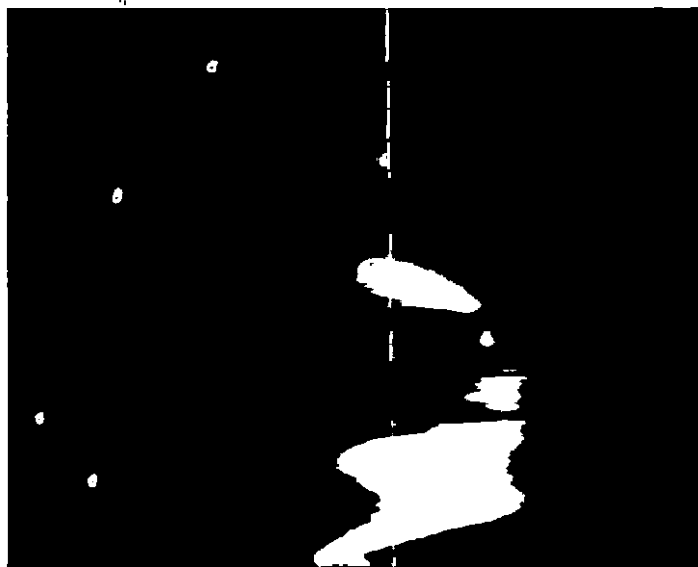

Fig.4 Iridescent *Pasteurella multocida* colonies under obliquely transmitted light in stereomicroscopic view (20 x)

4.2.2 Identification of Gram positive bacteria

First stage identification of gram positive bacteria was done based on the following characters of the organism like shape, acid fast staining, spore staining, motility, growth in air, growth anaerobically, catalase, oxidase, acid production from glucose and O/F reaction. The isolates were identified up to the family level. A total of 15 *Staphylococcus* sp. were isolated and identified, the details of which are given in table 7.

4.2.2.1 *Staphylococcus sp.*

Further identification of *Staphylococcus* sp. was made based on cultural characteristics and biochemical tests like growth anaerobically, oxidase, VP, coagulase, phosphatase, arginine, urease, novobiocin sensitivity, nitrate reduction tests, haemolysis on sheep blood agar, pigment production on BHIA and carbohydrate fermentation tests, the details of which are given in table 8 and 9. *Staphylococcus* isolates capable of fermenting mannitol *i.e.* is all isolates except S5 and S12 produced yellow colored colonies on mannitol salt agar (Fig.5). Colonies of S5 and S12 that are not capable of fermenting mannitol produced white colonies on mannitol salt agar (Fig.6).

On the basis of cultural characteristics and biochemical tests it was confirmed that, six isolates are *Staphylococcus simulans*, four isolates *Staphylococcus intermedius*, two *Staphylococcus gallinarum*, and one isolate each of *Staphylococcus lentus* and *Staphylococcus saccharolyticus* and *Staphylococcus chromogenes*. All the isolates were Gram-positive cocci, non motile, VP negative and nitrate reduction positive. The details are given in table 8 and 9.

Table 7. First stage identification of Gram positive bacteria

	Isolates														
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Test done Shape	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Acid fast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spores	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in air	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth anaerobically	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O/F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Genus	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*

C - Cocci, F - Fermentative

Table 8. Second stage identification of *Staphylococcus* sp.

Organism	Growth in MSA (colony colour)	Pigment production on BHIA	Haemolysis	Novobiocin	Urease	ADH	Nitrate	Phosphatase	Coagulase	VP	Oxidase	Growth anaerobically	Test done (Isolates)
Staphylococcus simulans	Yellow	-	-	S	+	+	+	+	-	-	-	+	S1
Staphylococcus intermedius	Yellow	-	-	S	+	+	+	+	+	-	-	+	S2
Staphylococcus simulans	Yellow	-	-	S	+	+	+	+	-	-	-	+	S3
Staphylococcus lentus	Yellow	-	-	R	-	+	+	+	-	-	+	+	S4
Staphylococcus saccharolyticus	White	-	-	S	-	+	+	+	-	-	-	+	S5
Staphylococcus simulans	Yellow	-	-	S	+	+	+	+	-	-	-	+	S6
Staphylococcus gallinarum	Yellow	-	-	R	+	+	+	+	-	-	-	+	S7
Staphylococcus simulans	Yellow	-	-	S	+	+	+	+	-	-	-	+	S8
Staphylococcus simulans	Yellow	-	-	S	+	+	+	+	-	-	-	+	S9
Staphylococcus simulans	Yellow	-	-	S	+	+	+	+	-	-	-	+	S10
Staphylococcus intermedius	Yellow	-	-	S	+	+	+	+	+	-	-	+	S11
Staphylococcus chromogenes	White	+	-	S	+	+	+	+	-	-	-	+	S12
Staphylococcus intermedius	Yellow	-	-	S	+	+	+	+	+	-	-	+	S 13
Staphylococcus intermedius	Yellow	-	-	S	+	+	+	+	+	-	-	+	S14
Staphylococcus gallinarum	Yellow	-	-	R	+	+	+	+	-	-	-	+	S15

Table 9.   Carbohydrate fermentation reactions of *Staphylococcus* sp.

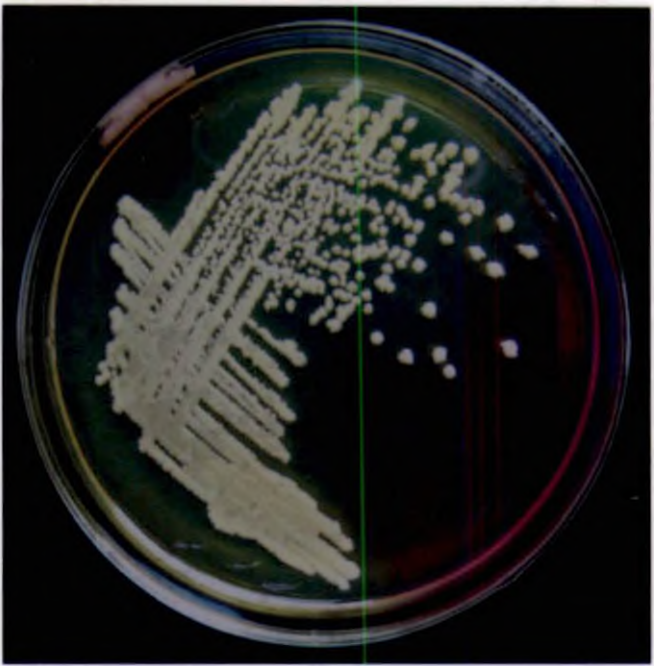| Carbohydrate	Isolates														
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S 13	S14	S15
Adonitol	-	-	+	+	-	-	-	+	-	-	+	+	-	+	+
Arabinose	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	+	-	+	-	-	-	+	-	-	-	+	+	+	-	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	-	+	+	+	-	+	+	+	+	+	-
Inositol	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+
Inulin	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+
Lactose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-
Mannitol	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mellibiose	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+
Raffinose	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-
Rhamnose	+	-	+	-	-	-	+	+	+	-	+	+	-	+	+
Salicin	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Sucrose	+	+	+	+	-	+	+	+	-	+	+	+	+	+	-
Trehalose	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	-	-	+	+	+	-	-	-	+	+	-	+

Fig.5 Mannitol fermenting *Staphylococcus* **sp.**
colonies on mannitol salt agar

Fig.6 Non mannitol fermenting *Staphylococcus* **sp.**
colonies on mannitol salt agar .

Staphylococcus gallinarum produced white colonies in BHIA (Fig.7). *Staphylococcus chromogenes* produced characteristic orange yellow pigment in BHIA (Fig.8).

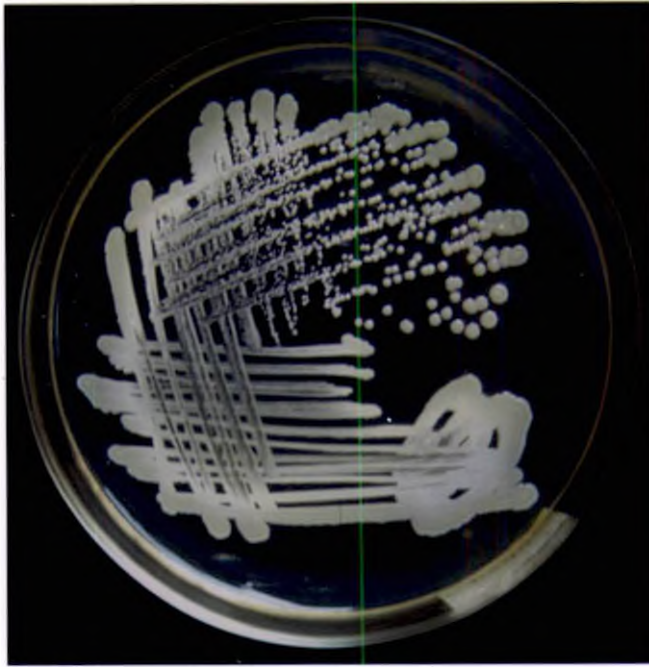All the *Staphylococcus* sp. isolates gave a positive Phosphatase test (Fig.9 and Fig.10).
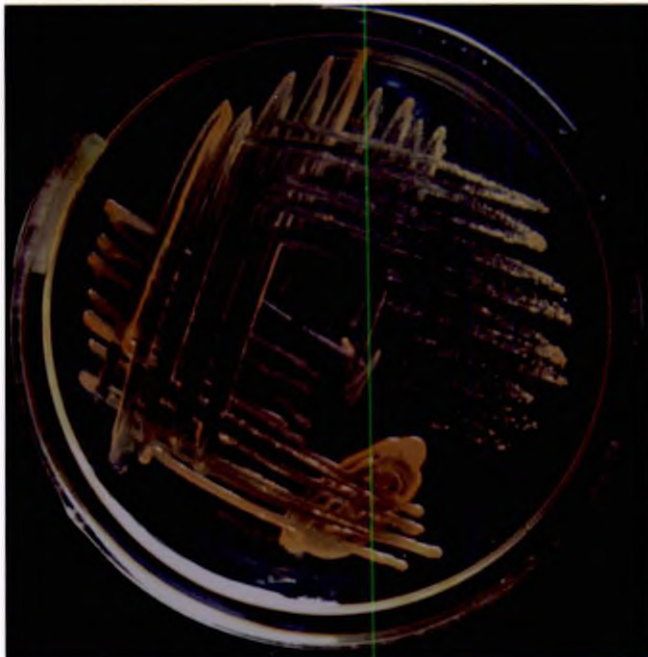
Fig.7 White colonies produced by
Staphylococcus gallinarum **on BHIA.**

Fig. 8 Orange yellow colonies produced by
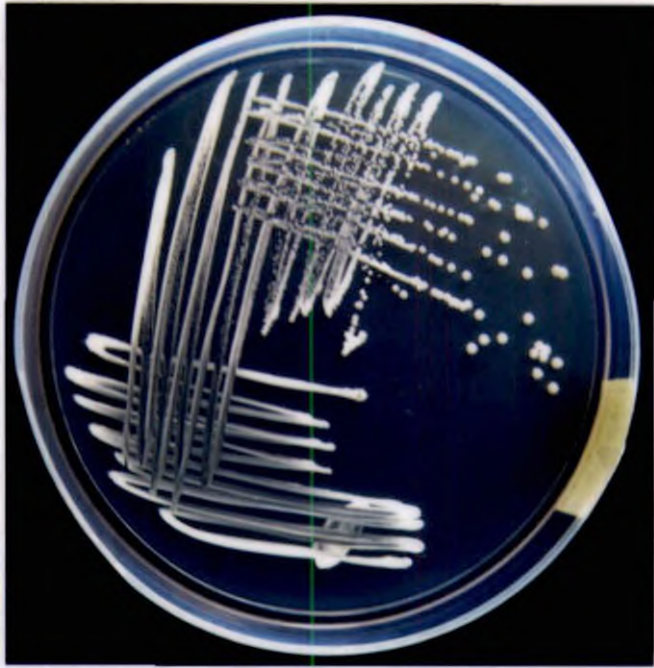*Staphylococcus chromogenes* **on BHIA.**

Fig. 9 *Staphylococcus* sp. colonies on
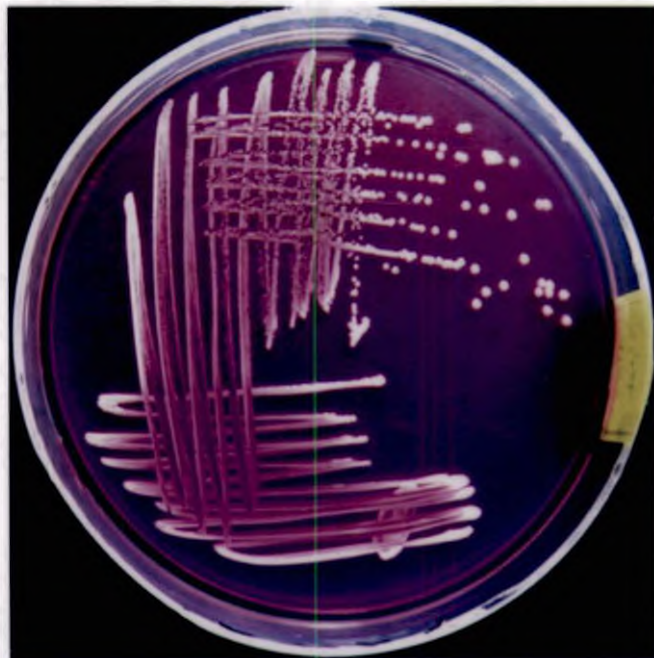phosphatase agar (before ammonia treatment).

Fig. 10 Pink *Staphylococcus* sp. colonies on
phosphatase agar (after ammonia treatment).

4.3. ANTIBIOGRAM

4.3.1 Antibiogram of *Escherichia coli*

Antibiogram of *Escherichia coli* isolates are given in table 10 and figure 11. The sensitivity shown by *E coli* isolates were cephalexin (83.33 per cent) amoxycillin-clavulanic acid (66.67 per cent), erythromycin (33.33 per cent), ciprofloxacin (16.67 per cent), clindamycin (16.67 per cent), tetracycline (16.67 per cent), cloxacillin (0 per cent) and co-trimoxazole (0 per cent). A low percentage of sensitivity was observed against the antibiotics like cloxacillin and co-trimoxazole, ciprofloxacin and clindamycin and a high percentage of sensitivity is observed against cephalexin. Isolate Ec 3 showed resistance to seven antibiotics tested (Fig.12). Multi drug resistance (resistance to at least three antimicrobials) was found among all *E. coli* isolates obtained in the study.

4.3.2 Antibiogram of *Pasteurella multocida*

Antibiogram of *Pasteurella multocida* was given in table 11 and figure 13. The antibiotic sensitivity obtained for *P. multocida* isolates were, chloramphenicol (100 per cent) enrofloxacin (100 per cent), furazolidone (100 per cent), pefloxacin (100 per cent), tetracycline (100 per cent), bacitracin (75 per cent), ciprofloxacin (75 per cent), co-trimoxazole (75 per cent), nitrofurantoin (75 per cent), penicillin (75 per cent), ampicillin (50 per cent), cloxacillin (50 per cent), gentamicin (50 per cent), streptomycin (0 per cent) and metronidazole (0 per cent).

Table 10.   Antibiogram of *E. coli* isolates

Isolate	Antibiotics								
	Ac	Cp	Cf	Cd	Cx	Co	E	T	Total resistant antibiotics
Ec 1	S	S	S	R	R	R	I	R	4
Ec 2	R	S	R	R	R	R	R	R	7
Ec 3	R	S	R	R	R	R	R	R	7
Ec 4	S	I	R	R	R	R	R	R	6
Ec 5	S	R	R	I	R	R	I	I	4
Ec 6	S	I	R	R	R	R	R	R	6
Ec 7	R	I	R	R	R	R	R	R	7
Ec 8	S	R	R	R	R	R	R	I	6
Ec 9	S	S	R	R	R	R	R	R	6
Ec 10	S	S	R	R	R	R	I	R	5
Ec 11	R	S	S	I	R	R	I	R	4
Ec 12	S	I	R	R	R	R	R	R	6
total no: of sensitive isolates	8	10	2	2	0	0	4	2	-
percentage sensitivity	66.67	83.33	16.67	16.67	0	0	33.33	16.67	-
percentage resistance	33.33	16.67	83.33	83.33	100	100	66.67	83.33	-

R – Resistant
S - sensitive

Table 11. **Antibiogram of** *Pasteurella multocida* **isolates.**

Antibiotic	Isolate						
	Pm 1	Pm 2	Pm 3	Pm 4	total no: of sensitive isolates	percentage sensitivity	percentage resistance
Ampicillin	R	R	S	S	2	50	50
Bacitracin	S	R	S	S	3	75	25
Chloramphenicol	S	S	S	S	4	100	0
Ciprofloxacin	S	R	S	S	3	75	25
Cloxacillin	R	S	S	R	2	50	50
Co-trimoxazole	S	S	S	R	3	75	25
Enrofloxacin	S	S	S	S	4	100	0
Furazolidone	R	R	R	S	1	25	75
Gentamicin	R	R	S	S	2	50	50
Metronidazole	R	R	R	R	0	0	100
Nitrofurantoin	R	S	S	S	3	75	25
Pefloxacin	S	S	S	S	4	100	0
Penicillin	R	S	S	S	3	75	25
Streptomycin	R	R	R	R	0	0	100
Tetracycline	S	S	S	S	4	100	0
Total sensitive antibiotics	7	8	12	11	-	-	-

R – Resistant
S - sensitive

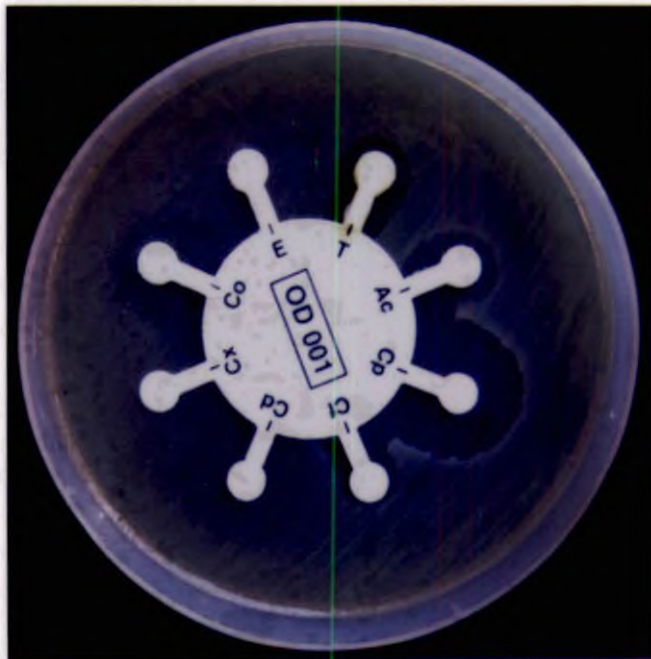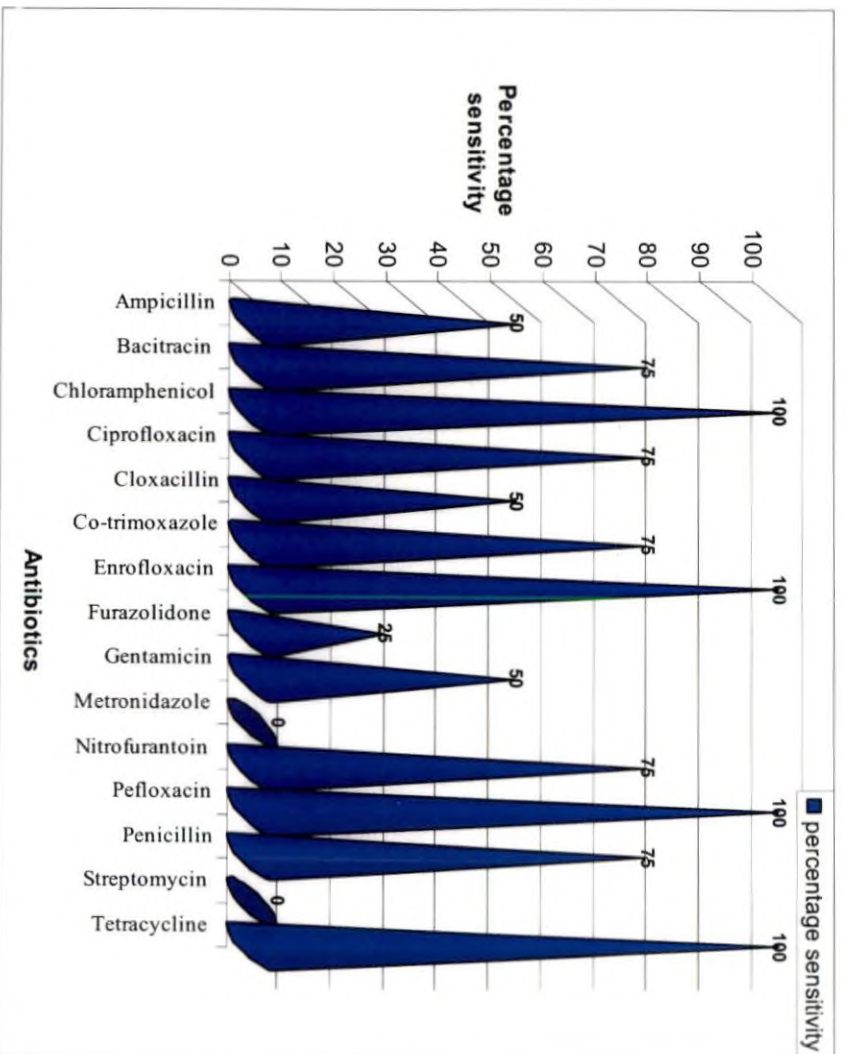**Fig 11. Percentage Sensitivity of *E. coli* Isolates to Antibiotics**
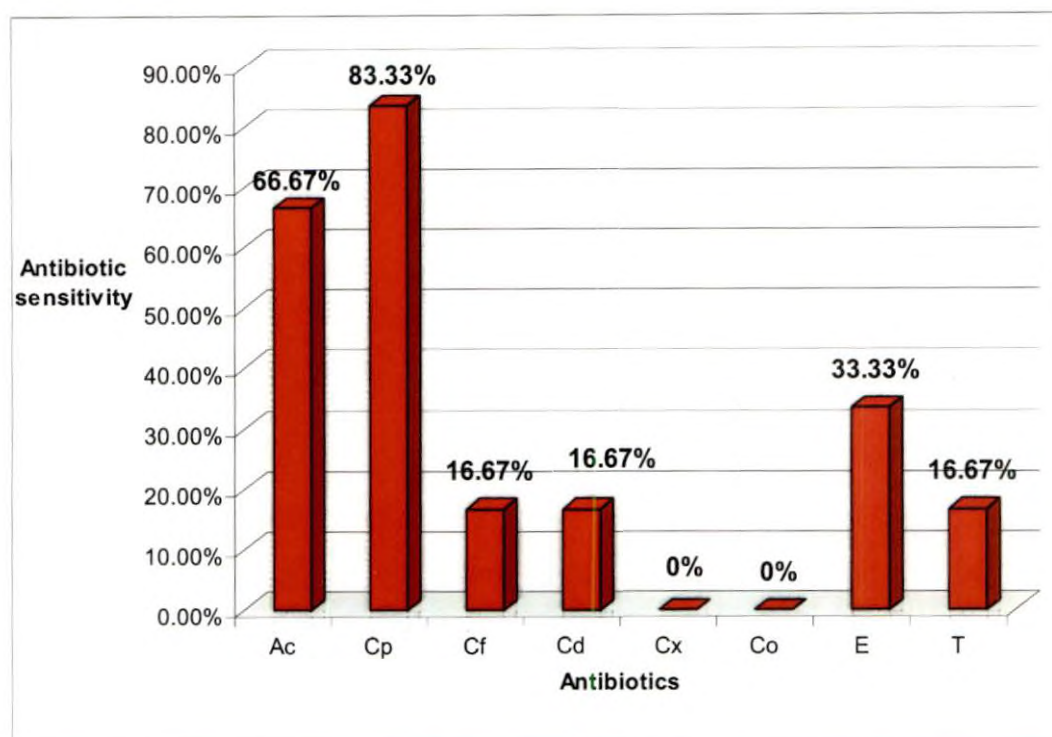
Fig.12 Antibiogram of *E. coli* 3 isolate showing resistance to seven antibiotics.

Fig 13. Percentage Sensitivity of *P. multocida* Isolates to Antibiotics

4.3.3 Antibiogram of *Staphylococcus sp.*

Antibiogram of *Staphylococcus* sp. was given in table 12 and figure 14. The sensitivity shown by *Staphylococcus* sp. isolates were amoxycillin-clavulanic acid (100 per cent), cephalexin (100 per cent), co-trimoxazole (93.33 per cent), clindamycin (86.67per cent), erythromycin (86.67 per cent), ciprofloxacin (73.33 per cent), tetracycline (66.67 per cent) and cloxacillin (13.33 per cent).

Out of 15 *Staphylococcus* sp. obtained all the isolates (100 per cent) were found to be sensitive to amoxycillin clavulanic acid (Ac) and cephalexin (Cp), 14 isolates (93.33 per cent) to found to be sensitive to co-trimoxazole, 11 isolates (73.33 per cent) to ciprofloxacin, 10 isolates (66.67 per cent) to tetracycline, 13 isolates (86.67 per cent) to both clindamycin and erythromycin and two isolates (13.33 per cent) to cloxacillin. Isolate S9 showed sensitivity to all the eight antibiotics (Fig.15)

4.4 PLASMID PROFILE OF *E. coli* ISOLATES

The plasmid DNA content of seven isolates of *Escherichia coli* (Ec 1 , Ec 2 , Ec 3, Ec 4, Ec 5, Ec 6 and Ec 7) was analyzed on agarose gel electrophoresis (Fig. 16). All the isolates harboured plasmids. The number of plasmids in each isolate and size of plasmids are given in table 13.

The number of plasmids varied from one to three and size varied from 1.54 K bp to 31.8 K bp.

A dendrogram was prepared based on the presence or absence of plasmids in *E .coli isolates* (Fig.17).
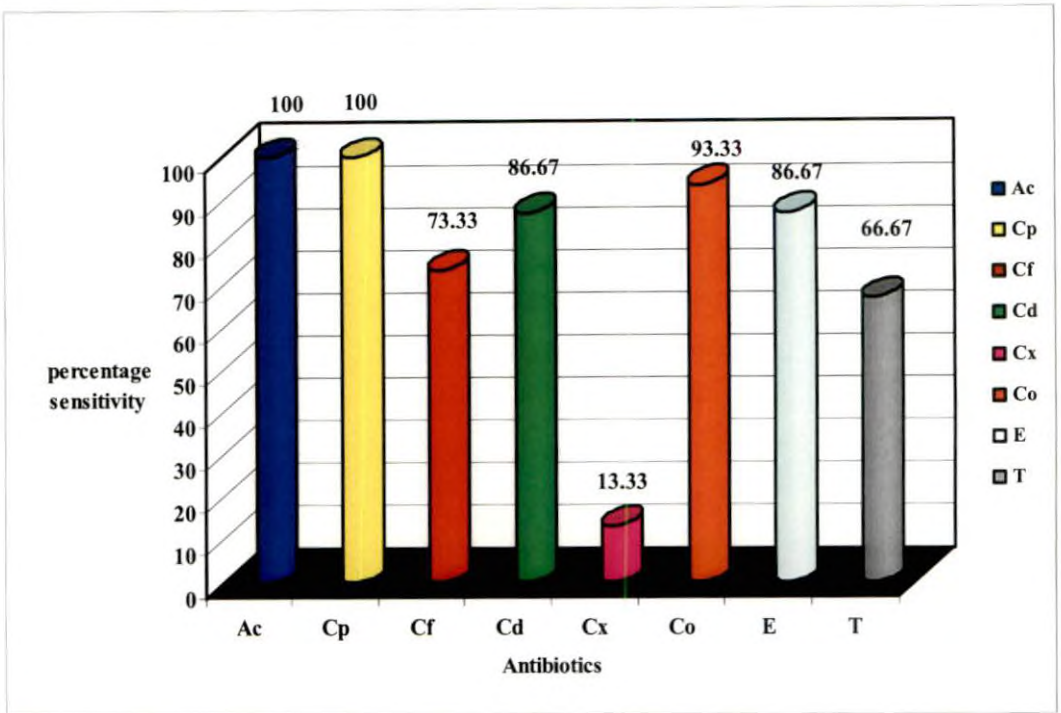
Table 12. Antibiogram of *Staphylococcus* sp.

Isolate no:	Antibiotics								
	Ac	Cp	Cf	Cd	Cx	Co	E	T	Total resistant antibiotics
S1	S	S	S	S	R	S	S	S	1
S2	S	S	R	S	R	S	S	S	2
S3	S	S	I	S	R	S	I	S	1
S4	S	S	I	S	R	S	I	S	1
S5	S	S	S	S	R	S	I	S	1
S6	S	S	S	S	R	S	I	S	1
S7	S	S	I	I	R	S	R	S	2
S8	S	S	I	S	R	S	I	R	2
S9	S	S	S	S	S	S	S	S	0
S10	S	S	I	S	R	S	I	R	2
S11	S	S	R	I	R	S	I	R	3
S12	S	S	S	R	R	R	I	R	4
S13	S	S	R	S	R	S	S	S	2
S14	S	S	R	S	R	S	S	S	2
S 15	S	S	S	R	S	I	R	R	3
Total sensitive isolates	15	15	11	13	2	14	13	10	-
Percentage sensitivity	100	100	73.33	86.67	13.33	93.33	86.67	66.67	-
Percentage resistance	0	0	26.67	13.33	86.67	6.67	13.33	33.33	-

Table 13. Plasmid profile of *E. coli* isolates

Sl no:	Isolate no:	Number of plasmids	Size in K Da	Number of resistant antibiotics
1	Ec 1	1	31.80	4
2	Ec 2	2	3.96 , 11.80	7
3	Ec 3	2	6.85 , 12.96	7
4	Ec 4	2	1.54 , 4.12	6
5	Ec 5	3	1.58 , 4.42 , 13.65	4
6	Ec 6	3	1.57 , 4.37 , 15.65	6
7	Ec 7	1	15.80	7

Fig 14. Percentage Sensitivity of *Staphylococcus sp.* Isolates to Antibiotics
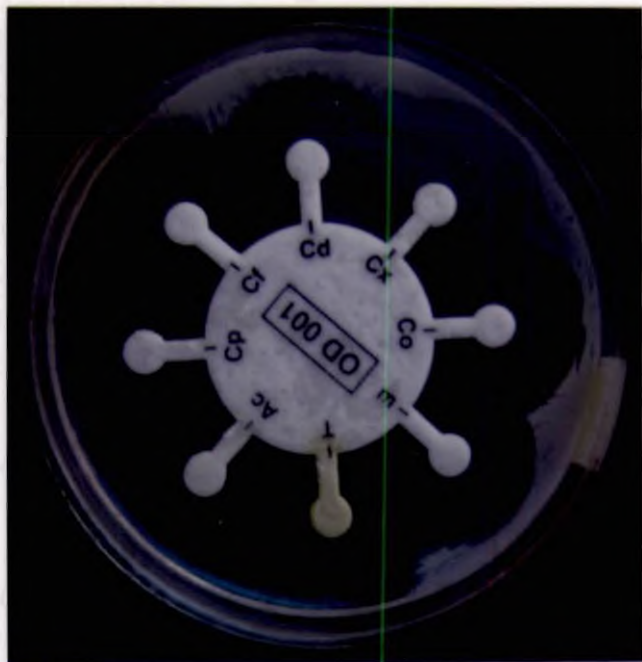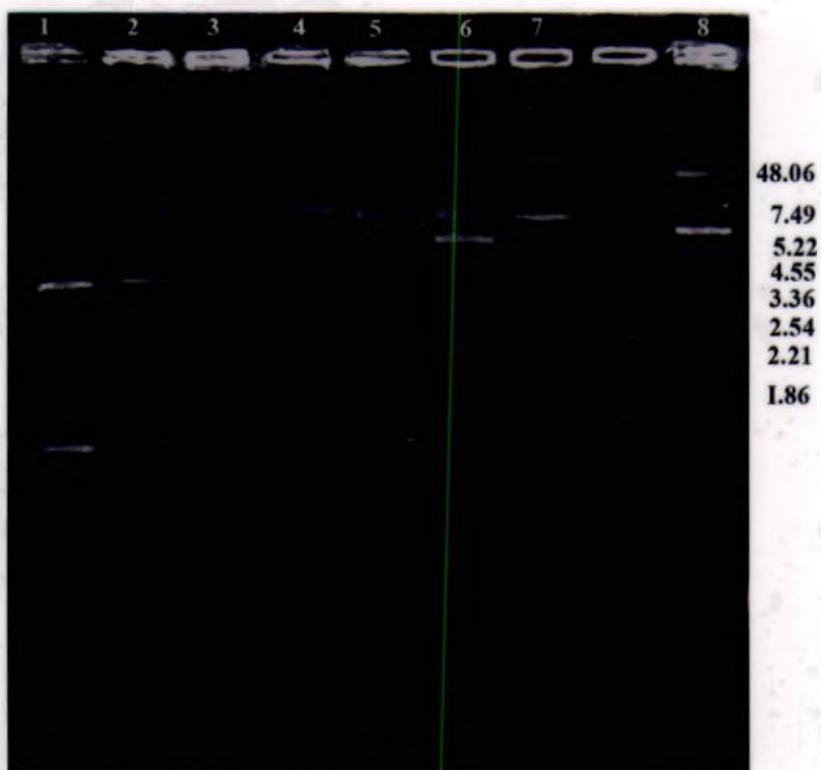
Fig.15 Antibiogram of *Staphylococcus simulans* (S-9) showing sensitiviy to eight antibiotics.

Fig.16. Plasmid profile of *E. coli* isolates

Lane 1 - Ec 4	Lane 5 - Ec 5
Lane. 2 - Ec 6	Lane 6 - Ec 3
Lane 3 - Ec 1	Lane 7 - Ec 2
Lane. 4 - Ec 7	Lane 8 - V517

Fig. 17. Dendrogram of *E. coli* isolates based on plasmid profile
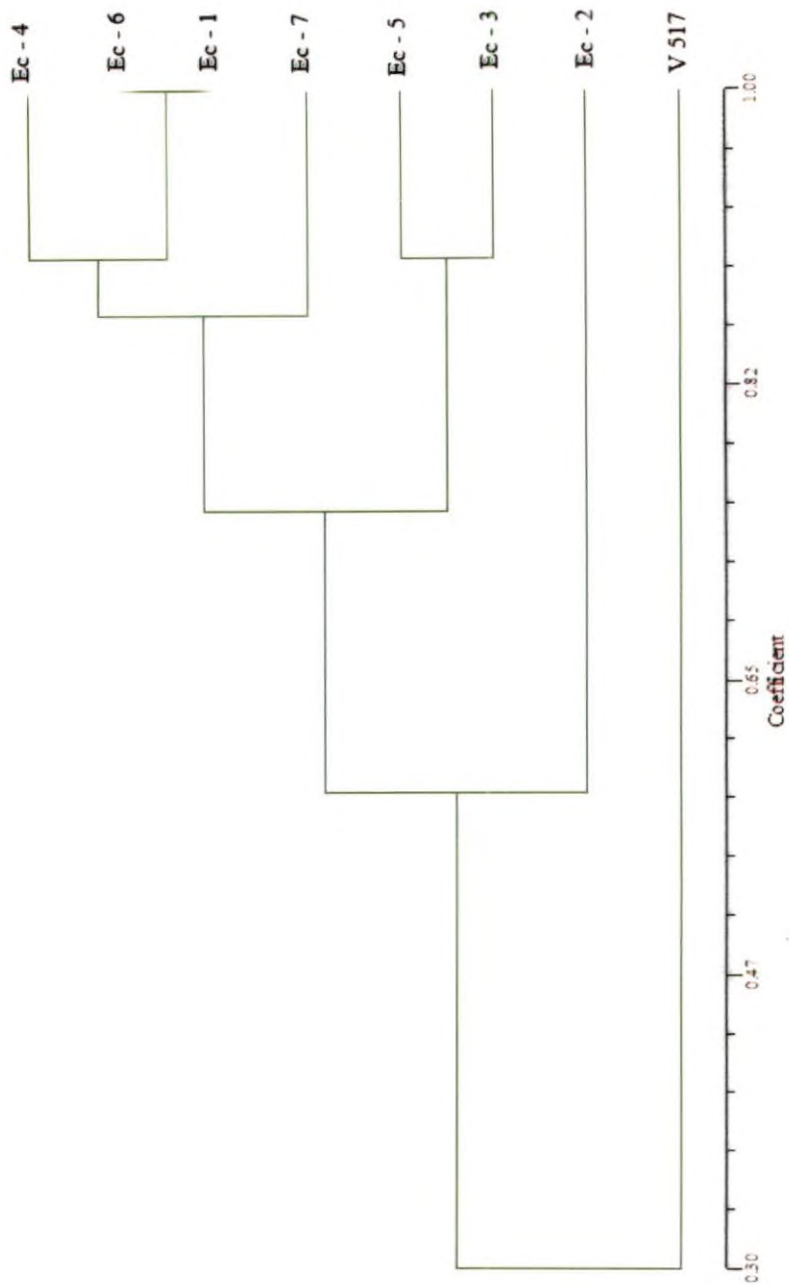
Discussion

5. DISCUSSION

5.1. ISOLATION OF ORGANISM

A total of 105 samples were collected by sacrificing birds showing clinical signs. Samples comprised of nasal swab, tracheal swab, air sac swab and lungs. All the samples were collected under sterile precautions and 31 bacterial isolates were obtained from them.

5.1.1 *Escherichia sp.*

A total of 12 different *Escherichia coli* isolates were obtained and identified. Similar studies were conducted by Manohar and Moorthy (2004). They obtained thirty two isolates of *E. coli* from 82 dead birds suspected for CRD from commercial farms in and around Bangalore. Pourbakhsh *et al.* (1997) concluded that both air sac and lung could act as the portal of entry for *E. coli* into systemic circulation, probably via damaged epithelium. In experimental studies he observed a gradual increase in bacterial counts in the trachea, lungs, air sac and liver following inoculation of pathogenic *E coli* into air sac. So in the *E. coli* infections observed in the study, the organism may have gained access to body via respiratory tract, colonizing the tract, causing respiratory disease and later might have entered systemic circulation causing colibacillosis.

All the *E. coli* isolates gave lactose fermenting rosy pink coloured colonies on Mac Conkey agar. They gave a characteristic metallic sheen on EMB agar. These isolates were identified mainly based on the IMViC test for which it gave a result of Indole (+), Methyl red (+), VP (-) and Citrate (-).The other biochemical tests used for confirmatory identification were motility, colony colour in Mac Conkey agar, urease, H_2S from TSI, ONPG, ADH, Lysine decarboxylase, Ornithine decarboxylase, nitrate reduction, haemolysis (α, β or -).

All the *Escherichia coli* isolates had the first stage and second stage biochemical identification tests and cultural characters consistent with the description by Barrow and Feltham (1993).

Carbohydrate fermentation reactions were carried out for 21 different sugars. The isolates showed mild variation in the sugar utilization tests. Barrow and Feltham (1993) explain that an indication of '+' sign in the identification table means 85-100 per cent are positive to that test and an indication of '-' sign means 0-15 per cent are positive to that test and the rest negative.

Five *E. coli* isolates Ec 3, Ec 4, Ec 5, Ec 10 and Ec 11 produced α-haemolysis on blood agar. α-Hemolysin of *E. coli* is an extra cellular toxin frequently produced by pathogenic isolates from human and animals (Cruz and Muller 1980). The haemolysis shown by five *E. coli* isolates obtained in the study could be an indication of its virulence.

5.1.2   *Pasteurella* sp.

A total of four isolates of *Pasteurella* sp. were obtained. All the isolates were obtained in blood agar by incubating at 37 ^{0} C under microaerophilic conditions provided by candle jar.

The gross lesions observed during post mortem examination were epicardial petechiae; enlargement, congestion and pinpoint white, multiple, necrotic foci of the liver; congested spleen; hemorrhagic tracheitis and diffuse hemorrhages in internal organs like proventriculus, lungs and intestine. Similar findings had been reported by Rhoades and Rimler (1989).

A total of four *Pasteurella sp.* isolates were obtained viz, Pm1, Pm 2, Pm 3 Pm 4. The isolates Pm1, Pm 2 and Pm 3 were isolated from cases of sudden death reported from outbreaks in ducks with a mortality percentage of 30. *Pasteurella multocida* 4 was isolated from turkey following a pasteurellosis outbreak that produced 40 per cent mortality in a turkey farm. No isolations of *P. multocida* could be made from respiratory infections among fowl. This reflects the high susceptibility of turkeys and ducks to pasteurellosis. According to Jordan and Pattison (1996) although all species of birds are susceptible to pasteurellosis, turkeys, ducks and geese are more susceptible than domestic fowl.

5.1.3   *Staphylococcus* sp.

A total of 15 *Staphylococcus* sp. were isolated and identified.

Sandhu (1988), isolated *Staphylococcus* from cases of air sacculitis and he could reproduce the disease in eight day old susceptible duckling by parenteral administration of organism.

On the basis of cultural characteristics and biochemical tests it was confirmed that, six isolates are *Staphylococcus simulans*, four isolates *Staphylococcus intermedius*, two *Staphylococcus gallinarum*, and one isolate each of *Staphylococcus lentus*, *Staphylococcus saccharolyticus* and *Staphylococcus chromogenes*. All the isolates were Gram-positive cocci, non motile, VP negative and nitrate reduction positive.

Four isolates *i.e.* S2, S11, S13 and S14 were coagulase positive, VP negative and produced yellow colonies in mannitol salt agar. According to Barrow and Feltham (1993) out of 26 *Staphylococcus* sp. described *Staphylococcus aureus, Staphylococcus intermedius* and *Staphylococcus hyicus* are the three coagulase positive species. And among coagulase positive Staphylococci, *Staphylococcus*

aureus gives a positive VP reaction and *Staphylococcus hyicus* gives negative result for mannitol fermentation test. As all the four coagulase positive isolates were VP negative and produced yellow colonies in mannitol salt agar, they were identified to be *Staphylococcus intermedius*.

Quinn *et al.* (2002) observe that coagulase production by *Staphylococcus* sp. acts as an important indicator of pathogenicity. So it could be concluded that four isolates of *Staphylococcus intermedius* obtained out of fifteen isolates (26.66 per cent) from respiratory tract of birds might be pathogenic organisms capable of inducing respiratory infection.

The eleven other isolates (73.33 per cent) were coagulase negative. The CoNS isolated were six isolates of *Staphylococcus simulans* (40 per cent), *two isolates* of *Staphylococcus gallinarum* (13.33 per cent), and an isolate each of *Staphylococcus lentus* (6.67 per cent), *Staphylococcus saccharolyticus* (6.67 per cent) and *Staphylococcus chromogenes* (6.67 per cent) were identified.

Among 79 isolates of *Staphylococcus* sp. isolated by Awan and Matsumoto (1998) from poultry 77 were coagulase-negative. Major species of CoNS isolated by them were *Staphylococcus lentus* (19 per cent), *Staphylococcus simulans* (18 per cent), *Staphylococcus cohnii* (13 per cent), *Staphylococcus gallinarum* (10 per cent), and *Staphylococcus captis* (7 per cent).

All the isolations were made from sacrificed birds showing clinical signs. As coagulase positive reaction directly indicates pathogenicity much importance was given only to coagulase positive *Staphylococcus* sp. Devriese (1990) reported that the presence of CoNS in animals has received little attention to date. Many studies have been initiated since 1958 as a result of the growing recognition that CoNS are clinically important (Smith *et al.*, 1958)

The results of the study conducted by Turkyilmaz and Kaya (2005) revealed that CoNS isolated from animals had virulence factors and might have an important role in the pathogenesis of infections. They concluded that CoNS species could be as dangerous as coagulase positive staphylococcus for both animals and humans. Considering these facts these CoNS isolates obtained cannot be regarded as mere resident flora and more studies including pathogenicity testing are required to establish its virulence.

This result of this study indicates that CoNS are more frequently isolated from staphylococcal infections, although they do not possess the virulent coagulase activity. So importance must be given to CoNS also, as given to coagulase positive staphylococcus and more studies are needed to find the virulence factors and role of them in producing infections in poultry.

Out of eleven CoNS, Six were identified as *Staphylococcus simulans* and all were from cases of clinical infection with respiratory signs. Gemmell and Dawson (1982) isolated CoNS like *Staphylococcus simulans* from various body sites as the primary etiological agent of clinical infection, indicating that the organism has virulence.

Staphylococcus chromogenes produced characteristic orange yellow colonies in BHIA. Quinn *et al.* (1994) reported that *Staphylococcus chromogenes* produce orange yellow pigment.

On mannitol salt agar all isolates except S5 and S12 produced yellow colonies, while colonies of S5 and S12 produced white colonies. The isolates S5 and S12 also gave a negative reaction for mannitol fermentation, and this may be the reason for white colonies produced in mannitol salt agar.

5.2   ANTIBIOGRAM

In vitro antibiotic susceptibility test was found to be the most ideal technique for the selection of effective antimicrobial agents in the treatment of respiratory diseases (Martel, 1996). He also opined that agar disc diffusion method was an inexpensive and flexible tool for determination of resistance of a panel of isolates.

5.2.1   Antibiotic Sensitivity of *E . coli* Isolates

The sensitivity shown by *E. coli* isolates were cephalexin (83.33 per cent) amoxycillin-clavulanic acid (66.67 per cent), erythromycin (33.33 per cent), ciprofloxacin (16.67 per cent), clindamycin (16.67 per cent), tetracycline (16.67 per cent), cloxacillin (0 per cent) and co-trimoxazole (0 per cent). A low percentage of sensitivity was observed against the antibiotics like cloxacillin and co-trimoxazole, ciprofloxacin and clindamycin and a high percentage of sensitivity was observed against cephalexin.

Similar observation was made by Giovanardi *et al.* (2005). They showed a high level of resistance to amoxycillin, enrofloxacin, tetracycline and trimethoprim-sulfamethoxazole among 39 *E. coli* O78 strains, and resistance to amoxycillin, enrofloxacin and tetracycline in 18 *E. coli* O139 isolates.

Multi drug resistance (resistance to at least three antimicrobials) was found among all *E. coli* isolates obtained in the study. Miles *et al.* (2006) also reported isolation of multi drug resistant *E. coli* from broilers. They observed a high frequency and proportion of multi drug resistance among avian isolates than in isolates from humans.

There is strong evidence that the use of antimicrobial agents can lead to the emergence and dissemination of resistant *E. coli* (Linton *et al.*, 1977). In intensively reared food animals, antibiotics may be administered to whole flocks rather than individual animals, and antimicrobial agents may be continuously fed to food animals such as broilers and turkeys as antimicrobial growth promoters. Therefore the antibiotic selection pressure for resistance is high in bacteria from poultry and consequently their faecal flora contains a relatively high proportion of resistant bacteria (Caudry and Stanisch, 1979).

Hence it could be concluded that the high level of resistance observed among poultry *E. coli* isolates obtained in the study might be due to incorporation of antibiotics in feed as growth promoters.

The tetracycline resistance observed was as high as 83 per cent. Miles *et al.* (2006) obtained a resistance of 82.4 per cent to tetracyclines among *E. coli* isolates from poultry, which lie closer to the value obtained in this study. The patterns of resistance to tetracycline have been attributed in part to widespread and lengthy use of tetracycline in the poultry industry (Piddock, 1996).

A resistance of 67 percent was observed against erythromycin in the study and it is lower than the percentage obtained (98 per cent) by Allan *et al.* (1993).

5.2.2   Antibiotic Sensitivity of *P. multocida*

The antibiotic sensitivity obtained for *P. multocida* isolates were, chloramphenicol (100 per cent) enrofloxacin (100 per cent), (100 per cent), (100 per cent), (100 per cent), bacitracin (75 per cent), ciprofloxacin (75 per cent), co-trimoxazole (75 per cent), nitrofurantoin (75 per cent), penicillin (75 per cent), ampicillin (50 per cent), cloxacillin (50 per cent), gentamicin (50 per cent), streptomycin (0 per cent) and metronidazole (0 per cent).

Seventy five percent of the isolates were found to be sensitive to penicillin. Morris *et al.*, (1989) reported that penicillin had the greatest activity against *P. multocida* isolated from turkeys. Williams and Horne (1993) reported a high penicillin sensitivity of 99.4 per cent among 176 isolates of *P. multocida.* Thus the penicillin resistance (25 percent) obtained is not consistent with the findings of other workers.

As 100 per cent sensitivity is shown to chloramphenicol, enrofloxacin furazolidone, pefloxacin, and, tetracycline these drugs may be used for treating pasteurellois in birds.

5.2.3 Antibiotic Sensitivity of *Staphylococcus sp.* Isolates

The sensitivity shown by *Staphylococcus* sp. isolates were amoxycillin-clavulanic acid (100 per cent), cephalexin (100 per cent), co-trimoxazole (93.33 per cent), clindamycin (86.67per cent), erythromycin (86.67 per cent), ciprofloxacin (73.33 per cent), tetracycline (66.67 per cent) and cloxacillin (13.33 per cent).

Out of 15 *Staphylococcus* sp. obtained all the isolates (100 per cent) were found to be sensitive to amoxycillin-clavulanic acid (Ac) and cephalexin (Cp), 14 isolates (93.33 per cent) were found to be sensitive to co-trimoxazole, 11 isolates (73.33 per cent) to ciprofloxacin, 10 isolates (66.67 per cent) to tetracycline, 13 isolates (86.67 per cent) to both clindamycin and erythromycin and two isolates (13.33 per cent) to cloxacillin.

A high level of resistance of 86.67 per cent was found against cloxacillin. Gemmell and Dawson (1982) found that resistance to penicillin was widespread among strains of several species of CoNS.

Amoxycillin clavulanic acid (Ac) and cephalexin (Cp) was found to be the most effective antibiotics against *Staphylococcus* sp. in the study.

5.3 PLASMID PROFILE OF *E. coli* ISOLATES

The plasmid DNA content of the seven isolates of *Escherichia coli* (Ec 1, Ec 2, Ec 3, Ec 4, Ec 5, Ec 6 and Ec 7) was analysed on agarose gel electrophoresis. All the isolates harboured plasmids.

The number of plasmids varied from one to three and size varied from 1.54 K bp to 31.8 K bp. Mini *et al.* (2005) reported that among plasmids isolated from 10 *E. coli* isolates, the number of plasmids varied from one to three in each isolate and the size of the plasmids ranged from 1.86 to 48.06 K bp and one isolate did not show any plasmid. The size range of plasmids they obtained lie more or less close to that obtained in this study.

Isolates Ec 1 and Ec 7 carried only one plasmid but showed resistance to four and seven antibiotics respectively out of eight antibiotics used in study. Isolate Ec 5 carried three plasmids but had shown resistance to only four antibiotics. These observations show that a correlation between the number of plasmids and antibiotic resistance could not be ascertained in this study.

The dendrogram prepared showed close relationship between the isolates Ec 1 and Ec 6 ; Ec 5 and Ec 3.

In conclusion, the results of this study provide evidence for significant antimicrobial resistance among bacterial isolates from birds. Long term prospective studies involving isolation, identification and antibiogram from more samples are required to identify novel pathogens causing respiratory disease in birds. Such

studies will provide data on temporal and spatial difference in antibiotic resistance patterns, which in turn will help the scientific community to design better disease control strategies.

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Summary

SUMMARY

Bacterial respiratory disease out breaks can occur either as a primary disease or as secondary complication to viral diseases, stress and other managerial faults. In all cases, the involvement of bacterial pathogens will increase mortality, decrease production and thereby cause economic losses. Therefore management of a sustainable poultry production system entails control of bacterial respiratory pathogens and it involves isolation and identification of the causative agent if present and the prevention of multiplication and spread of the same.

Considering the importance of these problems, this study was undertaken to isolate and identify of bacteria from the respiratory tract of poultry and to study the antibiogram of the isolates.

Samples were collected from birds maintained in farms attached to Kerala Agricultural University and cases of respiratory infections in poultry brought to Department of Veterinary Microbiology and Centre of Excellence in Pathology, College of Veterinary and Animal Sciences, Mannuthy for disease investigation. Birds showing respiratory signs were sacrificed, postmortem examination was conducted and samples such as nasal, tracheal and air sac swabs and lungs were collected after taking all sterile precautions.

A total of 105 samples were collected by sacrificing birds showing clinical signs. Isolation of causative bacteria was made by culturing on brain heart infusion agar, Mac Conkey agar and blood agar. For identification of isolates all the procedures were followed as described by Barrow and Feltham (1993). A total of 31 bacterial isolates were obtained from samples.

A total of 12 *E. coli* isolates were obtained and identified. All the *E. coli* isolates had the first stage and second stage biochemical identification tests and cultural characters consistent with the description by Barrow and Feltham (1993). The α- haemolysis shown by five *E. coli* isolates obtained in the study might be an indication of their virulence.

Four *Pasteurella* sp. isolates were isolated and identified. All the isolates were obtained in blood agar by incubating at 37 ^{0} C under microaerophilic conditions provided by candle jar. Identification of *Pasteurella* sp. up to species level was conducted based on biochemical tests and all the isolates were found to be *P. multocida.*

A total of 15 *Staphylococcus* sp. were isolated and identified. On the basis of cultural characteristics and biochemical tests it was confirmed that, six isolates are *Staphylococcus simulans*, four isolates *Staphylococcus intermedius*, two *Staphylococcus gallinarum*, and one isolate each of *Staphylococcus lentus* and *Staphylococcus saccharolyticus* and *Staphylococcus chromogenes*. All the isolates were Gram-positive cocci, non motile, VP negative and nitrate reduction positive.

Out of 15 *Staphylococcus* sp. isolated and identified 11 isolates (73.33 per cent) were coagulase negative. This result indicate that CoNS were more frequently isolated from staphylococcal infections, although they do not possess the virulent coagulase activity. So importance must be given to CoNS also, as given to CoPS and more studies need to be undertaken to find the virulence factors and their role in producing bacterial infections in poultry.

The sensitivity shown by *E. coli* isolates were cephalexin (83.33 per cent) amoxycillin-clavulanic acid (66.67 per cent), erythromycin (33.33 per cent), ciprofloxacin (16.67 per cent), clindamycin (16.67 per cent), tetracycline (16.67 per cent), cloxacillin (0 per cent), co-trimoxazole (0 per cent). A low percentage of

sensitivity was observed against the antibiotics like cloxacillin and co-trimoxazole, ciprofloxacin and clindamycin and a high percentage of sensitivity was observed against cephalexin.

Multi drug resistance (resistance to at least three antimicrobials) was found among all *E. coli* isolates obtained in the study. Hence it may be concluded that the high level of resistance observed among poultry *E coli* isolates obtained in the study may be due to incorporation of antibiotics in feed as growth promoters.

The antibiotic sensitivity obtained for *P. multocida* isolates were, chloramphenicol (100 per cent) enrofloxacin (100 per cent), furazolidone (100 per cent), pefloxacin (100 per cent), tetracycline (100 per cent), bacitracin (75 per cent), ciprofloxacin (75 per cent), co-trimoxazole (75 per cent), nitrofurantoin (75 per cent), penicillin (75 per cent), ampicillin (50 per cent), cloxacillin (50 per cent), gentamicin (50 per cent), streptomycin (0 per cent) and metronidazole (0 per cent).

 As 100 per cent sensitivity is shown to chloramphenicol, enrofloxacin, furazolidone, pefloxacin and tetracycline these two drugs may be used for treating pasteurellois.

Out of 15 *Staphylococcus* sp. obtained all the isolates (100 per cent) were found to be sensitive to amoxycillin clavulanic acid (Ac) and cephalexin (Cp), 14 isolates (93.33 per cent) to found to be sensitive to co-trimoxazole, 11 isolates (73.33 per cent) to ciprofloxacin, 10 isolates (66.67 per cent) to tetracycline, 13 isolates (86.67 per cent) to both clindamycin and erythromycin and two isolates (13.33 per cent) to cloxacillin.

Amoxycillin clavulanic acid (Ac) and cephalexin (Cp) were found to be the most effective antibiotics against *Staphylococcus* sp. in the study.

The plasmid DNA content of the seven isolates of *E. coli* was analysed on agarose gel electrophoresis. All the isolates harboured plasmids. The number of plasmids varied from one to three and size varied from 1.54 K bp to 31.8 K bp. A correlation between the number of plasmids and antibiotic resistance could not be ascertained in this study.

In conclusion, the results of this study provide evidence for significant antimicrobial resistance among bacterial isolates from birds. Long term prospective studies involving isolation, identification and antibiogram from more samples are required to identify novel pathogens causing respiratory disease in birds. Such studies will provide data on temporal and spatial difference in antibiotic resistance patterns, which in turn will help the scientific community to design better disease control strategies.

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BACTERIA ASSOCIATED WITH RESPIRATORY INFECTIONS IN POULTRY

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ABSTRACT

This study was undertaken to isolate and identify of bacteria from respiratory tract of poultry and to study the antibiogram of the isolates.

Birds showing respiratory signs were sacrificed, postmortem examination was conducted and samples such as nasal, tracheal and air sac swabs and lungs were collected after taking all sterile precautions.

A total of 105 samples were collected by sacrificing birds showing clinical signs. Isolation of causative bacteria was made by culturing on brain heart infusion agar, Mac Conkey agar and blood agar. For identification of isolates all the procedures were followed as described by Barrow and Feltham (1993). A total of 31 bacterial isolates were obtained from samples.

A total of 12 *Escherichia coli* isolates were isolated and identified, 4 *Pasteurella multocida isolates* and 15 *Staphylococcus* sp. Isolates were isolated and identified biochemically.

Out of 15 *Staphylococcus* sp. isolated and identified 11 isolates (73.33 per cent) were coagulase negative This result indicate that CoNS were more frequently isolated from staphylococcal infections although they do not possess the virulent coagulase activity. So importance must be given to CoNS also, as given to coagulase positive staphylococci and much study need to be diverted to find the virulence factors and role of them in producing bacterial infections in poultry.

Multi drug resistance (resistance to at least three antimicrobials) was found among all *E. coli* isolates obtained in the study. Hence it may be concluded that the high level of resistance observed among poultry *E coli* isolates obtained in the study may be due to incorporation of antibiotics in feed as growth promoters.

As 100 per cent sensitivity is shown to enrofloxacin and chloramphenicol by *P. multocida* isolates, these two drugs may be used for treating pasteurellois.

Amoxycillin clavulanic acid (Ac) and cephalexin (Cp) was found to be the most effective antibiotic against *Staphylococcus* sp. in the study.

The plasmid DNA content of the seven isolates of *E. coli* was analysed on agarose gel electrophoresis but correlation between the number of plasmids and antibiotic resistance could not be ascertained in this study.

In conclusion, the results of this study provide evidence for significant antimicrobial resistance among bacterial isolates from birds. Long term prospective studies involving isolation, identification and antibiogram from more samples are required to identify novel pathogens causing respiratory disease in birds. Such studies provide data on temporal and spatial difference in antibiotic resistance patterns, which in turn helps the scientific community to design better disease control strategies.

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