DETECTION OF PATHOGENIC HAEMOLYTIC BACTERIA IN RESPIRATORY TRACT INFECTIONS OF LIVESTOCK

APARNA. S.

Thesis submitted in partial fulfilment of the
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Department of Veterinary Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

DECLARATION

I hereby declare that the thesis entitled **"DETECTION OF PATHOGENIC HAEMOLYTIC BACTERIA IN RESPIRATORY TRACT INFECTIONS OF LIVESTOCK"** 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.

Mannuthy
22-08-05

APARNA.S

CERTIFICATE

Certified that this thesis entitled **"DETECTION OF PATHOGENIC HAEMOLYTIC BACTERIA IN RESPIRATORY TRACT INFECTIONS OF LIVESTOCK"** is a record of research work done independently by **Aparna. S,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

22/8/05

Dr. G. Krishnan Nair
(Chairman, Advisory Committee)
Associate Professor
Department of Veterinary Microbiology
College of Veterinary and Animal Sciences
Mannuthy

Mannuthy,
22-08-'05

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Aparna.S,** a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled **"DETECTION OF PATHOGENIC HAEMOLYTIC BACTERIA IN RESPIRATORY TRACT INFECTIONS OF LIVESTOCK"** may be submitted by Aparna.S, in partial fulfilment of the requirement for the degree.

Dr. G. Krishnan Nair
(Chairman, Advisory Committee)
Associate Professor
Department of Veterinary Microbiology
College of Veterinary and Animal Sciences
Mannuthy

Dr. V. Jayaprakasan
Associate Professor and Head
Department of Veterinary Microbiology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr.M.Mini
Assistant Professor
Department of Veterinary
Microbiology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr. N. Divakaran Nair
Assistant Professor (Selection Grade)
Centre of Excellence in Pathology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

15/10/05

External Examiner
(H.K. MUKHOPADHYAY)

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Introduction

1. INTRODUCTION

India is an agrarian country encompassing a major fraction of the livestock population of the world and ranks first in cattle and buffaloe population among the nations of the world. Income from the livestock sector contributes much to the Gross Domestic Product of our country.

Diseases of infectious origin have always remained a bane to our livestock sector. Respiratory tract infections have been considered as the most important health problem in humid tropics. The respiratory system is under constant assault from potentially dangerous agents, mainly the airborne pathogenic microbes and the opportunistic oropharyngeal flora. Experimental studies have established that there are chances of the normal flora being carried into the lungs via trachea. But in spite of this constant bacterial movement from the nasal flora and from the contaminated air, normal lungs remain sterile due to remarkably effective defensive mechanisms. Industrialization of livestock production has resulted in increased frequency of respiratory disease conditions. Usually the respiratory tract infections occur as a result of combination of environmental, managemental and infectious factors. It is a leading cause of economic loss to the farmers in India.

Haemolytic organisms like *Mannheimia, Staphylococcus, Arcanobacterium, Escherichia and Streptococcus* sp. are commensals in the respiratory tract of livestock and has also been documented as etiological agents of respiratory diseases. (Allan, 1977).

Biochemical tests are the most widely employed methods for identification of these bacterial organisms. They have their own merits as well as demerits. They are simpler to perform and cheaper. At the same time these tests are time consuming and variations in one or few biochemical tests may

result in failure to arrive at definite conclusions. On the other hand, nucleic acid based methods for detection; identification and characterization of microbes have been proved to be more accurate and easier. Molecular methods have helped to detect the genes associated/responsible for virulence/pathogenicity of microbes.

Mannheimia haemolytica has been found to be the causative agent of a devastating respiratory disease of cattle, namely, pneumonic pasteurellosis. The leukotoxin gene *lkta-* the major virulence factor of *Mannheimia haemolytica* has been found to be associated with the cytotoxicity and haemolytic activity of the organism. Fisher *et al.* (1999) have shown that polymerase chain reaction based identification of the *Mannheimia* strains possessing the *lkta* gene would help in the evaluation of the pathogenic potential of the organism.

Staphylococcus aureus has been shown to be a major haemolytic respiratory pathogen in a wide range of hosts. The genetic make up of *Staphylococcus aureus* isolated from respiratory infections of different hosts have not yet been completely understood. Random amplified polymorphic DNA-Polymerase chain reaction (RAPD-PCR) has been found to be an effective molecular tool in assessing the genetic relationship of *Staphylococcus aureus* isolates from different hosts. (Reinoso *et al.*, 2004).

Virulence in *Escherichia coli* is multifactorial and the ability to produce haemolysins is one of them. A high percentage of the extraintestinal isolates of *E.coli* produce haemolysins. The α-haemolysin production in *E.coli* of animal origin is determined by *hly* determinants on plasmids, while that of human isolates are of chromosomal origin. So haemolysin-producing *Escherichia coli* isolates from respiratory tract infections of livestock may harbour plasmids.

There is paucity of sufficient information on the etiological importance of haemolytic organisms in respiratory infections and the effect of antimicrobials on these organisms. No concerted effort has been made so far in this State to understand the involvement of pathogenic haemolytic bacteria in respiratory infections of livestock. Isolation and identification of these haemolytic organisms, determining their antimicrobial susceptibility and proving their pathogenicity in experimental animals have a great role to play in the effective control and prevention of these conditions.

Taking into consideration all the above factors, the present study was undertaken with the following objectives.

1. Isolation of haemolytic bacteria from livestock with respiratory infections and from apparently healthy animals.
2. Subjecting all Gram-negative haemolytic bacteria to Polymerase chain reaction (PCR) to detect the leukotoxin gene region of *Mannheimia haemolytica.*
3. Biochemical characterization of the haemolytic organisms.
4. Studying the antibiogram of the isolates.
5. Pathogenicity test of the isolates in mice.
6. RAPD-PCR of *Staphylococcus aureus* isolates
7. Plasmid profile analysis of *Escherichia coli* isolates

Review of Literature

2. REVIEW OF LITERATURE

Respiratory tract infections have always been a cause of trouble to the livestock sector. Bacterial etiology of respiratory tract infections has been well studied and understood. Among these many of the haemolytic bacteria have been incriminated as causative agents of respiratory tract infections and the involvement of *Mannheimia haemolytica* has been well documented. Perusal of literature has shown that other haemolytic organisms like *Staphylococcus aureus, Arcanobacterium pyogenes, Escherichia coli, Streptococcus* sp and *Klebsiella pneumoniae*, are also associated with respiratory tract infections in livestock.

2.1 MANNHEIMIA HAEMOLYTICA (PASTEURELLA HAEMOLYTICA)

Mannheimia haemolytica is a small non-motile, Gram negative, cocco-bacillary organism showing a weak zone of haemolysis on blood agar. It is associated with a devastating disease in cattle called Bovine pneumonic pasteurellosis or Shipping fever. In sheep it is associated with pneumonia and septicaemia. Besides its isolation from diseased animal tissue *Pasteurella haemolytica* is frequently isolated from the nasal passage of healthy cattle and sheep (Frank and Wessman, 1978). Mehrotra and Bhargava(1999) reported that *Pasteurella haemolytica* is associated with a septicaemic type of disease in domestic animals and in India, the disease has enzootic and epizootic proportions, which usually occur following some stress factors like, weather change, high humidity, transportation. The genus *Mannheimia* presently consists of five species *viz., Mannheimia haemolytica, Mannheimia glucosida, Mannheimia granulomatis, Mannheimia ruminalis* and *Mannheima varigenia.* (Angen *et al.*, 2002).

2.1.1 Incidence

Shreeve and Thompson (1970) reported that *Pasteurella haemolytica* serotype A: 2 were present in the nasopharynx and tonsils of apparently healthy animals. They also reported that the presence of organism in the nasopharynx of sheep coincided with the occurrence of the respiratory disease.

Al-Sultan and Aitken (1985) reported that *Pasteurella haemolytica* acquired from their dams, very soon after birth, could colonize the tonsils of lambs.

Allan *et al.* (1985) reported that *Pasteurella haemolytica* biotypeA was the most commonly isolated *Pasteurella* species from 80 calves examined at necropsy, from 40 outbreaks of respiratory disease. Nasopharyngeal swabs from incontact and apparently healthy calves indicated the wide spread presence of *Pasteurella haemolytica.*

Pasteurella haemolytica was responsible for the enzootic pneumonia in calves' upto six months of age (Frank, 1989). He reported that the massive rapid growth of *Pasteurella haemolytica* was responsible for the most acute form of pneumonic pasteurellosis, with fulminating fibrinous lobar pneumonia.

Gilmour and Gilmour (1989) reported that pasteurellosis of sheep in temperate climates was caused by *Pasteurella haemolytica* and rarely by *Pasteurella multocida.*

Pasteurella haemolytica was recovered from tonsil wash specimens for several weeks in the absence of clinical signs of disease, indicating that the tonsil was a site in which *Pasteurella haemolytica* could be carried for long periods by healthy calves (Frank and Briggs, 1992).

Whitley *et al.* (1992) opined that *Pasteurella haemolytica* was the primary causal agent of pneumonic pasteurellosis, fibrinous pneumonia or bovine respiratory disease in cattle.

Barbour *et al.* (1997) reported that *Pasteurella haemolytica* was one of the most prevalent bacteria in the lungs of unhealthy calves.

In the pathological and microbiological studies on pneumonic lungs from Danish calves, Tegtmeier *et al.* (1999) observed that *Pasteurella haemolytica* was one of the most commonly encountered lung pathogens.

Rowe *et al.* (2001) opined that *Mannheimia haemolytica* was the important cause of bacterial respiratory diseases associated with mortality in cattle, sheep and goats in U.K. They studied the growth morphology and long term survival of a representative isolate of *Mannheimia haemolytica* serotypeA1 and A2 in ovine and bovine tracheobronchial washings. They found that both strains survived for at least 244 days and 156 days in ovine and bovine tracheobronchial washing respectively.

2.1.2. Isolation and Identification

2.1.2.1 Isolation

Allan (1977) reported the isolation of *Pasteurella haemolytica* from 10 .3 per cent of non-pneumonic lung tissue of calves.

Isolation of *Pasteurella hemolytica* from an outbreak of pneumonia among a newly introduced herd of goats in a farm in Japan and serotyping of the isolates by rapid plate agglutination test was reported by Hayashidani *et al.* (1988).

Gilmour and Gilmour (1989) suggested that samples should be collected from cases which have not been given antibiotic therapy and should be taken from pleural and pericardial fluids, heart blood, lung lesions, liver, spleen and kidneys. They also suggested that *Pasteurella haemolytica* might be present in the lungs of sheep, in the absence of clinical disease.

Oberst *et al.* (1993) reported the storage of cultures of *Pasteurella haemolytica*- like organisms and other related Gram negative organisms in one milliliter of defibrinated sheep blood at -70^{0}C. They also observed that sheep blood agar was ideal for determining the colony mophology and hemolytic activity of *Pasteurella haemolytica.*

Kanwar *et al.* (1998) reported the isolation of *Pasteurella haemolytica* from a case of fibrinous pneumonia in goats and opined that *Pasteurella haemolytica* infection was comparatively less frequent than *Pasteurella multocida* infection in goats.

Vogel *et al.* (2001) isolated Mannheimia *haemolytica* from 15 per cent of cases of pneumonia in calves.

Odugleo *et al.* (2003) reported the isolation of *Pasteurella haemolytica* from pneumonic lung tissue by torching the surface with a hot spatula and smearing the cut surface onto blood agar plate.

Siji (2003) isolated *Mannheimia haemolytica* from cases of respiratory infections in cattle in Kerala.

2.1.2.2 Cultural and biochemical characterisation

Frank (1989) opined that low power magnification would best display the colony morphology and weak zone of β - hemolysis of *Mannheimia haemolytica* .

Pasteurella haemolytica were able to grow on Mac conkey agar and potassium cyanate (KCN) broth and were urease negative. Dextrose was uniformly fermented; lactose, raffinose and xylose were fermented variably. All the isolates produced hemolytic activity on blood agar medium (Mehrotra and Bhargava, 1999).

Fisher *et al.* (1999) reported that the identification of *Pasteurella haemolytica* was done on the basis of staining characteristics and biochemical reactions including oxidase production, nitrite reduction, fermentaton of glucose, mannitol and sucrose. They also opined that the hemolytic activity of the organism was correlated with the leukotoxin gene for most of the isolates studied. They also reported that the identification of the strains that possesed the leukotoxin gene might aid in the evaluation of the pathogenic potential of *Pasteurella* strains carried by wild and domestic animals. They isolated *Pasteurella haemolytica* on Columbia blood agar plates supplemented with five per cent ovine blood.

A Method for phenotypic characterisation of *Mannheima haemolytica*-like organisms using diatabs diagnostic tablets and correct identification of all strains belonging to *Mannheimia* sp was reported by Angen *et al.* (2002).

Odugleo *et al.* (2003) reported that those isolates on blood agar plates that were small, round and grey, β - hemolytic, Gram negative, showing yellow butt yellow slant reaction on TSI, a positive H_2S and nitrate test, catalase test and oxidase positive, indole and urease negative and which grew on Mac

Conkey agar, were considered to be *Mannheimia haemolytica* . They also reported the serotypng of the obtained isolates using rapid plate agglutination test

2.1.2.3 Molecular characterisation

Strathdee and Reggie (1988) showed that the structural gene of *Pasteurella haemolytica* leukotoxin determinant was highly homologous to that of the *Escherishia coli* hemolysin determinant .

Green *et al.* (1999) reported the designing of seven PCR primers of the leukotoxin gene region of *Pasteurella haemolytica.*

Detection of *Pasteurella haemolytica* by PCR was reported by Fisher *et al.* (1999) who opined that PCR procedure had the capability of detecting minute amounts of specific DNA and therefore had greater sensitivity than routine culture procedure for detecting bacteria.

2.1. 3 Antibiogram

Allan *et al.* (1985) conducted antibiogram of *Pasteurella haemolytica* A1 isolates and reported that they showed a sensitivity of 100 per cent for chloramphenicol, sulpha-TMP (98 per cent), oxytetracycline (80 per cent), ampicillin(85 per cent), penicillin (82 per cent), streptomycin (3 per cent) and lincomycin(1 per cent).

Mehrotra and Bhargava (1999) did drug sensitivity of 60 isolates of *Pasteurella haemolytica* and they found them to be resistant to conventional antibiotics.

Siji (2003) reported the isolation of *Mannheimia haemolytica* and showed that all the three isolates were susceptible to oxytetracycline and two were susceptible to amoxycillin, enrofloxacin and trimethoprim. One isolate was sensitive to chloramphenicol, gentamicin, ciprofloxacin and none of the isolates were sensitive to streptomycin.

2.1.4 Pathogenicity

Four virulence factors have been associated with *Pasteurella haemolytica,* fimbriae, a polysaccharide capsule, endotoxin and leukotoxin. The interactions of these virulence factors with components of the pulmonary alveolus were presumed to be responsible for the pathogenesis of pasteurellosis (Confer *et al.*, 1990).

Burrows and Winfield (1993) opined that the leukotoxin of *Pasteurella haemolytica* was associated with increased strain virulence in Bighorn sheep.

2.2 STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a Gram-positive organism occurring in irregular clusters resembling a bunch of grapes. They are known to show haemolysis on blood agar. They occur as commensals on skin and mucous membrane and act as opportunistic pathogens, causing pyogenic infections. Coagulase positive *Staphylococcus aureus* are important pathogens of domestic animals and the coagulase production correlates with its pathogenicity. Association of *Staphylococcus aureus* with respiratory infections has been well documented.

2.2.1 Incidence

Wikse and Baker (1996) reported *Staphylococus aureus* as one among the many organisms associated with the respiratory disease complex in cattle, sheep and goat.

Barbour *et al.* (1997) reported that *Staphylococcus aureus* was one of the most prevalent bacteria in the lungs of unhealthy calves.

In a study on nasal carriage of *Staphylococcus aureus* in dairy sheep Vautor *et al.* (2005) showed that ewe nasal carriage might represent a major reservoir for *Staphylococcus aureus* to contaminate other sites on sheep dairy farms.

2.2.2. Isolation and identification

2.2.2.1 Isolation

Allan (1977) isolated 52 bacterial species from 92 pneumonic lungs of cattle, out of which 7.6 per cent was *Staphylococcus aureus*.

Isolation of *Staphylococcus* sp. from the tonsils of healthy pigs and phage pattern study of the isolates was done by Shimizu *et al.* (1987)

Shimizu *et al.* (1990) showed that staphylococci were found in the tonsils of 121 (75.2 per cent) of 161 cattle and there were 15 different species, most predominant being *Staphylococcus simulans*, followed by *Staphylococcus aureus, Staphylococcus chromogenes* and *Staphylococcus epidermidis* and out of the 177 isolates only 25.4 per cent were coagulase negative.

Siji (2003) reported the isolation of *Staphylococcus aureus* from 18 cases of respiratory infection in cattle.

2.2.2.2. Cultural and biochemical characterisation

Staphylococcus aureus are usually haemolytic and often produce both the alpha hemolysin and beta hemolysin and so exhibit double haemolysis (Quinn *et al.,* 1994). They also reported the use of coagulase test, DNAase test, haemolysis, pigment production, alkaline phosphatase test, urease test, fermentation of mannitol and maltose, and aesculin hydrolysis, for characterisation of *Staphylococcus* sp.

2.2.2.3 Molecular characterisation

Brakstad *et al.* (1992) had reported the rapid detection of *Staphylococus aureus* by Polymerase chain reacton of the *nuc* gene.

The genetic heterogenicity among different *Staphylococcus aureus* isolates using Random amplified polymorphic DNA (RAPD) technique and Pulsed field gel electrophoresis (PFGE) was demonstrated by Ahmed *et al.* (1998).

In studies on molecular genotyping of *Staphylococcus aureus* strains Zef *et al.* (1999) observed that Repetetive element sequence based PCR (REP-PCR) was more superior to Arbitrarily primed-PCR since REP-PCR results obtained were highly reproducable. They also reported that REP-PCR was more useful than PFGE because of its high resolution power and ease of performance. They also opined that REP-PCR typing might be suitable for widespread use in the clinical microbiology laboratory for epidemiological typing of *Staphylococcus aureus* strains.

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

In the RAPD-PCR analysis study of *Staphylococcus aureus* strains isolated from bovine and human hosts, Reinoso *et al.* (2004) reported that RAPD profile resulted in DNA amplification fragments ranging in size from 300 to1900 bp and number of amplified fragments ranged from one to fourteen. They also showed that RAPD-PCR could be successfully applied to assess the genetic relationship of *Staphylococcus aureus* isolates from different hosts.

Vauter *et al.* (2005) reported that PFGE could be used to investigate the genetic diversity of the isolates of *Staphylococcus aureus* from nares and to understand genetic relatedness between isolates recovered from different sources.

2.2.3 Antibiogram

Gonlugur *et al.* (2003) showed that respiratory isolates of *Staphylococcus aureus* had an overall resistance of 61.9 per cent to erythromycin, followed by tetracycline (50.7 per cent), but had a low resistance of 10.3 percent to co-trimoxazole.

Siji (2003) reported that Staphylococcus *aureus* isolates weresensitive to enrofloxacin and ciprofloxacin (100 per cent), gentamicin (83.30 per cent), chloramphenicol (66.66 per cent), amoxycillin, streptomycin and oxytetracycline (50 per cent) and trimethoprim (16.66 per cent).

Vintov *et al.* (2003) in the study on phage types and antimicrobial resistance of 292 isolates of *Staphylococcus aureus* from bovines, obtained during the period of 1950-2000, showed that the Danish *Staphylococus aureus*

population had remained relatively unchanged over the last 50 years and the occurrence of antimicrobial resistance had remained low in Denmark in comparison to other countries in Europe.

2.2.4 Pathogenicity

Pak *et al.* (1999) reported pathogenecity testing of *Staphylococus aureus* isolates by intraperitoneal inoculation of 0.5ml of 10^8 -10^9organisms suspended in phosphate buffered saline to mice and observing them for a period of 15 days.

Production of coagulase by staphylococci was an important indicator of pathogenicity. Additional markers for pathogenecity were DNAase activity and protein A production (Quinn *et al.*, 2002)

2.3 ESCHERICHIA COLI

They are motile Gram-negative rods. Haemolytic activity on blood agar is a characteristic of certain strains of *Escherichia coli* (*E.coli*). Haemolytic activity is associated with increased pathogencicity of extraintestinal forms of *E. coli*. Alpha haemolysis is a useful marker for virulence in certain strains of *E. coli* and alpha haemolytic activity is associated with plasmids. The role of *E. coli* in causing respiratory infections in a wide variety of species has been documented by many researchers.

2.3.1. Incidence

Wikse and Baker (1996) reported *Escherichia coli* as one among the organisms associated with the respiratory disease complex in cattle, sheep and goat.

Escherichia coli was found in combination with *Staphyloccus* sp., *Pasteurella* sp., *Klebsiella* sp., and *Pseudomonas* sp., as causative agent of respiratory infections in bovines (Siji, 2003)

2.3.2 Isolation and identification

2.3.2.1 Isolation

Nasser and El-Sayed (1997) obtained nine isolates of *E. coli* from 20 calves exhibiting acute signs of bovine respiratory disease, from Italy.

Fecteau *et al.* (2001) reported the isolation of 25 *Escherichia coli* isolates from blood cultures from critically ill neonatal calves. They studied the virulence factors in *E.coli* and hypothesized that the *E. coli* strains involved in septicaemia on a particular farm were highly dependent on the *E. coli* present in calves in immediate environment.

In a study on respiratory tract infections of calves in Turkey, Aslan *et al.* (2002) could isolate *E. coli* (5 per cent) from 27 cases.

Siji (2003) reported the isolation of seven isolates of *E. coli* from 18 cases of respiratory infection in cattle.

Isolation and characterization of *E. coli* strain 078 from cases of septicaemia in sheep was reported by Kumar *et al.* (2005).

2.3.2.2 Cultural and biochemical characterisation

Pathogenic strains of *E. coli* are quite often haemolytic and as they are strong lactose fermenters, the colonies on Mac Conkey agar are bright pink. On Eosin methylene blue agar (EMB) they have a unique and

characteristic metallic sheen. The IMViC test (indole+/MR+/VP_/Citrate-)is a quick and presumptive method of identifying *E. coli*.(Quinn *et al.*,1994)

On characterization of *E. coli* 078 isolates from cases of septicaemia in sheep, Kumar *et al.* (2005) found that one of the isolates was positive for citrate utilisation test. They also found that *E. coli* 078 isolates lost the property of giving metallic sheen on EMB agar after subculturing even once and when such isolates were passaged in animals or cultured on five per cent sheep blood agar, the property of exhibiting metallic sheen on EMB was regained.

2.3.2.3 Plasmid characterisation

Guery *et al.* (1973) opined that plasmid deoxyribonucleic acid (DNA) ranging from $5x10^6$ to $65x10^6$daltons may be isolated from chromosomal DNA, by the preferential precipitation of high molecular weight chromosomal DNA in the presence of sodium lauryl sulphate and high concentration of sodium chloride.

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

Marcina *et al.* (1978) described a strain of *E. coli* that contained eight plasmid species ranging in size from $1.36x10^6$ to $35.8x10^6$daltons. This strain could be employed as a single source of covalently closed circular deoxyribonucleic acid molecules of different sizes, for use as reference in agarose gel electrophoretic analysis.

Birnboim and Doly (1979) reported a rapid alkaline extraction procedure for the isolation of plasmid DNA. The principle of the method

was selective alkaline denaturation of high molecular weight chromosomal DNA, while covalently closed circular DNA remained double stranded. Upon neutralization, chromosomal DNA re-natured to form an insoluble clot, leaving plasmid DNA in the supernatant. Large and small plasmid DNAs had been extracted by this method.

α-Hemolysin of *E. coli* is an extracellular toxin frequently produced by pathogenic isolates from human and animals (Cruz and Muller 1980). They also stated that α-hemolysin excreted by *E. coli* strain PM152 was controlled by three plasmid coded cistrons and also hemolysin production in *E coli* was controlled by a common genetic determinant which could be located on plasmids of different incompatability groups.

Mini *et al.* (2005) reported the plasmid isolation from 10 *E.coli* isolates and opined that the no: of plasmids from those strains varied from one to three and the size of the plasmids ranged from 1.86 kb to 48.06 kb and one isolate did not show any plasmid.

2.3.2.4 Molecular characterisation

Cruz and Zabata (1983) reported that there was a unique ubiquitous genetic determinant responsible for the hemolytic phenotype in *E. coli.*

Hemolytic determinant present in majority of hemolytic *E. coli* strains was of a common origin and differences in the expression of the determinant was related to the virulence level of the strains tested in a rat peritonitis model. (Welch and Falkow, 1984)

Virulence in *E. coli* was multifactorial and one of these was the ability to produce a hemolysin and a high percentage of extraintestinal isolates

produced hemolysin (Cavalieri *et al.*, 1984). They reported that virulence of hemolytic strains was lost by non-hemolytic mutants of the same strain. They also opined that hemolytic strains of *E. coli* were more toxic than non-hemolytic strains and also added that the α hemolysin production resulted from *hly* determinants on plasmids as in animal *E.coli* isolates or from *hly* determinant on the chromosome as in human isolates.

2.3. 3 Antibiogram

All *E.coli* 078 isolates from respiratory tract infections of sheep were found sensitive to ciprofloxacin, amoxycillin, gentamicin, chloramphenicol, streptomycin and oxytetracycline.(Kumar *et al.*,2005)

Siji (2003) reported that none of the antibiotics studied showed cent per cent sensitivity to *E. coli* isolates. Maximum sensitivity was shown to chloramphenicol, enrofloxacin and ciprofloxacin (85.17 per cent). Isolates had a sensitivity of 71.4 per cent to gentamicin, 46.86 per cent to amoxycillin, streptomycin and oxytetracycline and the least sensitivity was to trimethoprim (14.29 per cent).

2.3.4 Pathogenicity

Intraperitoneal infection of *E.coli* was induced on female rats by inoculating 10^6CFU of *E. coli* by May *et al.* (2000). They also reported that *hly* (α hemolysin) played key role in facilitating the pathogenecity of *E. coli* within the peritoneum and also inoculation of 10^8CFU of hly^+ variant was 100 per cent lethal, while the same size inoculum of the hly^- variant was non lethal to rat. They added that hemolysin production during peritonitis increased the lethality of infection in rat model. They also stated that one known *E. coli* virulence factor was the potent exotoxin α hemolysin(hly) and also hemolysin production

by *E. coli* within the peritoneum may not only alter the hosts ability to control the hemolytic strains itself but also other organisms and also wild type hemolytic strains demonstrate a significant competitive advantage over non hemolytic strains in a rat peritonitis model.

Knobl *et al.* (2001) studied the virulence properties of *E. coli* isolated from ostriches with respiratory disease and found that they possessed the same virulence factors present in *E. coli* isolated from poultry with chronic complicated respiratory disease.

Kumar *et al.* (2005) confirmed the pathogenecity of *E.coli* 078 cultures from clinical cases in sheep by intraperitoneal inoculation of live cultures to three mice in which it caused death within 24 hours of injection, with re-isolation of similar organisms from heart, blood, liver and spleen.

2.4 ARCANOBACTERIUM PYOGENES (ACTINOMYCES PYOGENES) (CORYNEBACTERIUM PYOGENES)

They are non-motile, non-spore forming, Gram-positive bacteria, which show a coryneform morphology and require enriched media for their growth. They are commonly present in the nasopharyngeal mucosa of cattle, sheep and pigs. It's a common cause of suppurative lesions in many domestic animals and is well associated with respiratory tract infections.

2.4.1 Incidence

Merchant and Packer (1971) stated that *Corynebacteriun pyogenes* were causative organisms of suppurative pneumonia in cow.

Barbour *et al.* (1997) reported that *Actinomyces pyogenes* was one of the most prevalent bacteria in the lungs of unhealthy animals.

In the pathological and microbiological studies on pneumonic lungs from Danish calves Tegtmeier *et al.* (1999) observed that *Actinomyces pyogenes was* one of the most commonly encountered bacterial pathogen in lungs.

A case of fibronecrotic rhinitis caused by a concurrent infection of *Fusobacterium necrophorum* and *Arcanobacterium pyogenes* in cow was reported by Semiya *et al.* (2004).

Arcanobacterium pyogenes have been reported to be responsible for chronic pneumonia, aspiration pneumonia and lung abscessation in cattle (Smith, 2000)

2.4.2 Isolation and identification

2.4.2.1. Isolation

Allan (1977) reported the isolation of *Corynebacterium pyogenes* from 9.6 per cent of the calves suffering from pneumonia and from seven per cent of non-pneumonic lung tissues of calves.

Funk *et al.* (1996) reported the isolation of 22 *Actinomyces pyogenes* isolates from hepatic abscesses in cattle and quantified their hemolysin production.

Vogel *et al.* (2001) isolated Arcanobacterium *pyogenes* from 46 per cent cases of pneumonia in calves.

Baksi *et al.* (2004) reported the isolation of *Corynebacterium pyogenes* as the pathogenic agent in an outbreak of chronic pneumonia from rabbits of 2-3 years, reared in nest box.

2.4.2.2 Cultural and biochemical characterisation

Funk *et al.* (1996) in their study on partial characterization of an *Actinomyces pyogenes* hemolysin showed that the hemolysin was oxygen stable, sensitive to treatment by protease, trypsin and amylase and destroyed by treatment at extreme temperature. Maximum hemolysis was produced in brain heart infusion agar incubated aerobically at six per cent carbondioxide and to lesser degree anaerobically in RPMI-1640 and no hemolysis was produced in the defined *Actinomyces pyogenes* medium. Significant leukotoxin activity was noticed in all samples screened for hemolysin activity.

At 48 h of incubation *Arcanobacterium pyogenes* produced tiny colonies of one millimetre surrounded by a narrow zone of complete haemolysis. A rapid presumptive test for *Arcanobacterium pyogenes* was to demonstrate its ability to pit a Loeffler serum slope in 24-48 hours, which indicated its proteolytic activity (Quinn *et al.*, 2002).

2.4.3 Antibiogram

Five antibiotics,viz., bacitracin, chlortetracycline, oxytetracycline, tylosin and virginiamicin were inhibitory to all strains of *Actinomyces pyogenes* and *Actinomyces pyogenes* like organism (Narayanan *et al* .,1998)

Trinh *et al.* (2002) opined that a large proportion of *Actinomyces pyogenes* field isolates were resistant to commonly used tetracycline, macrolide, and lincosamide antimicrobial agents.

Antimicrobial sensitivity of *Corynebacterium pyogenes* isolated from rabbits against 18 antimicrobials was studied and found that the organisms were highly sensitive to erythromycin and tetracycline (Baksi *et al.*, 2004).

Jost *et al.* (2004) suggested that ribosomal mutations *in Actinomyces pyogenes* conferred a unique spectrum of macrolide resistance to them.

2.4.4 Pathogenicity

Virulence factors of *Actinomyces pyogenes,* especially hemolysin, have been suggested to be important factors in synergestic interaction of *Actinomyces pyogenes* and *Fusobacterium necrophorum* in inducing liver abscesses. (Narayanan *et al.*, 1998).

Narayanan *et al.* (1998) reported that *Actinomyces pyogenes*- like organism had higher mean haemolytic and leukotoxic activities than *Actinomyces pyogenes.*

Imaizumi *et al.* (2001) reported that pyolysin (PLO) secreted by *Arcanobacterium pyogenes* was a novel member of the thiol activated cytolysin family of bacterial toxins and the N-terminal region of PLO was important for haemolytic activity.

2.5. KLEBSIELLA PNEUMONIAE

They are motile Gram-negative rods requiring no enriched media for their growth. They are opportunistic pathogens and are associated with respiratory infections in a wide variety of species.

2.5.1 Incidence

Allan (1977) reported *Klebsiella* sp. as one of the causative agents of respiratory tract infections in calves.

Incidence of *Klebsiella pneumoniae* as causative agents of respiratory tract infections in bovine was reported by Siji (2003).

2.5.2 Isolation and identifiifcation

2.5.2.1 Isolation

Isolation of five *Klebsiella pneumoniae* organisms from 18 cases of respiratory infection in cattle was reported by Siji(2003).

Brisse and Deujkeren (2005) reported the isolation, identification and antimicrobial susceptibility of 100 *Klebsiella* animal isolates.

2.5.2.2. Cultural and biochemical characterisation

Merchant and Packer (1971) reported that *Klebsiella pneumoniae* were short, plump rods with rounded ends and it had a heavy capsule. He also opined that in ordinary agar media it form white, mucus like colonies that are slimy to semifluid in consistency and it ferments a no: of carbohydrates.

Klebsiella pneumoniae had very mucoid colonies on primary isolation, indicative of the presence of a large capsule around individual cells. They are lactose fermenters producing pale pink colonies on Mac Conkey agar (Quinn *et al.*, 1994).

2.5.2.3 Molecular characterisation

Brisse and Deujkeren (2005) reported that *gyr*A PCR-RFLP was a rapid mehod to identify *Klebsiella* species and phylogenetic groups.

2.5.3 Antibiogram

Eguchi *et al.* (1988) studied the antimicrobial sensitivity of *Klebsiella* isolates from mare genital infections, using disc method and the agar dilution method. He found that all the isolates had a high degree of sensitivity to gentamicin, cephalexin, colistin, polymyxin B and furazolidone, while they showed a low sensitivity to other commonly used antimicrobial agents.

The *Klebsiella pneumoniae* isolated from respiratory tract infections was susceptible to enrofloxacin (100 per cent), chloramphenicol and ciprofloxacin (80 per cent) and amoxycillin, trimethoprim and oxytetracycline (60 per cent). Only 20 per cent sensitivity was shown to streptomycin (Siji, 2003).

Brisse and Deujkeren(2005) showed that most of the *Klebsiella* isolates were naturally resistant to ampicillin, due to constitutively expressed chromosomal class A β-Lactamase. They also reported that *Klebsiella* sp. had no intrinsic resistance to cephalosporins.

2.5.4 Pathogenicity

Klebsiella pneumoniae produce abundant capsular material, which may inhibit phagocytosis and enhance intracellular survival. Pathogenicity of the organism can be attributed to the release of endotoxins from them.(Quinn *et al* .,2002) .

2.6 STREPTOCOCCUS SP

They are Gram-positive cocci occuring in chains, fastidious, producing haemolytic transluscent colonies on blood agar. β - haemolytic streptococci are found to be more pathogenic and are incriminated as agents of pneumonia and respiratory tract infections.

2.6.1 Incidence

Wikse and Baker (1996) reported *Streptocccus* sp., as one among the organisms associated with the respiratory disease complex in cattle, sheep and goat.

2.6.2 Isolation and identification

2.6.2.1. Isolation

Fifteen isolates of β-haemolytic streptococci were obtained from 186 pneumonic swine lungs by L'Ecuyer *et al.* (1961).

Brown (1970) isolated α- haemolytic streptococci in pure cultures from 24 out of 62 cases of pneumonic swine.

Riley *et al.* (1973) isolated 10 tonsillar and four nasal, Group E streptococ*i* from swine. They reported the use of nasal and tonsillar swabbing and tonsillar biopsies to detect Group E streptococci in swine. They opined that although Group E streptococi were isolated irregularly and in small numbers, frequency was greater in the tonsils than in the nares and tonsillar swabbing was found to be more satisfactory than biopsy technique as a means of detecting, Group E streptococci.

Isolaton and identification of *Streptococcus pneumoniae* from a bison suffering from bronchopneumonia was reported by Ramachandra *et al.* (1983).

Nasser and El-Sayed (1997) obtained 13 isolates of β hemolytic Streptococci from 20 calves exhibiting acute signs of bovine respiratory disease, from Italy

In a study on respiratory tract infections of calves in Turkey, Aslan *et al.* (2002) could isolate haemolytic Streptococci (10 per cent)from 27 cases.

Siji (2003) reported eight isolates of *Streptococcus pyogenes* from 18 cases of respiratory infection in cattle.

2.6.2.2 Cultural and biochemical characterisation

Most streptococci produced small colonies of about one millimeter diameter after 48 hours of incubation and in case of the β- haemolytic streptococci the colonies appeared translucent. The group B streptococci produce a positive CAMP test with β- hemolytic *Staphylococcus aureus* (Quinn *et al.*, 1994)

2.6.3 Antibiogram

Siji (2003) reported that *Streptococcus pyogenes* isolates showed a 100 per cent sensitivity to enrofloxacin and ciprofloxacin, 75 per cent to chloramphenicol and gentamicin, 50 per cent to streptomycin and oxytetracycline, 37.5 per cent to amoxycillin and 25 per cent to trimethoprim.

2.6.4 Pathogenicity

The ability of streptococci to adhere to the upper respiratory tract is a pre-requisite for infection and pathogenicity and M proteins help in the adherance of the organism to the upper respiratory tract (Kurl ,1978)

β–haemolytic streptococci *are* generally more pathogenic than α-hemolytic streptococci. The virulence factors include streptolysins, hyaluronidase, DNAase, NADase, streptokinase and protease. (Quinn *et al.*,2002)

2.7. OTHER BACTERIA INVOLVED IN RESPIRATORY TRACT INFECTIONS

2.7.1. Incidence

Allan (1977) reported that potentially pathogenic bacteria could be isolated from normal pulmonary tissue in approximately 2/3 of animals and from similar proportion of calves having non fatal pulmonary diseases. They also reported the isolation of *Pasteurella multocida* from 11.5 per cent of calves suffering from pneumonia.

Barbour *et al.* (1997) reported that *Moraxella, Pseudomonas, Erysipelothrix* sp. and *Pasteurella multocida* are the most prevalent bacteria in the upper respiratory tract of unhealthy sheep.

2.7.2 Isolation and identification.

2.7.2.1 Isolation

Ishino *et al.* (1979) reported the isolation of *Pasteurella* sp. in combination with *Staphylococcus, Haemophilus, Streptococcus,* and

Corynebacterium sp. from cases of calf pneumonia occurring in mass rearing facilities.

Allen *et al.* (1991) suggested that nasopharyngeal swabs could be reliably used for research or diagnostic purposes to give a useful estimate of the pulmonary microbial flora in large groups of feedlot calves.

In an abattoir survey of pneumonia and pleurisy in swine from nine selected herds Hoie *et al.* (1991) isolated Pasteurella *multocida* from 54 per cent of the cases and *Actinobacillus pleuropneumoniae* from 11 per cent of the cases.

Walker (1996) opined that specimens for bacterial evaluation should be collected as close as possible to the actual site of infetion, either before antimicrobial agents are administered or three to four days after thier use had been discontinued. He also opined that samples of both normal and diseased lung tissue should be collected whenever possible. In an acute outbreak samples should be obtained from an animal, having acute form of disease than from a chronic case.

Naso pharyngeal swabs could be reliably used for research or diagnostic purposes to give a useful estimate of the pulmonary microbial flora in large groups of feedlot calves (Allen *et al.*, 1991)

In the pathological and microbiological studies on pneumonic lungs from Danish calves Tegtmeier *et al.* (1999) observed that *Hemophilus somnus* and *Pasteurella multocida* were some of the most commonly encountered bacterial pathogen in lungs.

Vogel *et al.* (2001) isolated *Pasteurella multocida* from 7.7 per cent and *Haemophilus somnus* from 23 per cent of cases of pneumonia in calves.

2.7.3 Antibiogram

Antimicrobial susceptibility studies were compared by the quantitative broth microdilution and the standard disk diffusion methods and found that microdilution method provided more clinically relevant information than that offered by antibiotic disc method for determining therapy of bacterial infections of animals (Cox *et al*., 1981).

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

2.7.4 Pathogenicity

Carter and Alwis (1989) reported that the inoculation of 0.1-0.2ml of washing of a swab from blood or bone marrow suspension containining *Pasteurella multocida* organisms to mouse subcutaneously or intramuscularly would cause the death of the mouse within 24 h.

Shivashankara *et al.* (2000) observed that *Pasteurella multocida* of bovine origin killed mice within 24h post inoculation.

Karunakaran (2004) reported that all the *Pasteurella multocida* isolates obtained from respiratory tract infections in ruminants were able to kill weaned mice at a concentation of $3x10^8$ organisms per ml within 24 hours, when injected by intraperitoneal route.

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and Tarsons and Genei brand plastic ware were used in this study. All chemicals used were of molecular biology grade, obtained from M/S Sigma-Aldrich, Bangalore Genei and Sisco Research Laboratories Private Limited (SRL). 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 HAEMOLYTIC BACTERIA

3.1.1 Collection of Samples

Nasal swabs from apparently healthy and clinically ill livestock; tracheal swabs and lung samples showing pneumonic lesions from slaughtered animals/ postmortem cases and blood samples from animals having respiratory infections formed the biomaterials for isolation trials.

Samples were collected from clinically ill livestock and at random from apparently healthy animals from in and around Thrissur district. Nasal swabs were collected at random from apparently healthy animals maintained at the Livestock, Pig, sheep and Goat farms, attached to the College of Veterinary and Animal Sciences, Mannuthy, from clinically ill animals presented at University Veterinary hospitals, and from animals slaughtered at Corporation slaughter house, Thrissur and Meat technology unit of the University. Tracheal swabs and lung samples showing pneumonic lesions were collected at random from animals slaughtered at the above slaughterhouses and also from the carcasses brought to the Department of Pathology for postmortem. Blood samples were obtained from animals that were clinically suspected for respiratory infections brought to University veterinary hospitals.

A total of 309 samples were taken, which comprised of 107 nasal swabs, 87 tracheal swabs, 75 lung samples and 40 blood samples. Of this 153 samples

were from cattle, 63 from goats, 12 from sheep and 81 from pigs. Details are given in Table 1.

3.1.2 Method of Collection of Sample

3.1.2.1 Nasal and Tracheal Swabs

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

Nasal swabs from apparently healthy and clinically suspected livestock were collected as follows. The nasal vestibule of each animal was thoroughly disinfected with 70 per cent alcohol and allowed to dry. Sterile swab was pushed about 10-15 cm into nasal passage and rotated to swab the mucosal lining of nasal fossa and brought to laboratory preserved over ice. Nasal and tracheal swabs from animals killed at local slaughterhouses were collected by swabbing the area with sterile swabs and brought to the laboratory preserved over ice as well as on transport enrichment medium specifically for the isolation of *Mannheimia haemolytica.*

3.1.2.2 Lung Samples

Representative portion of lung samples showing pneumonic lesions were collected from animals killed at slaughterhouses and from postmortem cases and carried to laboratory preserved over ice.

3.1.2.3 Blood Samples

Sterile vials containing 0.5 to one milliliter defibrinated ovine/bovine blood were used for collection.

Blood samples were collected from jugular vein of animals clinically suspected with respiratory infection. One to two drops of blood were added into

Table 1. Details of samples collected from different animal species and from different areas.

Type of sample	No. of samples collected	Species from which collected	Place of sample collection
Nasal swabs	107	Cattle, sheep, goat ,pig	1.University Veterinary hospitals 2.Thrissur corporation slaughter house and University Meat Technology Unit 3.Universitylivestock farms 4.Malappuram district 5.Kollam district 6.Thrissur district
Tracheal swabs	87	Cattle ,sheep, goat, pig	1.Corporation slaughter house 2.University Meat Technology Unit 3.Department of pathology
Lung samples	75	Cattle, sheep, goat, pig	1.Corporation slaughter house 2.University Meat Technology Unit 3.Department of pathology
Blood samples	40	Cattle, sheep, goat, pig	1.University Veterinary hospitals 2.Thrissur district 3.Kollam district 4.Malappuram district

the sterile vials containing 0.5 to one millilitre of defibrinated ovine /bovine blood.

3.2 MEDIA USED FOR ISOLATION

3.2.1 Materials

3.2.1.1 Blood Agar

Brain heart infusion agar supplemented with 5-10 per cent sterile ovine/ bovine blood was used for isolation. Brain heart infusion agar base (Hi-media) was prepared as per the manufacturer's instructions.

3.2.1.2 Transport enrichment medium

The transport enrichment medium was made by adding:

Bactoagar	3.0g
BHI brothbase	37.0g
Distilledwater	1000.0ml
Amikacin (10mg/ml of stock solution)	1.0ml
Gentamicin (10mg/ml of stock solution)	1.0ml
Potassium tellurite (0.4mg/ml of stock solution)	1.0ml
Bacitracin (6mg/ml of stock solution)	1.0ml
AmphotericinB(4mg/ml of stock solution in acetone)	1.0ml

Mixed well the first two ingredients in distilled water and autoclaved at 121^0 C at 15 lb pressure for15-20 minutes. Cooled to approximately 50^0 C, and then added the antibiotics per liter of BHI solution. Dispensed in 10 ml aliquots and stored at 4^{0}C.

3.2.1.3 Mannheimia haemolytica selective medium

The media was prepared as per Morris (1958). Brain heart infusion agar served as the base of the formulation. After autoclaving at 121^0 C at 15 lb pressure for 15-20 minutes and cooling to 50^0 C the basal medium was added with Neomycin 1.5µg/ml, novobiocin 2.5µg/ml, actidione 100µg/ml and sterile defibrinated ovine blood at 5 per cent level.

3.2.1.4 *Other media used*

Mannitol salt agar (Hi-media) for *Staphylococcus aureus,* Edward's medium (Hi-media) for *Streptococcus* sp, Mac Conkey's agar, and Eosin methylene blue agar(Hi-media) were used for identification of the bacteria. These media were prepared as per the manufacturer's instructions.

3.2.2 Method of Isolation

3.2.2.1 Nasal and Tracheal Swabs

Swabs were collected in duplicate one in transport medium and other in Brain heart infusion broth. The swab from transport medium was streaked on *Mannheimia haemolytica* selective medium and the swab from Brain heart infusion broth was inoculated on blood agar. The inoculated selective medium was incubated in a candle jar and the inoculated blood agar plate was incubated aerobically at 37^{0}C for 24-48 h.

3.2.2.2 Lung Samples

Lung samples showing pneumonic lesions were streaked onto the *Mannheimia haemolytica* selective media and blood agar and incubated at 37°C as above for 24-48 h.

3.2.2.3 Blood Samples

Blood samples collected in defibrinated ovine/bovine blood were streaked onto *Mannheimia haemolytica* selective media and blood agar plates and incubated at 37°C for 24 – 48 h as above.

3.3 IDENTIFICATION

Reference strains of *Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus pneumoniae* obtained from Institute of Microbial Technology, Chandigarh was employed for the study.

All haemolytic colonies on blood agar and on selective media were stained by Gram's method to study the morphological features. The Gram - positive haemolytic colonies obtained were subcultured on mannitol salt agar and on Edward's media for presumptive identification of *Staphylococcus* or *Streptococcus* sp. The Gram-negative isolates were subcultured on Mac Conkey's agar and eosin methyleneblue agar. Based on the cultural characteristics produced on these media the organisms were classified and subjected to further biochemical tests.

Further identification was done based on tests like catalase and oxidase, oxidative or fermentative utilization of glucose, indole production, methyl red and Voges-Proskauer reactions, urease activity, H_2S production, nitrate reduction, citrate utilization, gelatin liquefaction, beta-galactosidase activity, lysine and ornithine decarboxylase activities, and production of acid or gas or both from the carbohydrates (glucose, galactose, inositol, lactose, maltose, mannitol, mannose, salicin, sucrose, dulcitol, sorbitol, trehalose, xylose, and arabinose) as described

3.4 ANTIBIOGRAM

3.4. 1 Materials

Mueller–Hinton agar was used to study the antibiotic sensitivity pattern of the isolates. The following antibiotic discs with known concentrations as noted in micrograms (µg) or international units (IU) per disc were used.

1. Ampicillin (A) - 10 µg
2. Chloramphenicol (C) - 3 µg
3. Cloxacillin (Cx) - 5 µg
4. Co-trimoxazole (Co) - 25µg
5. Amoxycillin (Am) - 10µg
6. Gentamicin (G) - 30 µg
7. Cefotaxim(Cf) - 5µg
8. Nitrofurantoin (Nf) -300 µg
9. Penicillin G (P) -10 IU
10. Pefloxacin (Pf) - 5 µg
11. Streptomycin (S) -10 µg
12. Oxytetracycline (T) - 30 µg

3.4.2 Method

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

3.5 PATHOGENICITY TESTING OF ISOLATES

3.5.1 Materials

Swiss albino mice of six to eight weeks of age were procured from the Small Animal breeding station (SABS), College of Veterinary and Animal Sciences, Mannuthy.

Swiss albino mice of six to eight weeks of age were procured from the Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthy.

3.5.2 Method

Each mouse was inoculated intraperitoneally with 0.5 ml of inoculum containing approximately 3 x 10^8 organisms per ml in sterile normal saline. A control mouse was injected with 0.5 ml of sterile saline. A total of six mice were used for each isolate. All the animals were observed for signs of infection for a period of 14 days. Heart blood smears collected from dead mice were stained with Leishman's stain. Re-isolation of the inoculated organism from heart blood, lung, liver and spleen of the dead mice was carried out.

3.6 STORAGE OF ISOLATES

Storage of the isolates were done on blood agar slants kept at 4^0C

3.7 POLYMERASE CHAIN REACTION FOR DETECTION OF *Mannheimia haemolytica*

3.7.1 Buffers and Reagents for PCR

Buffers and reagents were obtained from Bangalore Genei.

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

Sodium chloride	80.00 g
Potassium chloride	2.00 g
Disodium hydrogen phosphate	11.33 g
Potassium di hydrogen phosphate	2.00 g
Distilled water	1000 ml

3.7.1.2 Primers for Mannheimia haemolytica specific PCR

Species-specific primers, to detect *Mannnheimia haemolytica* used *by* Green *et al.* (1999) were used. The sequences of the primers were as follows:

AMU (Upper primer) 5' GGG CAA CCG TGA AGA AAA AAT AG 3'
C3575 (Lower primer) 5' CGC CAT TTT GAC CGA TGA TTT C 3'

The primers were custom synthesized by IDT, USA.

3.7.1.3 PCR reaction buffer (10 X)

This includes 100 mM Tris–HCl pH 9.0, 15 mM $MgCl_2$ and 500 mM KCl.

3.7.1.4 Taq DNA polymerase

The *Taq* DNA polymerase enzyme with a conc. of 3U/µl.

3.7.1.5 Deoxy ribonucleotide triphosphate

Deoxy ribonucleotide triphosphate (dNTP) mix

10 mM (2.5mM each of dGTP, dCTP, dATP and dTTP in equal volume)

3.7.1.6 Magnesium chloride

Magnesium chloride with strength of 25 mM

3.7.1.7 DNA molecular size markers

(a) pBR-322 DNA/*Alu I* digest

With fragments of 908, 659, 656, 521, 403, 281, 257, 226, 100, 90,

63, 57, 49, 46, 19,15 and 11 base pairs

(b) 100 bp DNA Ladder

With fragments of 100,200,300,400,500,600,700,800,900,1000 base pairs

The molecular size markers were purchased from M/s Bangalore Genei (INDIA).

3.7.1.8 Reference Strain of Mannheimia haemolytica

Reference strain could not be obtained elsewhere from India.

3.7.1.9 Other Bacterial Strains

The following bacterial strains maintained in the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur were used in the present study for testing the specificity of the PCR

(i) *Leptospira icterohaemorrhagiae*

(ii) *Escherichia coli*

(iii) *Staphylococcus aureus* (Reference strain MTCC-1144 of *Staphylococcus aureus* obtained from Institute of Microbial Technology,Chandigarh was employed for the study).

3.7.2 Method

3.7.2.1 Preparation of samples for PCR analysis

3.7.2.1a Nasal and tracheal swabs and lung samples

Nasal and tracheal swabs and representative portion of lung samples were inoculated into 3 ml of brain heart infusion broth and incubated at 37° C for three

hours. The tubes containing inoculated broth were mixed thoroughly in a vortex mixer for 3 minutes. After that 1.5 ml of broth culture was transferred into an Eppendorf tube and centrifuged at 3000 X g for 15 min. The pellet was washed twice with sterile PBS (3.7.1.1) and resuspended in 200 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min, thawed and centrifuged at 3000 X g for 10 min and supernatant was stored at –20°C.

Lung samples, nasal and tracheal swabs directly streaked onto the selective medium and blood agar were examined after over night incubation. Colonies showing haemolysis and gram negative staining reaction were selected for PCR analysis. For this a pure colony was inoculated into five millilitres of BHI broth and incubated at 37 °C for 18 h. From this broth culture 1.5 ml was transferred to an Eppendorf tube and centrifuged at 3000 X g for 10 min, the supernatant was discarded, washed the pellet twice with sterile PBS (3.7.1.1) and final pellet was resuspended in 100 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The samples were thawed and centrifuged at 3000 X g for 5 min and supernatant was stored at -20°C for further use as template for PCR reactions.

3.7.2.1b Other bacterial strains

To determine the specificity of primer pairs they were tested against the DNA from other bacterial strains (3.7.1.9). The template DNA from other bacterial strains was prepared as described for *Mannheimia haemolytica*

3.7.2.1c Blood samples and blood smears

Blood samples and blood smears obtained from suspected cases were processed to prepare DNA. Two hundred microlitres of blood was taken in Eppendorf tubes. Sterile triple distilled water was added to make up the volume to 1.5 ml and centrifuged at 3000 X g for 15 min. The supernatant was discarded and cell pellet was washed twice with sterile PBS (3.7.1.1) and then resuspended

in 100 µl sterile triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The samples were then thawed and again centrifuged at 3000 X g for 10 min and supernatant was stored at -20°C for further use as template for PCR reactions.

Blood smears were scraped with a blade into an Eppendorf tube containing 1.5 ml sterile distilled water and the mixture was kept at 37°C for 30 min. It was then centrifuged at 3000 X g for 15 min, the cell pellet was washed twice with sterile PBS (3.7.1.1) and final pellet was resuspended in 50 µl sterile triple distilled water. The mixture was boiled for 10 min and immediately chilled over ice for 30 min, thawed and centrifuged at 3000 x g for 10 min and supernatant was stored at – 20 °C.

3.7.2.2 Reconstitution and Dilution of Primers

Primers obtained in lyophilized form were reconstituted in 100 µl of sterile triple glass distilled water to a concentration of 200 picomoles (pM/µl). The tubes were kept at room temperature with occasional shaking for one hour. They were spun briefly to pellet down the insoluble particles if any, and the stock solution was distributed into 10 µl aliquots and stored at -70°C. At the time of use the aliquots were thawed and further diluted 10 fold to obtain a concentration of 20 picomoles/µl before using for PCR.

3.7.2.3 Setting up of Mannnheimia haemolytica specific PCR

The PCR reaction was carried out with *Mannheima haemolytica* specific primers AMU and C3575 as described by Green *et al* (1999).

Polymerase chain reaction was performed in a total volume of 25-µl reaction mixtures. A master mix was prepared before setting up the PCR reaction by combining the following reagents in a 20-µl volume.

PCR reaction buffer	50 mM Kcl, 10 mM Tris hydrochloride, 1.5 mM $MgCl_2$
Primers	20 pM of each primer

dNTPs	200 µM of each dNTP
Taq polymerase	one unit

Preparation of 200-µl master mix for ten reactions was as follows:

Reagents	Quantity
PCR reaction buffer (10 X)	25µl
Forward primer	10µl
Reverse primer	10µl
dNTP mix	20µl
Taq DNA polymerase	3.3µl
Triple distilled water to make	200µl

To each PCR tube 20 µl of master mix and five µl of template DNA were added. One negative control without template DNA was included to monitor contamination, if any. The tubes were spun briefly and placed in the thermal cycler.

The programme of amplification was as follows:

Primers AMU and C3575

First nine cycles of Denaturation at 95°C for 30 sec, Annealing at 54°C for 60 sec and extension at 72°C for 2min. This was followed by 30 cycles of, denaturation at 95°C for 60 sec, annealing at 54°C for 35 sec, extension at 72°C for 2min and a final extension of 72°C for 7 min.

The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany).

3.8 RANDOM AMPLIFIED POLYMORPHIC DNA-PCR FOR *Staphylococcus aureus*

3.8.1 Buffers and reagents

Buffers and reagents used for PCR were the same as that used for *Mannheimia haemolytica* specific PCR except the primers. Specificity of the primers was checked in a manner similar to that for *Mannheimia haemolytica*, using other bacterial strains.

3.8.1.2 Primers

The RAPD PCR was carried out with primers as described by Williams *et al.* (1990) and Ahmed *et al.* (1998)

Primers of Williams et al. (1990)

OLP6 5' GAGGGAAGAG 3'

OLP11 5' ACGATGAGCC 3'

Primer of Ahmed et al. (1998)

RAPD 7 5'GTAGGATGCGA 3'

3.8.1.3 DNA molecular size markers

The markers were purchased from Bangalore Genei.

(a)100 bp DNA Ladder

With fragments of 100,200,300,400,500,600,700,800,900,1000 base pairs

(b)Low Range DNA Ruler

With fragments of 100,200,300,600,1000,1500,2000,2500,3000. base pairs

© pUC 18/*Sau3 A1*-Puc 18/*Taq 1* Digest

With fragments of 78,105,153,258,341,458,585,754,943,1444. base pairs.

3.8.1.4 Reference strain of Staphylococcus aureus

Reference strain MTCC-1144 of *Staphylococcus aureus* obtained from Institute of Microbial Technology, Chandigarh was employed for the study.

3.8.2 Method

3.8.2.1 Preparation of samples for PCR analysis.

3.8.2.1a Reference strain of Staphylococcus aureus

The reference strain of *Staphylococcus aureus* was grown overnight in blood agar plates at 37°C and a pure colony was inoculated into five millilitres of BHI broth and incubated at 37 °C for 18 h. From this broth culture 1.5 ml was transferred to an Eppendorf tube and centrifuged at 3000 Xg for 10 min, the supernatant was discarded, washed the pellet twice with sterile PBS (3.5.1.1) and final pellet was resuspended in 100 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The samples were thawed and centrifuged at 3000 X g for 5 min and supernatant was stored at – 20°C for further use as template for PCR reactions.

3.8.2.1b Clinical isolates of Staphylococcus aureus

Template DNA was prepared in the same manner as that for the reference strain .

3.8.2.2 Reconstitution and Dilution of Primers

It was done in a manner similar to that done for *Mannheimia haemolytica* primers.

3.8.2.3 Setting up of RAPD- PCR for Staphylococcus aureus

The PCR reaction was carried out with RAPD- PCR primers described by Williams *et al.* (1990) *i e.,* OLP6 and OLP11, and Ahmed *et al.* (1998) *i e.,* RAPD 7.

Polymerase chain reaction was performed in a total volume of 25 µl reaction mixture. A master mix was prepared before setting up the PCR reaction by combining the following reagents in a 20-µl volume.

PCR reaction buffer	50 mM Kcl, 10 mM Tris hydrochloride, 1.5 mM $MgCl_2$
Primers	20 pM of each primer
dNTPs	200 µM of each dNTP
Taq polymerase	one unit

Preparation of 200-µl master mix for ten reactions was as follows:

Reagents	Quantity
PCR reaction buffer (10 X)	25µl
Forward primer	10µl
dNTP mix	20µl
Taq polymerase	3.3µl
Triple distilled water to make	200µl

To each PCR tube 20 µl of master mix and five µl of template DNA were added. One negative control without template DNA was included to monitor contamination, if any. The tubes were spun briefly and placed in the thermal cycler.

The programme of amplification was as follows:

The programme of amplification was as follows:

(a) Primer OLP6 and Primer OLP11

A predenaturation at 94°C for 5 min followed by 40 cycles of denaturation at 93° C for 60 sec, annealing at 37° C for 90 sec, extension at 72° C for 1min and a final extension of 72° C for 8 min.

(b) Primer RAPD7

A Predenaturation at 94° C for 4 min followed by 40 cycles of denaturation at 94° C for 45 sec, annealing at 25° C for 45 sec, extension at 72°C for 2 min.

The PCR amplification was carried out in an automated thermal cycler as for *Mannheimia haemolytica* specific PCR.

3.9 DETECTION OF PCR PRODUCTS

3.9.1 Materials for Submarine Agarose Gel Electrophoresis

3.9.1.1 0.5 M EDTA (pH 8.0)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 70 ml of triple glass distilled water. The pH was adjusted to 8.0 with 1 N NaOH. The volume was made upto 100 ml, filtered, autoclaved at 121^0 C at 15 lb pressure for 15 min and stored at room temperature.

3.9.1.2 Tris Borate EDTA buffer (TBE) (10 X) pH 8.2

Tris base	108.0 g
Boric acid	55.0 g
0.5 M EDTA, pH (8.0)	40 ml

3.9.1.3 Agarose Gel (1.5 percent)

Agarose low EEO (Genei)	1.5 g
TBE buffer	100 ml

3.9.1.4 Ethidium Bromide stock solution

Ethidium bromide (SRL)	10 mg
Triple distilled water	1 ml

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

3.9.1.5 Gel loading buffer (6 X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Sucrose	40.00g
Distilled water	100 ml

Stored at 4 °C.

3.9.2 Method

The amplified products of PCR were detected by electrophoresis in 1.5 per cent agarose gel in TBE buffer (1 X). Agarose was dissolved in TBE buffer (1 X) by heating and cooled to 50°C. To this, ethidium bromide was added to a final concentration of 0.5 µg/ml. The clean, dry gel platform edges were sealed with adhesive tape and the comb was kept in proper position before pouring melted agarose. Once the gel was set, the comb and adhesive tape were removed gently and placed the gel tray in buffer tank. Poured TBE buffer (1 X) till it covered the top of the gel completely.

Five microlitres of amplified product was mixed with one microlitre of 6 X gel loading buffer (3.7.1.5) and samples were loaded into respective slots carefully. The pBR 322/ *Alu I*digest for *Mannheimia haemolytica* specific PCR, 100 B.P DNA ladder, Low Range DNA Ruler and pUC 18/*Sau3 A1*-Puc 18/*Taq 1* Digest for RAPD PCR were used as DNA molecular size markers.

Electrophoresis was carried out at 5V/cm for one hour (or) until the bromophenol blue dye migrated more than two-third of the length of the gel. DNA fragments were viewed on a transilluminator and photographed using a gel documentation system. (Bio-Rad, USA) .

3.10 PLASMID PROFILE OF *Escherichia coli* ISOLATES

3.10.1 Isolation of Plasmid DNA

3.10.1.1 Materials

3.10.1.1a Luria Bertani Broth

Yeast extract	5 g
NaCl	10 g
Tryptone	10 g
Distilled water to	1000 ml

Autoclaved at 121^0 C at 15 lb pressure for 15 min

3.10.1.1b 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 sterilised by autoclaving at 121^0 C at 15 lb pressure for 15 min..

3.10.1.1c 3M Sodium acetate solution (pH 4.8)

Sodium acetate	40.81 g
Triple distilled water	70 ml

Adjusted pH with glacial acetic acid and stored at 4^0 C

3.10.1.1d 1N NaOH

NaOH	4 g
Triple distilled water to	100 ml

Stored at room temperature.

3.10.1.1e Tris EDTA glucose(TEG) buffer pH (8.0)

Tris (0.25 M)	2.5 ml 1M Tris
Glucose (50mM)	9.008 g
EDTA (10mM)	2ml of 0.5 M EDTA

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

3.10 .1.1f Tris EDTA buffer (TE) pH (7.8)

Tris	1 ml of 1M Tris
EDTA	0.2 ml of 0.5 M EDTA

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

3.10.1.1g 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 .

3.10.1.1h Phenol: Chloroform: Isoamyl alcohol (25:24:1)

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

3.10.1.1i Chloroform: Isoamyl alcohol (24: 1)

Chloroform	24 ml
Isoamyl alcohol	1 ml

Mixed and stored in amber coloured bottles

3.10.1.1j Ethanol 70 percent

Ethanol	70 ml
Distilled water	30 ml

Mixed and stored in amber coloured bottles

3.10.1.1k Ribonuclease A

Ribonuclease A 10 mg

Distilled water 2 ml

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

3.10.1.2 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 was 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. Washed the DNA pellet twice with 70 per cent ethanol, dried and resuspended in 20 µl of TE Buffer. Ribonuclease A (10mg/ml) 2 µl was

added and incubated at 37 °C for half an hour and then stored at –20°C. Plasmid DNA from *Escherichia coli* V517 maintained in the Department of microbiology was prepared in a similar manner.

Electrophoresis

The isolated plasmid DNA was analysed by submarine gel electrophoresis using 0.8 percent 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 bottom of the gel. DNA fragments were viewed on a transilluminator and photographed using a gel documentation system (Bio-Rad, USA).

Results

4. RESULTS

4.1 ISOLATION OF HAEMOLYTIC BACTERIA

Blood agar used as the principal medium for isolation trials, supported the growth of reference strains of *Staphylococcus aureus, Streptococus pyogenes* and *Streptococcus pneumoniae* and all the different types of bacteria. The *Mannheimia haemolytica* specific transport medium and selective medium supported the growth of most of the Gram-negative organisms, but prevented the growth of Gram- positive organisms and other contaminant bacteria. *Mannheimia haemolytica* could not be isolated from any of the nasal and tracheal swabs, lung samples and blood samples cultured on selective medium, as well as blood agar. Pooled sample of nasal swabs collected from animals with respiratory tract infections were also cultured on blood agar and *Mannheimia haemolytica* selective media. One of the pooled samples cultured on blood agar gave an isolate with characteristics almost similar to *Mannheimia haemolytica,* but showed variations for ornithine decarboxylase activity and utilization of sugars like trehalose and salicin. Since no reference strain of *Mannheimia haemolytica* was available for comparison, the isolate could not be confirmed as *Mannheimia haemolytica.*

From the samples cultured on blood agar medium, pure and mixed cultures of different haemolytic organisms could be obtained. A total of 20 haemolytic bacteria could be isolated in pure culture. Out of the 20 isolates obtained, 65 per cent *i.e.* 13 were Gram-positive organisms and 35 per cent *i.e.* seven were Gram-negative organisms. Out of the 13 Gram- positive isolates inoculated on mannitol salt agar 10 of them gave yellow coloured colonies and the medium changed from pink to yellow. They were presumptively identified as *Staphylococcus* sp. Two of the Gram-positive isolates showed growth on Edward's medium and were presumptively identified as *Streptococcus* sp. All the seven Gram- negative isolates produced pink coloured growth on Mac Conkey's

agar and all of them showed growth on eosin methylene blue agar, but only six gave the typical metallic sheen. The six isolates which gave metallic sheen on eosin methylene blue agar were subjected to Eijkman's test and were found positive and thus presumptively identified as *Escherichia coli.* The other Gram-negative isolate which produced mucoid colonies with semifluid consistency on blood agar was subjected to further characterization presuming that it was *Klebsiella.* A single Gram-positive isolate with pin point haemolytic colonies gave coryne-form arrangement on staining and did not show any growth on any of the above-mentioned media other than blood agar. It was presumed as *Arcanobacterium* species and was further characterized.

Upon further biochemical characterization the species level identification of all the 20 isolates could be made. The different bacterial isolates were *Staphylococus aureus-* eight isolates (40 per cent), *Staphylococcus epidermidis-* two isolates (10 per cent), *Escherichia coli-* 6 isolates (30 per cent), *Klebsiella pneumoniae-* one isolate(5 per cent), *Streptococcus pyogenes* –one isolate(5 per cent), *Streptococcus agalactiae*-one isolate (5 per cent), *Arcanobacterium pyogenes* -one isolate(5 per cent)(Fig.1). The *Staphylococcus* and *Escherichia coli* isolates were designated as following. Bovine isolates of *Staphylococcus aureus* were named as SAb1, SAb2, SAb3 and SAb4, the caprine isolates as SAc1, SAc2, SAc3 and the porcine isolate as SAp1. The bovine isolate of *Escherichia coli* was designated as EB1, caprine isolates as EC1, EC2, EC3 and porcine isolates as EP1 and EP2. The bovine isolates of *Staphylococcus epidermidis* were designated as SEb1 and SEb2.

The caprine isolates of *Staphylococcus aureus* SAc1, SAc2 and SAc3 were obtained from tracheal swabs of three goats died of pneumonia. They were isolated in combination with three *Escherichia coli* isolates EC1, EC2 and EC3. The bovine isolate of *Staphylococcus aureus* SAb1 was obtained from lung sample of a cow died of pneumonia and the porcine isolate of *Staphylococcus aureus* SAp1 was obtained from lungs of a pig died of pneumonia. The *Staphylococcus epidermidis* isolates were obtained from tracheal swabs of

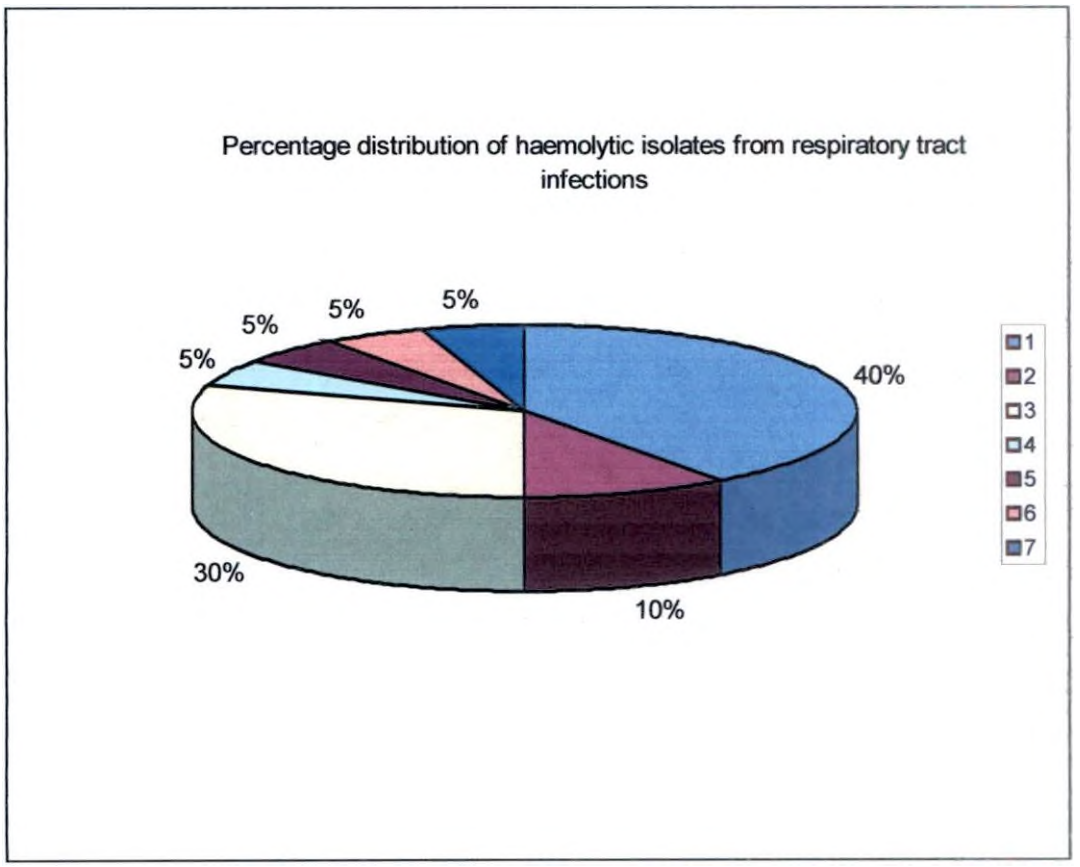

Fig .1. Percentage distribution of haemolytic isolates from respiratory tract Infections

1. *Staphylococcus aureus*
2. *Staphylococcus epidermidis*
3. *Escherichia coli*
4. *Klebsiella pneumoniae*
5. *Streptococcus pyogenes*
6. *Streptococcus agalactiae*
7. *Arcanobacterium pyogenes*

apparently healthy cattle slaughtered in Thrissur Corporation slaughterhouse. The bovine *Escherichia coli* EB1 was isolated from the tracheal swab of cow died of respiratory infection. The porcine *E.coli* isolate EP1 was from tracheal swab of a case of respiratory infection and EP2 was isolated from lungs of a case of pneumonia. The *Klebsiella* isolate was from the lungs of an apparently healthy animal slaughtered at the University Meat Plant. *Streptococcus pyogenes* was isolated from the lungs of a pig died of pneumonia. *Streptococcus agalactiae* was isolated from the lungs of one of the goats died of pneumonia from which *E. coli* and *Staphylococcus aureus* were isolated from tracheal swab. The *Arcanobacterium pyogenes* isolate was obtained from the lung sample of a calf died of pneumonia.

Most of the isolates were obtained in pure cultures. Mixed cultures of bacteria were also obtained. From three cases *Staphylococcus aureus* was isolated in combination with *Escherichia coli*. Pure cultures of *Staphylococcus aureus* and *Escherichia coli* were obtained from five and three different samples each respectively, from samples collected from animals having respiratory infection. *Klebsiella pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae* and *Arcanobacterium pyogenes* were obtained in pure cultures.

All the isolates were subcultured and maintained in the pure form.

4.2 IDENTIFICATION OF THE ORGANISM

4.2.1 *Staphylococcus* sp.

Staphylococcus aureus was identified based on cultural characteristics and biochemical tests. *Staphylococcus aureus* isolates produced haemolysis on blood agar and showed yellow coloured colonies on mannitol salt agar. (Fig.2.). Further identification was done on the basis of Gram's staining, morphology, motility, catalase, oxidase, O/F test, VP, MR and nitrate reduction tests. Isolates of *Staphylococcus aureus* and

Staphylococcus epidermidis were differentiated based on sugar fermentation tests and coagulase activity, which is characteristic of the pathogenicity of former. The *Staphylococcus aureus* isolates were coagulase positive, whereas *Staphylococcus epidermidis* were found coagulase negative. Details of characterisation of *Staphylococus aureus* and *Staphylococcus epidermidis* are given in tables 2 and 3.

4.2.2 *Escherichia coli*

Escherichia coli isolates produced haemolysis on blood agar and gave lactose fermenting pink coloured colonies on Mac Conkey agar. They gave a characteristic metallic sheen on EMB agar. *E. coli* isolates were identified mainly based on the IMViC test for which it gave a result of Indole (+), Methyl red (+), VP (-) and Citrate (-). Presumptive identification was done on the basis of Eijkman's test. All the six isolates were found positive for Eijkman's test. Details of characterization of *E. coli* isolates are given in tables 4 and 5.

4.2.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae isolates produced haemolysis on blood agar and lactose fermenting pink coloured colonies on Mac Conkey agar. On EMB agar mucoid colonies with semifluid consistency was produced. Identification was based on IMViC test for which it gave a result of (-+-+). Urease test was found positive. Details of characterization are given in tables 6 and 7.

4.2.4 *Streptococcus* sp

Streptococcus pyogenes and *Streptococcus agalactiae* isolates produced growth on Edward's medium, showing haemolytic, transparent, small colonies. They were β haemolytic on blood agar. (Fig .3.). Differentiation of *Streptococcus pyogenes* and *Streptococcus agalactiae* was done based on hydrolysis of hippurate, sensitivity to bacitracin and sugar fermentation tests. *Streptococcus agalactiae* gave a positive result for hydrolysis of hippurate, while Streptococus *pyogenes* gave a negative result. *Streptococcus pyogenes* was found sensitive to bacitracin whereas *Streptococcus agalactiae* was found resistant. Details are presented in table 8.

4.2.5 *Arcanobacterium pyogenes*

Arcanobacterium pyogenes produced small, haemolytic colonies on blood agar. It was catalase (-) and oxidase (+). Growth was found enhanced by CO_2. Pitting of Loeffler's slope, which is characteristic for Arcanobacterium, *pyogenes* was found positive for the isolate. Details are given on table 9.

4.3 ANTIBIOGRAM

Staphylococcus aureus isolates showed a sensitivity of 100 per cent to ampicillin and pefloxacin, 87.5 per cent to cloxacillin, gentamicin, cefotaxim, penicillin G, streptomycin and oxytetracycline, 75 per cent to chloramphenicol and erythromycin, 37.5 per cent to co-trimoxazole and 25 per cent to amoxycillin (Table 10, Fig. 4.).

Staphylococcus epidermidis isolates were 100 per cent sensitive to ampicillin, chloramphenicol, cloxacillin, co-trimoxazole, gentamicin,

Fig. 2. *Staphylococcus aureus* colonies on Mannitol Salt Agar

Fig. 3. Haemolytic colonies of *Streptococcus pyogenes* on blood agar

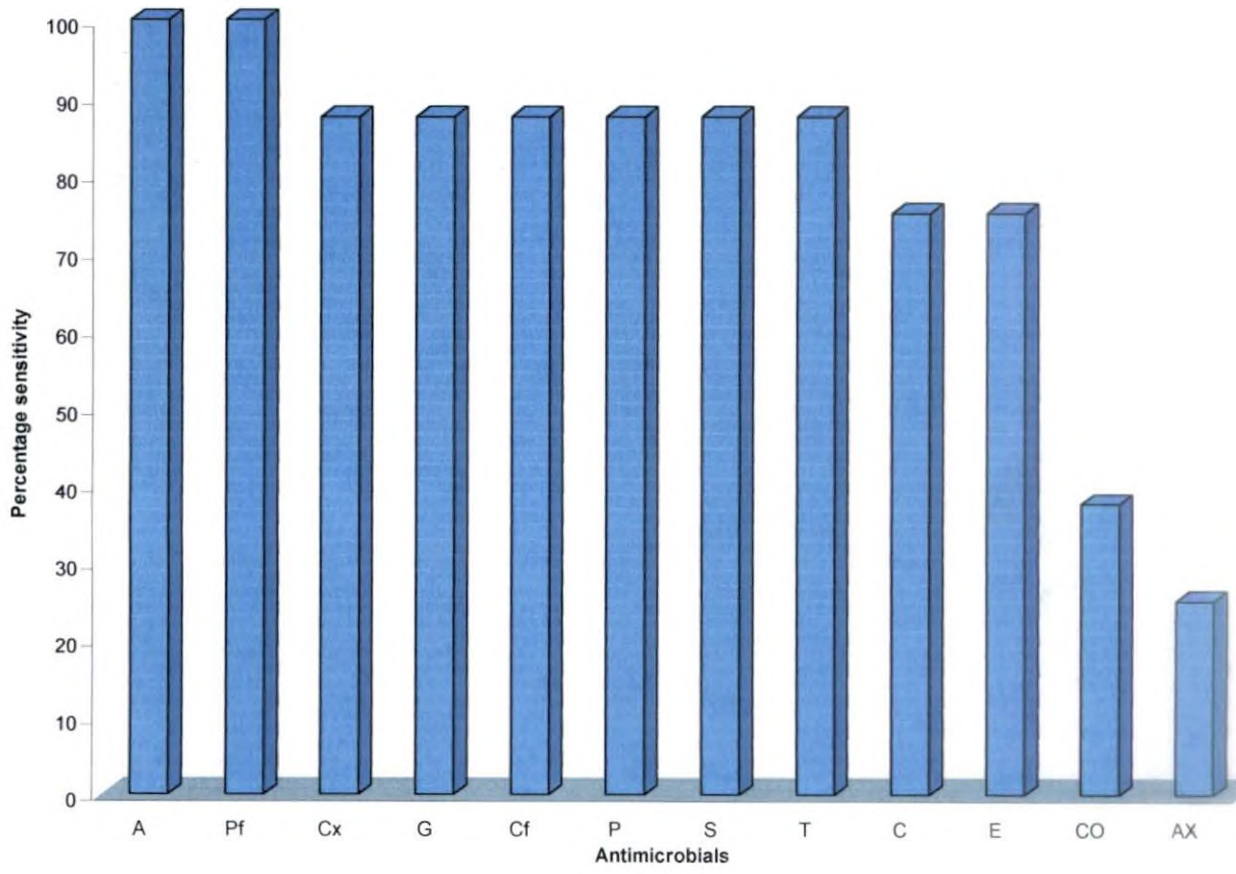

Fig. 4. Percentage distribution of antimicrobial sensitivity pattern of *Staphylococcus aureus* isolates

1. **A - Ampicillin**
2. **Pf - Pefloxacin**
3. **Cx - Cloxacillin**
4. **G - Gentamicin**
5. **Cf - Cefotaxim**
6. **P - Penicillin G**
7. **S - Streptomycin**
8. **T - Oxytetracycline**
9. **C - Chloramphenicol**
10. **E - Erythromycin**
11. **CO - Co-trimoxazole**
12. **Ax - Amoxycillin**

Table 2. Identification of *Staphylococcus* sp

Test	*Staphylococcus aureus*								*Staphylococcus epidermidis*	
	SAb1	SAb2	SAb3	SAb4	SAc1	SAc2	SAc3	SAp1	SEb1	SEb2
Gram's staining	Gram + ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram+ve
Morphology	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters	Cocci in irregular clusters
Capsule	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent
Motility	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
Growth in air	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Growth anaerobically	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Hemolysis on blood agar	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Growth on Mac Conkey agar	-ve	-ve	-ve	- ve	-ve	-ve	-ve	-ve	-ve	-ve
O/F of glucose	F	F	F	F	F	F	F	F	F	F
VPtest	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
MR	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Nitrate reduction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Coagulase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve

Table 3. Identification of *Staphylococcus* sp -sugar fermentation tests

Test	*Staphylococcus aureus*								*Staphylococcus epidermidis*	
	SAb1	SAb2	SAb3	SAb4	SAc1	SAc2	SAc3	SAp1	SEb1	SEb2
Acid from										
Lactose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Fructose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Trehalose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Xylose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cellobiose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Raffinose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Mannose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve

Table 4. Identification of *Escherichia coli*

Test	*Escherichia coli isolates*					
	EB1	ECI	EC2	EC3	EP1	EP2
Gram's reaction	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve
Morphology	rods	rods	rods	rods	rods	rods
Motility	+ve	+ve	+ve	+ve	+ve	+ve
Growth in air	+ve	+ve	+ve	+ve	+ve	+ve
Growth anaerobically	+ve	+ve	+ve	+ve	+ve	+ve
Growth on Mac Conkey's agar	Pink coloured colonies	Pink coloured colonies	Pink coloured colonies	Pink coloured colonies	Pink coloured colonies	Pink coloured colonies
Haemolysis	+ve	+ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	-ve	-ve	-ve	-ve
O/F of glucose	F	F	F	F	F	F
Simmons citrate	-ve	-ve	-ve	-ve	-ve	-ve
Urease	-ve	-ve	-ve	-ve	-ve	-ve
MR	+ve	+ve	+ve	+ve	+ve	+ve
VP	-ve	-ve	-ve	-ve	-ve	-ve
Indole	+ve	+ve	+ve	+ve	+ve	+ve

Table 5. Identification of *Escherichia coli*- sugar fermentation reactions

Test	ESCHERICHIA COLI					
	EB1	EC1	EC2	EC3	EP1	EP2
Adonitol	-ve	-ve	-ve	-ve	-ve	-ve
Arabinose	+ve	+ve	+ve	+ve	+ve	+ve
Cellobiose	-ve	-ve	-ve	-ve	-ve	-ve
Dulcitol	-ve	-ve	-ve	-ve	-ve	-ve
Inositol	-ve	-ve	-ve	-ve	-ve	-ve
Lactose	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	-ve	-ve	-ve	-ve	-ve	-ve
Raffinose	+ve	+ve	+ve	+ve	+ve	+ve
Rhamnose	+ve	+ve	+ve	+ve	+ve	+ve
Salicin	-ve	+ve	-ve	-ve	-ve	-ve
Sorbitol	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	-ve	-ve	-ve	+ve	-ve	-ve
Trehalose	+ve	+ve	+ve	+ve	+ve	+ve
Xylose	+ve	+ve	+ve	+ve	+ve	+ve

Table 6. Identification of *Klebsiella pneumoniae*

Test	Reaction/ results
Gram's reaction	Gram - ve
Morphology	Rods
Motility	-ve
Growth in air	+ve
Growth anaerobically	+ve
Growth on Mac Conkey's agar	Pink coloured colonies
Haemolysis	+ve
Catalase	+ve
Oxidase	-ve
O/F of glucose	F
Simmons citrate	+ve
Urease	+ve
MR	+ve
VP	-ve
Indole	-ve

Table 7. Identification of *Klebsiella pneumoniae*- sugar fermentation tests

Test	Reaction
Adonitol	+ve
Arabinose	+ve
Cellobiose	+ve
Dulcitol	+ve
Inositol	+ve
Lactose	+ve
Maltose	+ve
Mannitol	+ve
Raffinose	+ve
Rhamnose	+ve
Salicin	+ve
Sorbitol	+ve
Sucrose	+ve
Trehalose	+ve
Xylose	+ve

Table 8. Identification of *Streptococcus* sp

Test	*Streptococcus pyogenes*	*Streptococcus agalactia*
Gram's reaction	+ve	+ve
Shape	cocci	cocci
Spore	-ve	-ve
Motility	-ve	-ve
Growth in air	+ve	+ve
Growth anaerobically	+ve	+ve
Catalase	-ve	-ve
Oxidase	-ve	-ve
O/F of glucose	F	F
Haemolysis	β hemolysis	β hemolysis
Requirement of CO_2 for growth	-ve	-ve
VP	-ve	+ve
Hydrolysis of hippurate	-ve	+ve
Sensitivity to bacitracin	+ve	-ve
H_2O_2 Production	-ve	-ve
Sugar Fermentation		
Ribose	-ve	+ve
Arabinose	-ve	-ve
Mannitol	-ve	-ve
Sorbitol	-ve	-ve
Adonitol	+ve	-ve
Sucrose	+ve	+ve
Lactose	+ve	+ve
Trehalose	+ve	-ve
Raffinose	-ve	-ve

Table 9. Identification of *Arcanobacterium pyogenes*

Test	Reaction/Result
Gram's reaction	+ve
Shape	rod
Spore	-ve
Motility	-ve
Growth in air	+ve
Growth anaerobically	+ve
Catalase	-ve
Oxidase	+ve
O/F of glucose	-
CO_2 required for growth	+ve
CO_2 improves growth	+ve
VP	-ve
Nitrate	+ve
Indole	-ve
Haemolysis	+ve
Pitting of Loeffler's slope	+ve
Fermentation of	
Lactose	+ve
Maltose	-ve
Mannitol	-ve
Raffinose	-ve
Salicin	-ve
Sorbitol	-ve
Sucrose	-ve
Trehalose	-ve
Xylose	+ve
Melibiose	-ve

cefotaxim, pefloxacin and 50 per cent sensitive to amoxycillin, penicillin, streptomycin, oxytetracycline and erythromycin (Table 10).

Escherichia coli isolates showed 100 per cent sensitivity to ampicillin, chloramphenicol, gentamicin, pefloxacin, streptomycin, penicillin, cefotaxim and oxytetracycline. It showed a sensitivity of 66.6 per cent to co-trimoxazole and cloxacillin, 33.3 per cent to erythromycin and 16.6 per cent to amoxycillin (Table 11, Fig. 5).

Klebsiella pneumoniae was found sensitive to ampicillin, chloramphenicol, co-trimoxazole, gentamicin, cefotaxim and pefloxacin. Resistance was shown to cloxacillin, amoxycillin, penicillin, streptomycin, oxytetracycline and erythromycin (Table 12).

Streptococcus pyogenes and *Streptococcus agalactiae* isolates were found sensitive to ampicillin, chloramphenicol, co-trimoxazole, amoxycillin, gentamicin, cefotaxim, pefloxacin, penicillin, streptomycin and oxytetracycline. It showed resistance only to cloxacillin and erythromycin (Table 13).

Arcanobacterium pyogenes was found sensitive to ampicillin, chloramphenicol, co-trimoxazole, amoxycillin gentamicin, cefotaxim, penicillin, pefloxacin, and streptomycin. It showed resistance to cloxacillin, oxytetracycline and erythromycin (Table 12).

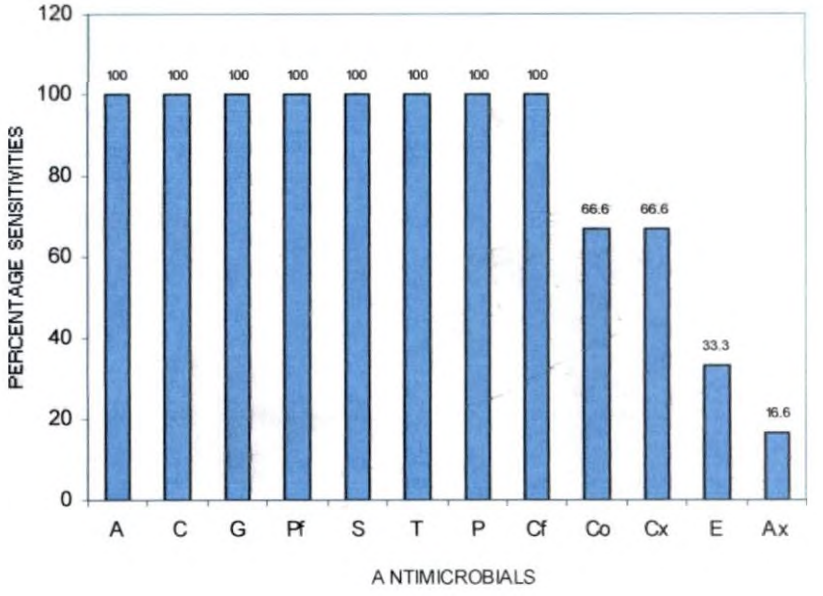

Fig. 5. Percentage distribution of antimicrobial sensitivity pattern of *Escherichia coli* isolates

1. A - **Ampicillin.**
2. C - **Chloramphenicol**
3. G - **Gentamicin**
4. Pf - **Pefloxacin.**
5. S - **Streptomycin**
6. T - **Oxytetracycline**
7. P - **Penicillin G**
8. Cf - **Cefotaxim**
9. Co - **Cotrimoxazole**
10. Cx - **Cloxacillin**
11. E - **Erythromycin**
12. Ax - **Amoxycillin**

Table 10. Antimicrobial sensitivity pattern of *Staphylococcus* isolates

Antimicrobials	ISOLATES									
	SAb 1	SAb2	SAb3	SAb4	SAc1	SAc2	SAc3	SAp1	SEb1	SEb2
Ampicillin	S	S	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	R	R	S	S	S	S	S	S
Cloxacillin	S	S	S	S	R	S	S	S	S	S
Co-trimoxazole	S	R	R	S	R	S	R	R	S	S
Amoxycillin	R	R	R	R	R	S	S	R	S	R
Gentamicin	S	S	S	R	S	S	S	S	S	S
Cefotaxim	S	S	S	S	R	S	S	S	S	S
Penicillin-G	S	S	S	S	R	S	S	S	S	R
Pefloxacin	S	S	S	S	S	S	S	S	S	S
Streptomycin	R	S	S	S	S	S	S	S	S	R
Oxytetracycline	S	S	R	S	S	S	S	S	S	R
Erythromycin	S	R	S	S	S	S	R	S	S	R

Table.11 Antimicrobial sensitivity pattern of *Escherichia coli* isolates

Antimicrobials	ISOLATES					
	EB1	EC1	EC2	EC3	EP1	EP2
Ampicillin	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S
Cloxacillin	R	R	S	S	S	S
Co- trimoxazole	S	S	R	R	S	S
Amoxycillin	R	S	R	R	R	R
Gentamicin	S	S	S	S	S	S
Cefotaxim	S	S	S	S	S	S
Penicillin-G	S	S	S	S	S	S
Pefloxacin	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S
Oxytetracycline	S	S	S	S	S	S
Erythromycin	R	S	R	R	S	R

Table 12. Antimicrobial sensitivity pattern of *Klebsiella* and *Arcanobacterium* isolates.

Antimicrobials	*Klebsiella pneumoniae*	*Arcanobacterium pyogenes*
Ampicillin	S	S
Chloramphenicol	S	S
Cloxacillin	R	R
Co- trimoxazole	S	S
Amoxycillin	R	S
Gentamicin	S	S
Cefotaxim	S	S
Penicillin-G	R	S
Pefloxacin	S	S
Streptomycin	R	S
Oxytetracycline	R	R
Erythromycin	R	R

Table13.Antimicrobial sensitivity pattern of *Streptococcus* isolates

Antimicrobials	*Streptococcus pyogenes*	*Streptococcus agalactia*
Ampicillin	S	S
Chloramphenicol	S	S
Cloxacillin	R	R
Co-trimoxazole	S	S
Amoxycillin	S	S
Gentamicin	S	S
Cefotaxim	S	S
Pefloxacin	S	S
Streptomycin	S	S
Oxytetracycline	S	S
Erythromycin	R	R
Penicillin-G	S	S

4.4 PATHOGENICITY TESTING

The three *Staphylococcus aureus* isolates *viz.*, SAc1, SAc2 and SAb1 caused death of mice within 24 hours post inoculation. A concentration of $1.5x10^8$ organisms per 0.5 ml was able to kill mice within 24 h, when injected by the intra peritoneal route. The reference strain of *Staphylococcus aureus* MTCC 1144 and the other five isolates did not cause the death of mice even after an observation period of 14 days. On sacrifice and subsequent postmortem of those mice which were inoculated with reference strain and the other five isolates which did not cause death, it was found that all these isolates produced gross lesions in internal organs like congestion of lungs and petechial haemorrhages in liver. Postmortem of the mice, which were dead within 24h after inoculation of *Staphylococcus aureus,* showed severe petechial haemorhages in the liver and generalized congestion. Re-isolation of haemolytic *Staphylococcus aureus* was possible for all the eight isolates from heart blood, lung, liver and spleen of mice, on bovine blood agar.

The two *Staphylococcus epidermidis* isolates could not cause the death of mice in 14 days observation period. On sacrifice and postmortem no gross lesions could be observed and re -isolation was not possible on bovine blood agar.

All the six *E. coli* isolates caused the death of mice within 24 h after the intra peritoneal inoculation of 0.5 ml of $1.5x10^8$ organisms. On postmortem of the mice the gross lesions observed in the internal organs of dead mice were petechiae in the liver and congestion of lungs and pericardium. Re- isolation of haemolytic *E. coli* was done from the heart blood, lung, liver and spleen on bovine blood agar, in all cases.

Klebsiella pneumoniae isolate caused death of mice after 72 h post inoculation, via intra peritoneal route. Death was caused by the injection of 0.5ml of $1.5x10^8$organisms. On postmortem the gross lesions in the internal organs of dead mice were, petechiae in the liver and pericardium and congestion of lung and spleen. The organism could be re-isolated from the heart blood, lung, liver and spleen of dead mice.

The reference strain of *Streptococus pyogenes* as well as the isolates of *S. pyogenes* and *S. agalactiae* could not cause the death of mice in the 14 days observation period. Generalized congestion was observed on postmortem. The organisms could be re-isolated in all cases from liver and spleen on bovine blood agar.

Arcanobacterium pyogenes isolate could not cause the death of mice in the 14 days observation period after the inoculation of 0.5ml of $1.5x10^8$ organisms. Generalized congestion was observed in the internal organs on posmortem. Re-isolation of the haemolytic *Arcanobacterium pyogenes* was possible from liver and spleen.

4.5 STORAGE OF ISOLATES

Storage of all the isolates was done on blood agar slants at 4^0C and these were sub cultured to blood agar plates once in a month. All the isolates stored in this manner could be revived and were found to be pure.

4.6 POLYMERASE CHAIN REACTION FOR DETECTION OF MANNHEIMIA HAEMOLYTICA

4.6.1 *Mannheimia haemolytica* specific PCR.

All Gram-negative isolates from cattle, sheep, goat and pig were subjected to specific amplification by *Mannheimia haemolytica* specific PCR. None of the isolates gave a positive result. One of the haemolytic *E.coli* isolates obtained from cattle gave a band between 300 and 400 base pair region. The observation was made on agarose gel (1.5 per cent) electrophoresis of the amplified PCR product along with a negative control and a molecular size marker (pBR322 DNA/*AluI* digest) in 1X TBE buffer. *Mannheimia haemolytica* would produce a band of 563 base pair.

4.6.2 Specificity of primers

There was no amplification when primers AMU and C3575 were used to amplify the DNA prepared from *Leptospira icterohaemorrhag*iae. *Escherichia coli* and *Staphylococcus aureus*.

Hence, the primer pair AMU and C3575 were selected for the amplification of *Mannheimia haemolytica* DNA from clinical samples.

4.6.3 Amplification of *Mannheimia haemolytica* DNA from clinical samples

4.6.3.1 Blood samples and blood smears

The blood samples and blood smears from clinical cases were processed and used to amplify with primers AMU and C3575. No amplification was observed at 563 bp region.

4.6.3.2 Nasal, tracheal swabs and lung samples

Nasal swabs collected from apparently healthy and clinically ill ruminants, tracheal swabs and lung samples collected were processed and tested for amplification with primers AMU and C3575. Pooled nasal swabs were also

used for amplification. One pooled sample of nasal swabs showed a band at 563 bp region.

None of the other nasal swabs, tracheal swabs, and lung samples were found positive by *Mannheimia haemolytica* specific PCR (Fig. 6).

4.7 RANDOM AMPLIFIED POLYMORPHIC DNA- POLYMERASE CHAIN REACTION FOR *Staphylococcus aureus*

The RAPD PCR was used as a tool to assess the genetic relationship of eight *Staphylococcus aureus* strains isolated from bovine, porcine and caprine hosts. The PCR was carried out with RAPD primers described by Williams *et al.* (1990) *i.e.,* OLP6 and OLP11, and Ahmed *et al.* (1998), *i.e.,* RAPD7. A set of reproducible bands produced from a particular oligonucleotide was defined as a profile.

The RAPD profiling resulted in DNA amplification fragments ranging in size from 300 to 1500 bp. The number of amplified fragments ranged from 1 to 6.

4.7.1 RAPD profile for OLP 6

The RAPD profiles obtained for the eight isolates of *Staphylococcus aureus* were found different. The fragments of different isolate ranged in size from 300 bp to 1500 bp. The number of fragments obtained for each isolate varied from one to six. Seven different profiles were obtained for the amplification with primer OLP 6. The reference strain of *Staphylococcus aureus* and the bovine isolate SAb4 presented almost similar pattern. Uniformity was noticed for the profiles of bovine isolate SAb3 and the caprine isolate SAc2. Unique patterns were shown by the bovine isolates SAb1, SAb2, the caprine isolate SAc1, SAc3 and the porcine isolate SAp1 (Fig. 7 and 8).

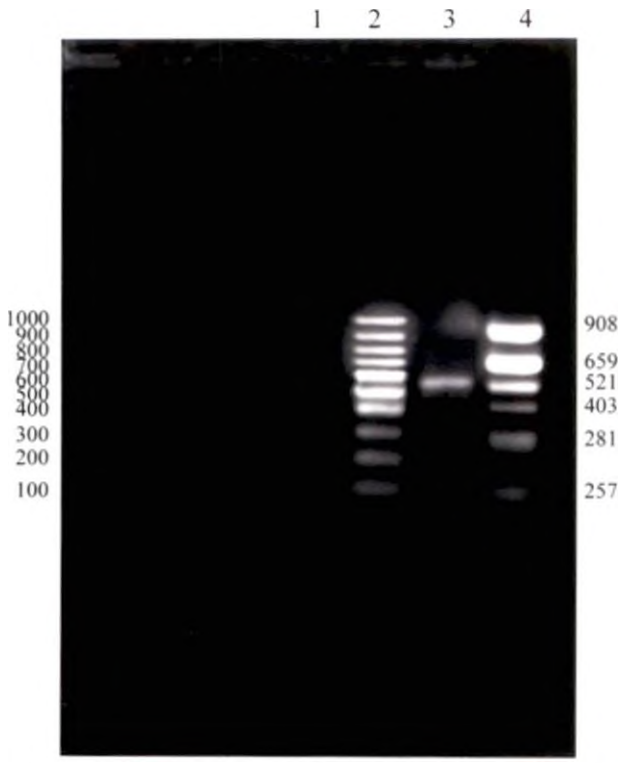

Fig. 6. *Mannheimia haemolytica* specific PCR of pooled nasal swab

Lane 1. Negative control
Lane 2. 100 bp DNA ladder
Lane 3. Pooled nasal swab
Lane 4. pBR 322 DNA/ *Alu 1* Digest

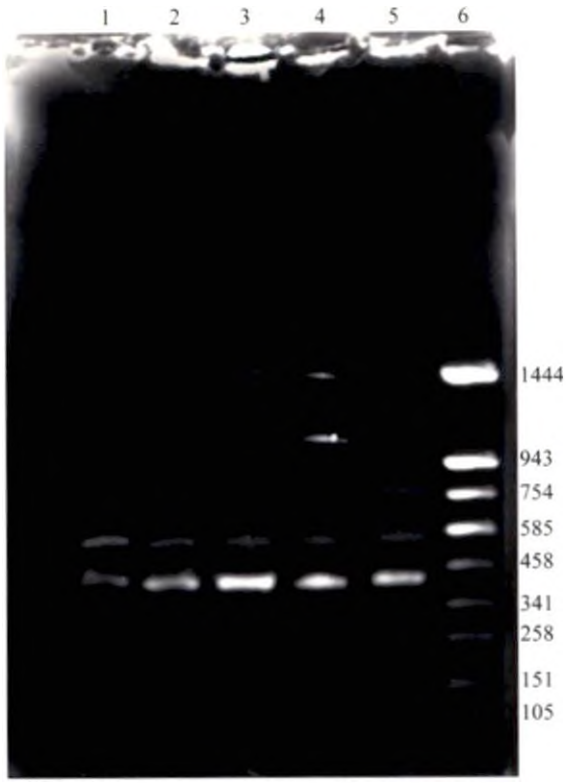

Fig. 7. RAPD –PCR of *Staphylococcus aureus* using primer OLP-6

Lane 1 SAb3
Lane 2 SAc3
Lane 3 SAc2
Lane 4 SAc1
Lane 5 Reference strain
Lane 6 pUC18/*Sau3A 1*- pUC18/*Taq1* Digest

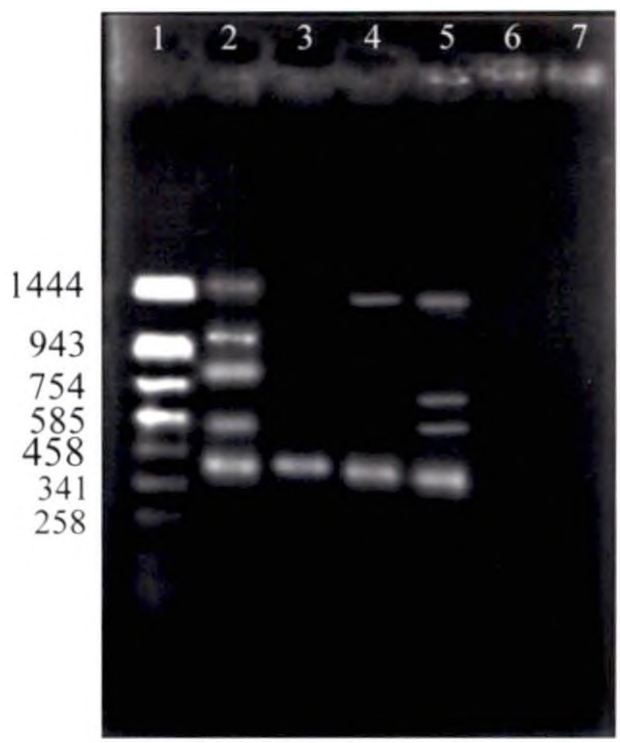

Fig. 8. RAPD-PCR of *Staphylococcus aureus* using primer OLP-6

Lane 1 pUC18/*Sau3A 1*- pUC18/*Taq1* Digest
Lane 2 SAp1
Lane 3 SAb1
Lane 4 SAb2
Lane 5 SAb4

4.7.2 RAPD profile for OLP 11

Seven different profiles were obtained. However, similarities were noticed between the profiles of some isolates. The fragments ranged in size from 300 to 1500 bp. The number of fragments for each isolate ranged from two to six. The reference strain of *Staphylococcus aureus* and the caprine isolate SAc1 was found to have similar profiles. The profiles of porcine isolate SAp1 and bovine isolate SAb1 were found similar. However the bovine isolates SAb3, SAb2, SAb4 and the goat isolates SAc2 and SAc3 showed unique patterns (Fig 9 and 10).

4.7.3 RAPD profile for RAPD 7

All the isolates yielded the same profile. A single band was obtained for all the eight isolates and the reference strain, between the 400 and 500 bp region of the 100 bp DNA ladder. Since all the isolates gave the same pattern, representation is given by a single figure *i.e.*, fig. 11

4.8 PLASMID PROFILE OF *Escherichia coli* ISOLATES

The plasmid profiles of the six *E. coli* isolates were analysed. Among the six *E. coli* isolates only two isolates harboured plasmids. The bovine isolate EB1 harboured two plasmids of size 48.06 kb and 7.49 kb and a single plasmid of size 48.06 kb was isolated from the caprine isolate EC1 (Fig.12).

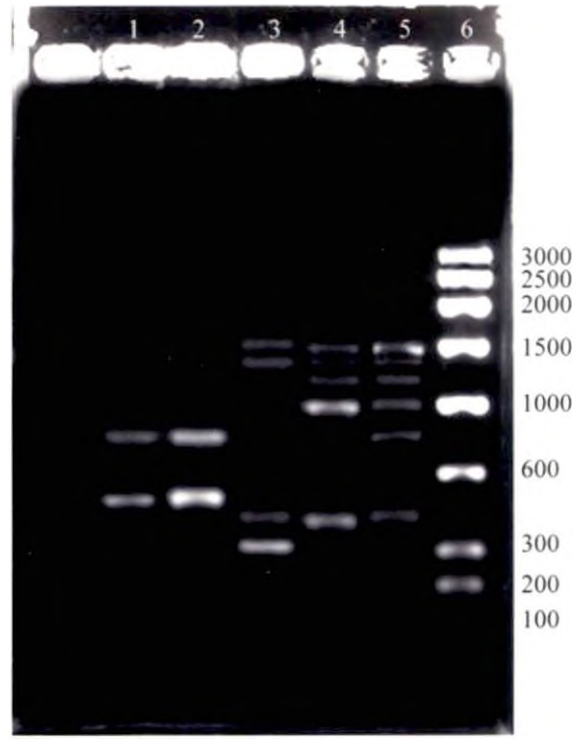

Fig. 9. RAPD –PCR of *Staphylococcus aureus* using primer OLP-11

Lane 1 Reference strain
Lane 2 SAc1
Lane 3 SAc2
Lane 4 SAc3
Lane 5 SAb3
Lane 6 Low Range DNA Ruler

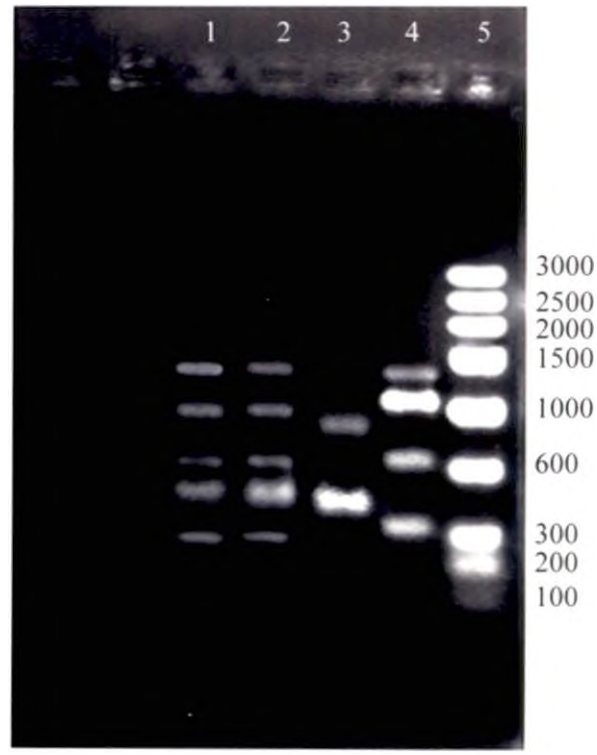

Fig. 10. RAPD –PCR of *Staphylococcus aureus* using primer OLP-11

Lane 1 SAp1
Lane 2 SAb1
Lane 3 SAb2
Lane 4 SAb4
Lane 5 Low Range DNA Ruler

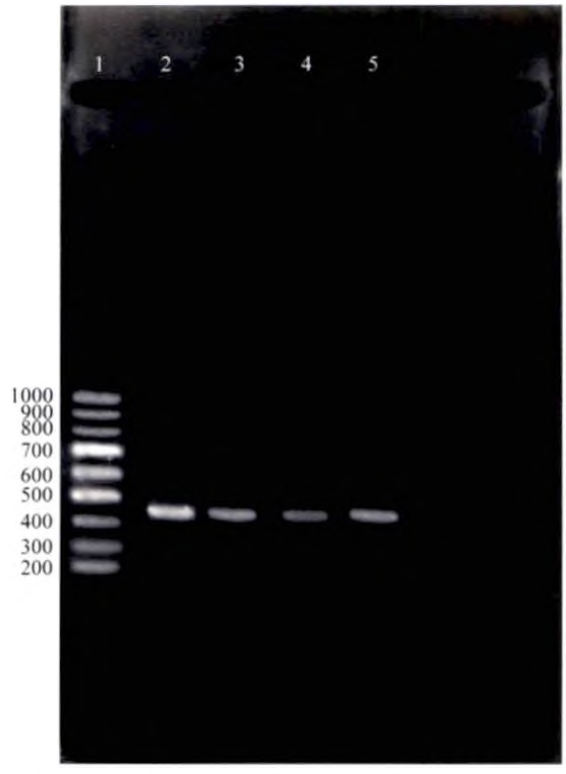

Fig. 11. RAPD –PCR of *Staphylococcus aureus* using primer RAPD-7

Lane 1 100 bp DNA ladder.
Lane 2 SAp1
Lane 3 SAb1
Lane 4 SAb2
Lane 5 Reference strain

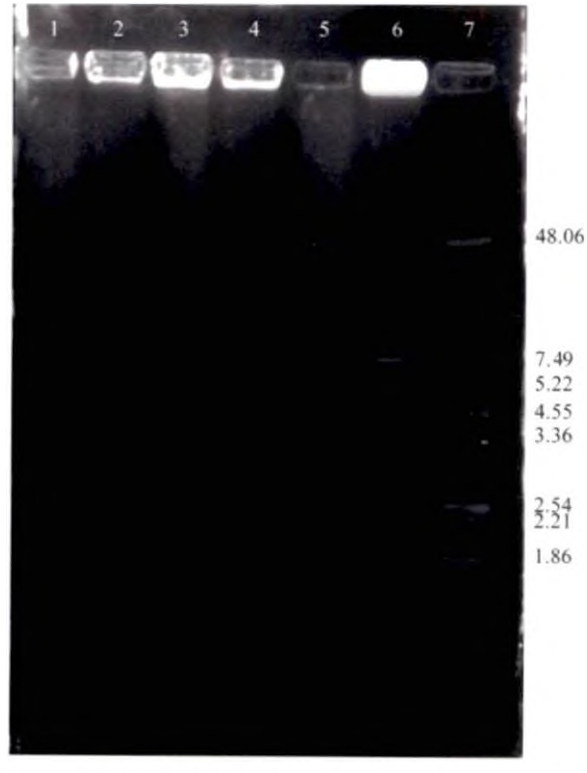

Fig. 12. Plasmid profile of *Escherichia coli* isolates

Lane 1 EC2
Lane 2 EC3
Lane 3 EP2
Lane 4 EP1
Lane 5 EC1
Lane 6 EB1
Lane 7 *E. coli* V517

Discussion

5. DISCUSSION

Respiratory tract infections associated with bacterial organisms have always been a baffling problem in the livestock sector round the world. Many of the haemolytic organisms have been found to be etiological agents of the respiratory tract infections. An effective treatment and control over these infections mainly depends on the timely and proper diagnosis, which in turn depends on accurate isolation, identification and determination of the antimicrobial sensitivities of these haemolytic organisms. In addition to conventional cultural and biochemical identification methods, molecular techniques have been found to be an efficient tool for detection and identification of these organisms directly from clinical specimens. The present study envisages isolation and identification of the haemolytic organisms, determining their antibiogram and testing the pathogenicity of isolates in mice.

5.1 ISOLATION OF HAEMOLYTIC BACTERIA

Isolation of haemolytic organisms was attempted from nasal swabs of apparently healthy and clinically ill livestock, tracheal swabs and lung samples with pneumonic lesions from slaughtered animals and postmortem cases, and blood samples from animals having respiratory infections. Allen *et al.* (1991) suggested that nasopharyngeal swabs could be reliably used for research or diagnostic purposes to give a useful estimate of the pulmonary microbial flora in large groups of feedlot calves.

Kanwar *et al.* (1998) reported about the involvement of *Pasteurella haemolytica* as a causative organism in pneumonic lesions encountered in lungs of animals killed at local slaughterhouse. In this study attempts were made to

isolate *Mannheimia haemolytica* from lung samples showing pneumonic lesions.

Primary isolation of organisms from nasal swabs, tracheal swabs and lung samples were tried on *Mannheimia haemolytica* selective medium as well as on ovine/bovine blood agar. Blood agar was found to be the most suitable medium for the isolation of haemolytic organisms eventhough many of the contaminant bacteria over grew. Morris *et al.* (1958) reported the use of a selective medium for the isolation of *Mannheimia haemolytica.* The selective medium supported the growth of most of the Gram-negative organisms and inhibited the growth of Gram-positive organisms. It was effective in inhibiting the growth of many of the contaminant bacteria. Odugleo *et al.* (2003) used ordinary sheep blood agar for the isolation of *Mannheimia haemolytica.* *Mannheimia haemolytica* could not be isolated from nasal swabs, tracheal swabs, lung samples or blood samples in either blood agar or in selective medium. Isolation was also tried from pooled sample of nasal swabs collected from animals with respiratory tract infection. A single isolate obtained from the pooled sample showed characteristics similar to that of *Mannheimia haemolytica.* But the results of ornithine decarboxylase activity and utilization of sugars like salicin and trehalose was not in accordance to that given by Barrow and Feltham (1993). Moreover, no reference strain was available to make a comparison and confirm the isolate as *Mannheimia haemolytica.* The other media used, such as mannitol salt agar, Edward's medium, Mac Conkey's agar and eosin methyleneblue agar, were found effective in presumptive identification of *Staphylococcus*, *Streptococcus* and *Escherichia* sp. This was in accordance to Quinn *et al.*, 1994.

In this study, it was found that *Staphylococcus aureus* and *Escherichia coli* were the most important haemolytic bacteria associated with respiratory tract infections. They formed 40 per cent and 30 per cent respectively of the total isolates. Wikse and Baker (1996) had already reported *Staphylococcus*

aureus and *E. coli* as important organisms associated with respiratory tract infections. Staphylococci are considered to be opportunistic pathogens found on the skin and mucous membrane, capable of localizing in any part of the tissue. Under suitable conditions and they can invade the lungs causing pneumonia, abscess and death. Nasser-El- Sayed (1997) had reported the isolation of *E. coli* from cases of respiratory tract infection in cattle, sheep and goat.

In the present study three of the *Staphylococcus aureus* isolates were obtained in combination with *Escherichia coli* isolates, from cases of death in goats due to pneumonia in a farm. It may be explained that the outbreak of pneumonia might be caused by combined infection of *Staphylococcus aureus* and *Escherichia coli.* Siji (2003) had earlier reported the isolation of *Staphylococcus aureus* in combination with *E. coli* from cases of respiratory tract infection in bovines.

Staphylococcus epidermidis were isolated from the tracheal swabs of cattle slaughtered at the Thrissur Corporation slaughter house. Allan (1977) and Siji (2003) had earlier reported the isolation of *Staphylococcus epidermidis* from cattle with respiratory tract infection.

Klebsiella pneumoniae was isolated from 5 per cent of the total cases. The organism was isolated from the lungs of a cow died of pneumonia. The isolation correlates with the findings of Allan (1977), Siji (2003) and Brisse and Deujkeren (2005). Allan (1977) reported *Klebsiella* sp. as one of the causative agents of respiratory tract infections in calves. Incidence of *Klebsiella pneumoniae* as causative agents of respiratory tract infections in bovine was reported by Siji (2003).

Streptococcus pyogenes has been isolated from 5 per cent of the total cases. It was isolated from the lungs of a pig, which died due to pneumonia. L'Ecuyer *et al.* (1961) and Brown. (1970) isolated haemolytic streptococci from

lungs of swine with pneumonia. Riley *et al.* (1973) reported the use of nasal and tonsillar swabbing and tonsillar biopsies to detect Group E streptococci in swine. They found that nasal and tonsillar swabbing as more satisfactory than biopsy as a means of detecting Group E streptococci.

Streptococcus agalactiae was isolated from 5 per cent of the cases. The single isolate was obtained from the lungs of a goat died due to pneumonia. Perusal of literature has not shown any reference regarding the involvement of *Streptococcus agalactiae* in respiratory tract infections.

Arcanobacterium pyogenes was isolated from 5 per cent of the cases,The isolation was consistent with the findings of Merchant and Packer (1971), Barbour *et al.* (1997), Tegtmier *et al.* (1999) and Semiya *et al.* (2004). Merchant and Packer (1971) stated that *Corynebacteriun pyogenes* were causative organisms of suppurative pneumonia in cow. Barbour *et al.* (1997) reported that *Actinomyces pyogenes* was one of the most prevalent bacteria in the lungs of unhealthy animals. Smith *et al.* (2000) had reported that *Arcanobacterium pyogenes* is responsible for chronic pneumonia, aspiration pneumonia and lung abscessation in cattle. Allan (1977) reported the isolation of *Corynebacterium pyogenes* from 9.6 per cent of the calves suffering from pneumonia and from seven per cent of non-pneumonic lung tissue of calves. In the present study isolation was made from a case of pneumonia in calf. Vogel *et al.* (2001) isolated *Arcanobacterium pyogenes* from 46 per cent of cases of pneumonia in calves. No *Arcanobacterium pyogenes* could be isolated from nasal swabs or tracheal and lung samples of apparently healthy animals.

5.2 IDENTIFICATION

All the isolates as well as the reference strains of *Staphylococcus aureus, Streptococcus pyogenes,* and *Streptococcus pneumoniae* produced haemolytic colonies on blood agar.

Eight isolates of *Staphylococcus aureus* were identified based on cultural and biochemical characteristics described by Barrow and Feltham (1993). All the isolates were Gram-positive cocci, non motile, grew on mannitol salt agar producing yellow coloured colonies and the medium changed from pink to yellow. All the isolates were catalase positive, oxidase negative, VP+, MR+, and nitrate reduction positive. They were found fermentative for OF test. All were found coagulase positive, suggestive of their virulent nature. Quinn *et al.*(2002) reported that production of coagulase by staphylococci was an important indicator of their pathogenicity. The isolates differed among themselves slightly in the utilization of certain sugars like xylose, raffinose and cellobiose. *Staphylococcus epidermidis* was isolated from the tracheal swabs of cattle slaughtered at the Corporation slaughterhouse. *Staphylococus epidermidis* isolates varied from *Staphylococcus aureus* isolates only for the sugar tests and was found coagulase negative. This is in concordance with Barrow and Feltham (1993) and Quinn *et al.* (1994).

All the *Escherichia coli* isolates had the first stage and second stage biochemical identification tests and cultural characterization consistent with the findings of Barrow and Feltham (1993). The isolates showed slight variation in the sugar utilization tests *i.e.,* for the utilization of salicin and sucrose. All the isolates gave a positive Eijkmans test. One of the isolates (EB1) lost the property of showing metallic sheen on EMB agar on subculturing. This finding was in concordance with the findings of Kumar *et al.* (2005) where all the *E. coli* 078 isolates lost the property of giving metallic sheen on EMB agar after subculturing once. They also opined that when such isolates were passaged on

animals (lambs or mouse) or cultured on 5 per cent sheep blood agar the property of exhibiting metallic sheen on EMB agar was regained.

Klebsiella pneumoniae isolate was haemolytic. The finding was not in concordance with Barrow and Feltham (1993) and Quinn *et al.* (2002). They have not mentioned about the haemolytic properties of *Klebsiella pneumoniae.* On blood agar media it produced white mucus like colonies, which were slimy and semifluid in consistency. The findings agree with those given by Merchant and Packer (1971) who reported that *Klebsiella pneumoniae* produce mucoid colonies which are slimy and semifluid in consistency.

Both *Streptococcus pyogenes* and *Streptococcus agalactiae* produced small colonies that were β-haemolytic on blood agar and haemolytic transparent small colonies on Edward's medium. As per Quinn *et al.* (1994) differentiation of *Streptococcus pyogenes* and *Streptococcus agalactiae* was done based on hydrolysis of hippurate and sensitivity to bacitracin and sugar fermentation tests. *Streptococcus agalactiae* gave a positive result for hydrolysis, whereas *Streptococcus pyogenes* gave a negative result for it. *Streptococcus pyogenes* was sensitive to bacitracin whereas *Streptococcus agalactia* was resistant.

Arcanobacterium pyogenes produced tiny colonies on blood agar after incubation for a period of 24 h. After 48 h incubation a narrow zone of complete haemolysis was seen around the colonies. This is in accordance with the findings of Quinn *et al.* (1994). Narayanan *et al.* (1998) reported the use of sheep blood agar for the primary isolation of *Arcanobacterium pyogenes* which was incubated in 5 per cent CO_2 at 39^0C for 30-48 h. The growth was obtained on incubation of the plates in a candle jar. So we could infer that CO_2 incubation enhanced the growth of *Arcanobacterium pyogenes* which is consistent with the findings of Barrow and Feltham(1993) and Narayanan *et al.* (1998). It produced a positive CAMP test with *Staphylococcus aureus,* which is in accordance with the findings of Quinn *et al.* (1994).

5.3 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 bovine 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. In the present study antibiogram has shown that most of the *Staphylococcus aureus* isolates were having low sensitivity to co-trimoxazole and amoxycillin. Maximum sensitivity was shown to ampicillin and pefloxacin. Gonlugur *et al.* (2003) showed that respiratory isolates of *Staphylococcus aureus* had an overall resistance of 61.9 per cent to erythromycin, followed by tetracycline (50.7 per cent), but had a low resistance of 10.3 percent to co-trimoxazole which is not consistent with the findings of the present study.

Staphylococcus epidermidis isolates were 100 per cent sensitive to ampicillin, chloramphenicol, cloxacillin, co-trimaxazole, gentamicin and 50 per cent sensitivity was shown to amoxycillin, penicillin, streptomycin, oxytetracycline and erythromycin. Siji (2003) reported that *Staphylococcus epidermidis* isolate was 100 per cent sensitive to enrofloxacin, gentamicin and ciprofloxacin.

Escherichia coli isolates showed least sensitivity to amoxycillin followed by erythromycin. The isolates showed complete resistance to none of the 12 antimicrobials used. Isolates showed 100 per cent sensitivity to ampicilllin, chloramphenicol, gentamicin, cefotaxim, pefloxacin, streptomycin, penicillin and oxytetracycline. Siji (2003) reported that none of the antibiotics studied showed cent per cent sensitivity to *E. coli* isolates. According to her, maximum sensitivity was to chloramphenicol, enrofloxacin and ciprofloxacin and least

sensitivity was to trimethoprim. The findings of the present study is consistent with the findings of Kumar *et al.* (2005) who opined that all the *E. coli* 078 isolates from respiratory tract infections were found sensitive to ciprofloxacin, gentamicin, amoxycillin, chloramphenicol, streptomycin, sulpha, tetracycline and oxytetracycline except one which was resistant to tetracycline.

Klebsiella pneumoniae isolate showed sensitivity to ampicillin, chloramphenicol, co-trimoxazole, gentamicin, cefotaxim and pefloxacin. This is not in concordance with the findings of Brisse and Deujkeren (2005) who reported that *Klebsiella* isolates were naturally resistant to ampicillin, due to constitutively expressed chromosomal class A β-lactamase. Resistance was shown to cloxacillin, amoxycillin, penicillin, streptomycin, oxytetracycline and erythromycin. High degree of sensitivity of *Klebsiella* isolate to gentamicin was reported by Eguchi (1988). Siji (2003) had reported that *Klebsiella pneumoniae* isolates had 100 per cent sensitivity to enrofloxacin and was least sensitive to streptomycin.

Streptococcus pyogenes and *Streptococcus agalactiae* showed almost same pattern of sensitivity to antimicrobials. They were found sensitive to ampicillin, chloramphenicol, co-trimoxazole, amoxycillin, gentamicin, cefotaxim, pefloxacin, penicillin, streptomycin and oxytetracycline. Resistance was noted to cloxacillin and erythromycin. According to Siji (2003) least sensitivity of *Streptococcus pyogenes* was to trimethoprim, followed by amoxycillin.

Arcanobacterium pyogenes isolate was sensitive to ampicillin, chloramphenicol, amoxycillin, gentamicin, cefotaxim, penicillin, streptomycin, pefloxacin and co-trimoxazole. The isolate showed resistance to cloxacillin, erythromycin, streptomycin and oxytetracycline. Resistance of *Arcanobacterium pyogenes* to oxytetracycline has previously been reported by Narayanan *et al.* (1998) and Trinh *et al.* (2002). However, Baksi *et al.* (2004)

had shown that *Corynebacterium pyogenes,* isolated from rabbits had high sensitivity to tetracycline and erythromycin. Resistance to macrolide antibiotics of *Arcanobacterium pyogenes* had been reported by Trinh *et al.* (2002) and Jost *et al.* (2004), which is in concordance with the findings of the present study.

5.4 PATHOGENICITY

In the present study, three of the *Staphylococcus aureus* isolates caused death of the mice within 24 h post inoculation of $1.5x10^8$ organisms/0.5 ml via the intraperitoneal route. However, the reference strain of *Staphylococcus aureus* and rest of the five isolates could not cause death of mice even after the 14 days observation period. Even then all the isolates could be re-isolated from heart blood, liver, lungs and spleen of mice, on bovine blood agar. From this it could be inferred that that the reference strain and five isolates which could not kill the mice were of low virulence, when compared to the isolates which caused the death of mice. However, the caprine isolate SAc1, which killed mice in 24 h, was found resistant to five out of all 12 antimicrobials used and it was the isolate, which showed maximum resistance. So the antibiotic resistance may be correlated with the virulence of the isolate. However the other two isolates SAc2 and SAb1, which killed mice, showed no such correlation. Pak *et al.* (1999) had reported the pathogenicity testing of *Staphylococcus aureus* isolates by intraperitoneal inoculation of 0.5 ml of 10^8- 10^9 organisms and observing them for a period of 15days.

Staphylococcus epidermidis isolates could not cause the death of mice in the 14 days observation time. No gross lesions were observed on postmortem and re-isolation was not possible. Perusal of literature has not shown any reference regarding the virulence of *Staphylococcus epidermidis* in mice. However, it may be inferred from this finding that *Staphylococcus epidermidis* were not that pathogenic to cause infection in mice. They are opportunistic

pathogens and might have caused infection under condition of stress or due to other concurrent infections.

Death of mice was caused by all the six *E. coli* isolates within 24 h after the intraperitoneal inoculation of 0.5ml of $1.5x10^8$ organisms. Gross lesions were observed on postmortem and re-isolation was possible from heart blood, lungs, liver and spleen, on bovine blood agar. Kumar *et al.* (2005) confirmed the pathogenicity of *E. coli* 078 cultures by intraperitoneal inoculation of live cultures to mice which caused death within 24 h of inoculation, with re-isolation of similar organisms from heart blood, liver and spleen. May *et al.* (2000) reported intraperitoneal infection of *E. coli* in rats by inoculating 10^6 CFU/ml. They reported that α-haemolysin played key role in facilitating the pathogenicity of *E. coli* within the peritoneum and also inoculation of 10^8CFU/ml of α-haemolytic variant of *E. coli* was 100 per cent lethal, while the same size inoculum of *hly*⁻ variant was non lethal to rat, thereby indicating haemolysin production during peritonitis increased the lethality of infection in rat model. All the *E. coli* isolates of present study were α-haemolytic. Death of mice within 24 h post intraperitoneal inoculation of 10^8 organisms can be correlated with virulence associated with haemolysin production.

Klebsiella pneumoniae isolate caused death of mice after 72 h post inoculation intraperitoneally of 0.5 ml of 1.5 $x10^8$ organisms. Gross lesions were observed in different internal organs and re-isolation was possible. Perusal of literature has not shown any reference regarding the pathogenicity of *Klebsiella pneumoniae* in mice on intraperitoneal inoculation.

The reference strain of *Streptococcus pyogenes* as well as the isolates of *Streptococcus pyogenes* and *Streptococcus agalactiae* could not cause the death of mice in the 14 days observation period. Generalized congestion was observed in the internal organs on postmortem and the organism could be re-

isolated from liver and spleen on bovine blood agar. The isolates obtained might not be that virulent to cause the death of mice.

Arcanobacterium pyogenes isolate could not cause the death of mice within the 14 days observation period. Generalized congestion was observed in the internal organs on post mortem. Re-isolation of haemolytic *Arcanobacterium pyogenes* was possible from liver and spleen. Narayanan *et al.* (1998) had reported that haemolysin production in *Arcanobacterium pyogenes* is an important virulence factor. However, the isolate was not that virulent enough to cause death of mice, but the production of lesions on internal organs and re-isolation of the organisms is an indication of the low level virulence and invasiveness of the organism.

5.5 STORAGE OF ISOLATES

All the isolates were stored on blood agar slants kept at 4^0C and were subcultured to blood agar plates once in a month. It was found that isolates could be stored in pure culture even upto 3 months on blood agar slants at 4^0C.

5.6 POLYMERASE CHAIN REACTION FOR DETECTION OF *Mannheimia haemolytica*

5.6.1 *Mannheimia haemolytica* specific PCR

All the Gram-negative isolates from cattle, sheep, goat and pig were subjected to specific amplification by *Mannheimia haemolytica* specific PCR. None of the isolates gave a positive result.

One of the α-haemolytic *E. coli* isolate obtained (EB1) gave a band between 300 and 400 bp region. Strathdee and Reggie (1989) had reported that the structural gene of *Pasteurella haemolytica* leukotoxin determinant was

highly homologous to that of the *E. coli* haemolysin determinant. It may be because of the high homology that α- haemolytic *E. coli* gave a band. The primers used were designed for the amplification of the leukotoxin gene region of *Mannheimia haemolytica.* The primers might have amplified the haemolysin determinant of *E. coli* due to the high homology. No positive result of PCR was obtained from blood sample, nasal and tracheal swabs and lung samples. However, a pooled sample of nasal swabs obtained from cattle having respiratory tract infections gave a band at the 563 bp regions. Isolation trials from that pooled sample yielded an isolate which was having almost all the characteristics similar to *Mannheimia haemolytica* but showed variation for ornithine decarboxylase activity, fermentation of salicin and trehalose. But PCR of that isolate could not yield a positive result. Moreover, since no reference strain of *Mannheimia haemolytica* was available the results could not be compared and confirmed.

5.7 RAPD –PCR FOR *Staphylococcus aureus.*

To assess the genetic relationship of *Staphylococcus aureus* isolates from different hosts RAPD-PCR of the eight *Staphylococcus aureus* isolates was done. Reinosso *et al.* (2004) had reported the RAPD-PCR assay with three primers to assess the genetic relationship of *Staphylococcus aureus* from different hosts. The RAPD-PCR of the four bovine, three caprine and the single porcine isolate of *Staphylococcus aureus* yielded different profiles for the three different primers. However, the PCR with the primer RAPD-7 produced similar profile for all the isolates. It resulted in a single DNA amplification fragment for all the isolates, which was found between the 400 and 500 bp region of the 100 bp DNA ladder. The reference strain of *Staphylococcus aureus* also yielded the same profile. This was not consistent with the findings of Ahmed *et al.* (1998), where multiple bands were obtained for RAPD-PCR using the same primer for the human isolates of *Staphylococcus aureus* from the nasal cavity.

However, the bovine, porcine, caprine isolates as well as the reference strain yielded a single band in the present study.

The RAPD-PCR using the primer OLP-6 gave multiple bands for each isolate, and the isolates differed among themselves in the profiles. The fragments ranged in size from 300 bp to 1500 bp. This is in accordance to the findings of Reinosso *et al.* (2004) where they obtained fragments of size ranging from 300 bp to 1500 bp. The number of fragments obtained in this study for each isolate varied from one to six. Reinosso *et al.* (2004) reported that the number of amplified fragments ranged from one to 14. No host specific patterns were observed in the present study. Similarities of profile between isolates from different hosts were observed.

The RAPD-PCR using the primer OLP-11 also gave multiple bands. The amplified fragments ranged in size from 300 to 1500 bp and the number of fragments varied from two to six. Similarities were noticed between profiles of caprine and bovine hosts and also between bovine and porcine hosts. No host specific patterns were observed for the isolates with this primer also.

From this study it could be inferred that there is considerable genetic relationship between *Staphylococcus aureus* isolates of different host species and also there is noticeable genetic diversity of the isolates within the host species. This may be due to the transfer of bacteria through the common environment shared by the different animal hosts.

5.8 PLASMID PROFILE OF *Escherichia coli* ISOLATES.

The plasmid DNA content of each of the six isolates of *Escherichia coli* was analysed on agarose gel electrophoresis. Of the six isolates only two of them harboured plasmids. One bovine isolate EB1 yielded two plasmids of size 48.04 kb and 7.49 kb. The caprine isolate EC2 yielded a single plasmid of size

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

The bovine isolate EB1, which harboured two plasmids, was found resistant to three out of the 12 antibiotics used. The caprine isolate which harboured a single plasmid also showed resistance to three out of the 12 antimicrobials used. The other isolates which did not harbour any plasmid also showed similar antimicrobial resistance. So a correlation between the presence of plasmids and antibiotic resistance could not be ascertained in this study.

The pathogenicity test conducted in mice revealed that all the isolates, even those, which did not harbour plasmids, were pathogenic. So this observation could not establish a correlation between the presence of plasmids and virulence.

Summary

6. SUMMARY

Respiratory infections have been considered as one of the most important health problems of the livestock sector in the humid tropics. Association of bacteria with respiratory diseases has been known for many decades. Various haemolytic bacteria are reported to be associated with respiratory tract infections of livestock. Of these *Mannheimia haemolytica* has been incriminated as a major etiological agent of respiratory tract infections in livestock. However the true role-played by haemolytic bacteria in respiratory diseases is unclear.

The present study was undertaken with a view to understand the role of haemolytic bacteria in respiratory tract infections of livestock, by the isolation and identification of the organisms, determining their antimicrobial sensitivities and testing the pathogenicity of the isolates in mice. The study also envisaged the detection of *Mannheimia haemolytica* by Polymerease chain reaction, determining the genetic relationship of *Staphylococcus aureus* isolates from different animal hosts using RAPD-PCR technique and also analysis of plasmid profiles of *Escherichia coli isolates.*

Samples were collected from clinically ill livestock and at random from apparently healthy animals from in an around Thrissur district. A total of 309 samples were taken, which comprised of 107 nasal swabs, 87 tracheal swabs, 75 lung samples and 40 blood samples. Of this, 153 samples were from cattle, 63 from goats, 12 from sheep and 81 from pigs.

The samples were cultured on blood agar media and on *Mannheimia haemolytica* selective medium. *Mannheimia haemolytica*

could not be isolated from any of the nasal and tracheal swabs, lung samples and blood samples cultured on *Mannheimia haemolytica* selective medium as well as on blood agar. But pooled nasal swabs when cultured on blood agar gave an isolate with characteristics almost similar to *Mannheimia haemolytica*, but showed variations for ornithine decarboxylase activity and utilization of sugars like trehalose and salicin. But, no reference strain was available to make a comparison and confirm the isolate as *Mannheimia haemolytica*.

From the samples cultured on blood agar media, a total of 20 haemolytic bacteria could be isolated. Out of the 20 isolates 65 per cent (13) were gram positive and 35 per cent (7) were gram negative. The different isolates were *Staphylococcus aureus* 40 per cent (8), *Staphylococcus epidermidis* 10 per cent (2), *Escherichia coli* 30 per cent, (6) *Klebsiella pneumoniae* 5 per cent (1), *Streptococcus pyogenes* 5 per cent(1), *Streptococcus agalactiae* 5 per cent(1) and *Arcanobacterium pyogenes* 5 per cent(1). The bovine *Staphylococcus aureus* isolates were designated as SAb1, SAb2, SAb3 and SAb4, caprine isolates as SAc1, SAc2, SAc3 and the porcine isolate as SAp1. The *Escherichia coli* isolates from bovine was designated EB1, caprine isolates as EC1, EC2, EC3 and pocrine isolates as EP1 and EP2. The bovine isolates of *Staphylococcus epidermidis* were designated as SEb1 and SEb2.

The different bacteria were identified based on morphological, cultural and biochemical characteristics as described by Barrow and Feltham (1993).

Antimicrobial sensitivities of the different isolates were studied on Muller Hinton agar medium. Antimicrobial pattern of different isolates to ampicillin, chloramphenicol, cloxacillin, co-trimoxazole, amoxycillin, gentamicin, cefotaxim, penicillin, pefloxacin, erythromycin, streptomycin and oxytetracycline, were determined. *Staphylococcus aureus* isolates

had high sensitivity to ampicillin and pefloxacin. The least sensitivity was shown to amoxycillin. *Staphylococcus epidermidis* isolated was 100 per cent sensitive to ampicillin, chloramphenicol, cloxacillin, co-trimoxazole, gentamicin, cefotaxim and pefloxacin. *E.coli* isolates showed 100 per cent sensitivity to ampicillin, chloramphenicol, gentamicin, pefloxacin, streptomycin, penicillin cefotaxim and oxytetracycline. Least sensitivity was shown to amoxycillin. *Klebsiella pneumoniae* was found sensitive to ampicillin, chloramphenicol, co-trimoxazole, gentamicin, cefotaxim and pefloxacin. Resistance was shown to cloxacillin, amoxycillin, penicillin, streptomycin, oxytetracycline and erythromycin. *Streptococcus pyogenes* and *Streptococcus agalactiae* isolates were found sensitive to ampicillin, chloramphenicol, co-trimoxazole, amoxycillin, gentamicin, cefotaxim, pefloxacin, penicillin, streptomycin and oxytetracycline. It showed resistance only to cloxacillin and erythromycin. *Arcanobacterium pyogenes* isolates showed resistance only to cloxacillin and erythromycin and oxytetracycline. Overall antibiotic resistance was maximum to erythromycin, followed by amoxycillin and cloxacillin. Almost all the isolates had high sensitivity to pefloxacin and ampicillin, followed by gentamicin and chloramphenicol.

The three *Staphylococcus aureus* isolates SAc1, SAc2 and SAb1 caused death of mice within 24h post inoculation of $1.5x10^8$ organisms/ml. The reference strain and the rest of the five isolates could not cause death of mice. But all the isolates produced gross lesions in the internal organs like congestion of lungs and petechial haemorrhages in liver and re-isolation of the organisms was possible. *Staphylococcus epidermidis* isolates did not cause death of mice in the 14 days observation time, gross lesions were not produced and re-isolation was not possible.

All the six *E.coli* isolates caused death of mice in 24h post inoculation of 0.5 ml of $1.5x10^8$ organisms. On postmortem of the mice

the gross lesions observed in the internal organs of dead mice were petechiae in the liver and congestion of lungs and pericardium and re-isolation was possible. *Klebsiella pneumoniae* caused death of mice after 72 h post inoculation of $0.3x10^8$ organism's intra peritoneally. Gross lesions were observed on internal organs and re-isolation was done from heart blood, lungs, liver and spleen. *Streptoccoccus pyogenes*, *Streptococcus agalactiae* and *Arcanobacterium pyogenes* isolates eventhough did not cause the death of mice, gross lesions were observed on internal organs and re-isolation was possible.

All the isolates were stored on blood agar slants at 4^0C and were subcultured to blood agar plates, once in a month. The isolates stored in this manner could be revived and were found to be pure.

All the Gram-negative isolates were subjected to specific amplification by *Mannheimia haemolytica* specific PCR. None of the isolates gave a positive result. A pooled sample of nasal swabs collected from cattle having respiratory infection gave a positive result for *Mannheimia haemolytica* specific PCR with a band at 563 bp region of the pBR 322/*Alu*1Digest. But PCR of the pooled sample culture did not yield a positive result. Moreover, no reference strain was available to make a comparison and confirm the result.

RAPD-PCR was conducted to assess the genetic relationship of *Staphylococcus aureus* obtained from different animal hosts, using three different primers *viz.*, OLP-6, OLP-11 and RAPD-7. The primers OLP-6 and OLP-11 gave multiple bands for all the *Staphylococcus aureus* isolates. The number of fragments ranged from 300 bp to 1500 bp. However some similarities were observed between profiles from bovine, caprine and porcine hosts. No host specific patterns were obtained. For the primer RAPD-7 all the isolates yielded a single band between the 400 and 500 bp region of the 100 bp DNA ladder. From the study it was

inferred that there was considerable genetic relationship between *Staphylococcus aureus* isolates of different host species and also there was noticeable genetic diversity of the isolates within the host species. This may be due to the transfer of bacteria through the common environment shared by the different animal hosts.

The plasmid DNA content of each of the six *E.coli* isolates was analysed. Of the six isolates only two of them harboured plasmids. The bovine isolate EB1 yielded two plasmids of size 48.04 kb and 7.49 kb. The caprine isolate EC1 yielded a single plasmid of size 48.06. No correlation was observed between the virulence, antibiotic resistance and presence of plasmids in the *E.coli* isolates studied.

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DETECTION OF PATHOGENIC HAEMOLYTIC BACTERIA IN RESPIRATORY TRACT INFECTIONS OF LIVESTOCK

APARNA. S.

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Kerala Agricultural University, Thrissur

2005

Department of Veterinary Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ABSTRACT

A study was undertaken to elucidate the role of haemolytic bacteria in respiratory tract infections of livestock. This envisaged the isolation and identification of the haemolytic bacteria, determining their antibiogram patterns and testing the pathogenicity of the isolates in mice. The study also envisaged the detection of *Mannheimia haemolytica* by polymerase chain reaction, determining the genetic relationship of *Staphylococcus aureus* isolates from different animal hosts using RAPD-PCR technique and also analysis of plasmid profiles of *Escherichia coli* isolates.

Samples were collected from clinically ill livestock and at random from apparently healthy animals from in an around Thrissur district. A total of 309 samples were taken which consisted of nasal swabs, tracheal swabs, lung samples and blood samples. Samples were cultured on blood agar and on *Mannheimia haemolytica* selective medium. *Mannheimia haemolytica* could not be isolated from any of the samples. But pooled nasal swabs when cultured on blood agar gave an isolate with characteristics almost similar to *Mannheimia haemolytica*, but showed variations for ornithine decarboxylase activity and utilization of sugars like trehalose and salicin. As, no reference strain was available it was not possible to make a comparison and confirm the isolate as *Mannheimia haemolytica*. From the samples cultured on ordinary blood agar medium a total of 20 haemolytic bacterial isolates could be obtained. The different bacterial isolates were *Staphylococcus aureus* (40 per cent), *Staphylococcus epidermidis* (10 per cent), *Escherichia coli* (30 per cent), *Klebsiella pneumoniae* (5 per cent), *Streptococcus pyogenes* (5 per cent), *Streptococcus agalactiae* (5 per cent) and *Arcanobacterium pyogenes* (5 per cent).

The haemolytic bacteria were identified based on morphology, cultural characteristics and biochemical tests. Antimicrobial sensitivity pattern of the isolates showed that almost all the isolates had high sensitivity to pefloxacin and ampicillin. Antimicrobial resistance was shown maximum to erythromycin.

The three *Staphylococcus aureus* isolates, all the *E. coli* isolates and *Klebsiella* isolate caused death of mice. Rest of the five *Staphylococcus aureus* isolates, *Streptococcus pyogenes* , *Streptococcus agalactiae* and *Arcanobacterium pyogenes* did not cause death of mice but produced internal lesions and could be re-isolated. *Staphylococcus epidermidis* isolates could neither cause death, nor produce internal lesions and could not be re-isolated.

None of the nasal and tracheal swabs, lung samples and blood samples gave a positive result for *Mannheimia haemolytica* specific PCR. A pooled sample of nasal swabs from cattle with respiratory infection gave a positive result for it. But PCR of the culture could not yield a positive result. Moreover, no reference strain was available to make a comparison and confirm the result.

RAPD-PCR of the *Staphylococcus aureus* isolates showed that there was considerable genetic relationship between *Staphylococcus aureus* isolates of different species and also there was noticeable genetic diversity of the isolates within the host species.

Plasmids could be isolated only from two of the six isolates of *Escherichia coli* studied. Plasmid profile analysis of the isolates could not ascertain any correlation between the virulence, antibiotic resistance and presence of plasmids.