DETECTION OF *Pasteurella multocida* IN DOMESTIC RUMINANTS BY ISOLATION AND POLYMERASE CHAIN REACTION

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

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DECLARATION

I hereby declare that the thesis entitled **"DETECTION OF *Pasteurella multocida* IN DOMESTIC RUMINANTS BY ISOLATION AND POLYMERASE CHAIN REACTION"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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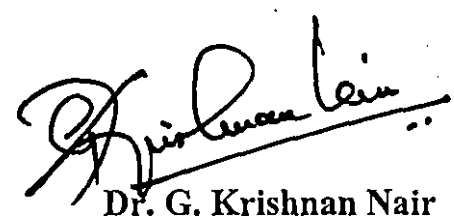
CERTIFICATE

Certified that this thesis entitled **"DETECTION OF *Pasteurella multocida* IN DOMESTIC RUMINANTS BY ISOLATION AND POLYMERASE CHAIN REACTION"** is a record of research work done independently by **Sunitha Karunakaran,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.



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Sunitha Karunakaran

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Introduction

1. INTRODUCTION

The largest world population of cattle and buffalo exists in India. These animals are the most important segments of livestock economy, contributing to the major portion of milk, meat, skin and manure production. They are susceptible to large number of *infectious and non-infectious* diseases.

Haemorrhagic septicaemia (HS) has a wide distribution in tropical countries like Asia and Africa. Asian countries like India rank HS as one of the most important contagious bacterial diseases affecting cattle and buffaloes. The disease is prevalent in almost all states of the country.

On the basis of distribution of the disease, three distinct categories of countries have been identified. India comes under the category 'A' where the disease is endemic and is of utmost economic importance (OIE Animal Health, 2002). In India, during the last four decades, it had been found that HS accounted for 46 to 56 per cent of all bovine deaths.

Resource allocation for prevention and control of HS reflects the correct estimate of its economic impact. Epidemics of HS may occur as alarming and devastating disease problems in cattle and buffalo and jeopardize not only the economic return of production animals, but also affect draught power of animals.

Two specific serotypes of *Pasteurella multocida* are responsible for the highly fatal acute septicaemic disease principally affecting cattle and buffaloes. In Asia HS is caused by serotype B:2 and in Africa by serotype E:2. In India B:2 is the most prevalent serotype. The disease is characterized by a rapid course, oedematous swelling in the head, throat and brisket region, swollen and haemorrhagic lymphnodes and presence of numerous subserous petechial haemorrhages. *Pasteurella multocida* affects and causes great economic loses in

a wide spectrum of hosts, including domestic ruminants like sheep and goats, besides cattle and buffaloes. In sheep and goats cases of pneumonia due to *P. multocida* had been reported.

There are reports about the significant association of *P. multocida* in the nasopharynx of apparently normal bovines and outbreak of the disease. Hence detection of *P. multocida* in apparently healthy domestic ruminants can be considered as an indicator of the susceptibility of the herd for HS.

Although *Pasteurella multocida* are demonstrated *insitu* by direct microscopy, isolation of bacteria is difficult, both from clinically infected and from apparently healthy animals. In the case of live animals, choice of clinical material for isolation is limited when compared to dead animals. Moreover, contaminant flora often overgrows specific pathogens when samples are taken from a contaminated site of the animal such as nose or throat, making the isolation trials more difficult.

The limitations faced in the isolation and identification of bacteria can be overcome by employing techniques like nucleic acid based assays, which serve as alternate methods of bacterial identification.

Nucleic acid based assays allow detection of organisms, both dead and live, in clinical samples with utmost sensitivity and specificity, thereby decreasing the time required for bacterial identification. Polymerase Chain Reaction (PCR) has been practically useful in this regard, with the use of primer sequences designed to facilitate identification at any level of specificity, strain, species or of all members of a domain.

Occurrence of *Pasteurella multocida* causing haemorrhagic septicaemia in apparently healthy domestic ruminants in this state has not been systematically studied so far, eventhough HS has been diagnosed clinically and rarely by laboratory methods like isolation. Several of the samples received in the

Department of Microbiology were found to be positive for bipolar organisms by direct microscopic examination, but isolation trials were found to be negative in most of the cases. Hence the present study is under taken with a view to understand the occurrence of *Pasteurella multocida* organisms causing HS in apparently healthy and clinically ill domestic ruminants in a specified area of Thrissur district *viz.*, Ollukkara block, covering one per cent of total ruminant population in the area. Objectives of the present study include,

(1) Detection of *Pasteurella multocida* by polymerase chain reaction, using *Pasteurella multocida* species specific (PM- specific) and Type-B specific (HS-B specific) primers.

(2) Isolation of *P. multocida* from apparently healthy and clinically ill ruminants in Ollukkara block area, Thrissur District.

(3) Detection of *P. multocida* in clinical samples brought to department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy from areas in and around Thrissur by polymerase chain reaction (PCR), using PM-specific and HS-B specific primers.

(4) Isolation of *P. multocida* from clinical samples brought to the department from areas in and around Thrissur.

(5) Conventional characterization of isolates based on morphological, cultural, biochemical characters and antibiogram.

(6) Characterisation of the isolates obtained, by Multiplex PCR and Repetitive Extragenic Palindromic sequence PCR (REP-PCR).

(7) Plasmid profile analysis of the isolates.

(8) Detection of *P. multocida* in clinical samples by Nested PCR.

Review of Literature

2. REVIEW OF LITERATURE

Haemorrhagic Septicaemia (HS) is an acute, septicaemic disease principally affecting cattle and buffalo. It is characterized by a rapid course, oedematous swelling in the head, throat and brisket region, swollen and haemorrhagic lymphnodes and presence of numerous subserous petechial haemorrhages (Carter and De Alwis, 1989).

The disease is a primary pasteurellosis caused by two specific serotypes of *Pasteurella multocida, viz.*, Asian and African serotypes. *Pasteurella multocida* is a Gram-negative facultatively anaerobic organism belonging to genus *Pasteurella.* All isolates of *Pasteurella multocida* associated with HS have been found to belong to type B of Carter (1955), type 6 of Namioka and Murata (1961 a, b, 1964) and type 2 of Heddleston *et al.* (1972). Asian serotype is designated 6:B or B:2 and African serotypes as 6:E or E:2 respectively.

2.1 NOMENCLATURE

Burrill (1883) gave the first name to the organism. He called it *Micrococcus gallicidus.*

Zopf (1885) gave the name *Micrococcus cholerae gallinarum* to the bacterium.

Trevisan (1887) suggested the name *Pasteurella cholerae gallinarum*, in the paper where he named the genus *Pasteurella.* The origin of combination *Pasteurella multocida* goes back to Kitt (1893). He used the name *Bacterium bipolare multocidum.*

Lehmann and Neumann (1899) gave the name *Bacterium multocidum.*

Topley and Wilson (1929) suggested *Pasteurella septica* as the suitable name to indicate the bacteria of septicaemia and also because the organism from different hosts behaved as if they belonged to a single species.

Rosenbusch and Merchant (1939) proposed the name *Pasteurella multocida,* which was later, listed as the type species of the genus (Mannheim and Carter, 1984). The genus found its place in the family *Pasteurellaceae,* under section five (facultatively anaerobic Gram-negative bacilli), in the ninth edition of Bergey's Manual of Systematic Bacteriology (Manheim, 1984).

Rimler and Rhoades (1989) suggested the same name to represent the heterogenous species of the organism.

The binomial nomenclature *Pasteurella multocida* is universally accepted as the name of the etiological agent of haemorrhagic septicaemia (Holmes, 1998).

2.2 HISTORY

Bollinger (1878) gave the first detailed report of an acute pasteurellosis, which affected deer, cattle and swine. He reproduced the disease in cattle.

Pasteur (1880) described the organism that caused fowl cholera.

Kitt (1885) isolated and described an organism similar to the one that caused fowl cholera, from a severe outbreak of a septicaemia in cattle, horses and swine.

Hueppe (1886) placed the organism causing similar diseases in several animal species under one name *Bacillus septicaemiae haemorrhagicae.*

Trevisan (1887) proposed the generic name *Pasteurella* in commemoration of the work of Pasteur on these bacteria.

Buchanan *et al.* (1966) listed the numerous names that have been proposed over the years for the heterogenous species *Pasteurella multocida.*

Pasteurella multocida is a heterogenous species that contains strains isolated from a variety of diseases and many animal species. These strains vary greatly with respect to pathogenicity, host prediliction, cultural and biochemical characteristics and antigenic nature.

Mutters *et al.* (1985) have proposed a reclassification of the genus pasteurella on the basis of DNA homology. They proposed three subspecies of *P. multocida.* The causal agents of HS would under this proposal, be designated *P. multocida* subspecies *multocida.*

Bisgaard *et al.* (1991) reclassified German, British and Dutch isolates of so called *Pasteurella multocida* obtained from pneumonic calf lungs. Taxonomic relationship of 131 strains previously identified as *Pasteurella multocida* obtained from calf pneumonia was investigated by extended phenotypic and limited genotypic characterization. Twenty-four strains were classified as *Pasteurella multocida* subspecies *multocida,* 15 strains as *P. avium* biovar 2 and 13 strains as *P. canis* biovar 2. Sixty-five and five strains were tentatively classified as ornithine negative *P. multocida* subspecies *multocida* and *P. multocida* subspecies *septica* respectively. Genetic investigations showed that ornithine negative strains of *P. multocida* were related on species level.

2.3 PREVALENCE

2.3.1 Prevalence Outside India

The disease of buffaloe called "barbone" in Italy was considered to be haemorrhagic septicaemia (Oreste and Armanni, 1887).

Since the turn of the century, there have been few reports of HS in European countries, other than Italy.

Haemorrhagic septicaemia is known to occur occasionally in American bison and there were outbreaks in 1912 (Mohler and Eichhorn, 1913), 1922 (Gochenour, 1924) and 1965 (Heddleston *et al.*, 1967). The strain serotype B:2

recovered from one of early outbreaks in bison was available and has been used in numerous studies.

Pasteurellae related to or resembling the HS serotypes has been reported in numerous instances from diseased or healthy animals and birds. A type B isolate but possessing somatic antigen 11 instead of 6 which is found in HS serotypes, was reported from a wound in a bovine animal in Australia (Bain 1951, 1959, Bain and Jones 1958).

Although acute pasteurellosis had been reported in Africa, it was Perreau (1961) who recognized that the similar outbreaks prevalent in Chad were typical HS, caused by a distinct variety of *P. multocida*. Perreau isolated strains from a number of outbreaks in Africa and these were identified by Carter as a new capsular type, *viz.*, type E (Carter, 1961).

Namioka and Bruner (1963) reported the known occurrence of Type B HS strains in Egypt.

Kasali (1972) reported the case of HS in an African Buffalo (*Syncerus nanus*) in Nigeria.

De Alwis and Panangala (1974) stated that in Srilanka HS was a per acute or acute disease caused exclusively by *P. multocida* serotype 6:B.

Mustafa *et al.* (1978) reported that HS was wide spread in Sudan.

Serotypes B:2 (6:B) and E:2 (6:E) had been recovered from diseases in Egypt and Sudan. (Shigidi and Mustafa, 1979)

Francis *et al.* (1980) referred to an outbreak of HS in Zambia caused by type E *Pasteurella multocida* that resulted in the death of more than 10,000 cattle in less than one year.

Bastianello and Jonker (1981) reported the serogroup E *Pasteurella multocida* in South Africa.

Chandrasekaran *et al.* (1981) conducted biochemical and serological studies of *Pasteurella multocida* isolated from cattle and buffaloes in Malaysia.

De Alwis (1981) studied the pattern of mortality in cattle and buffaloes in Srilanka due to haemorrhagic septicaemia. Mortality in buffaloes were 45.2 per cent and cattle 15.8 per cent respectively.

In a Srilankan survey conducted by Bain *et al.* (1982), the percentage of herds with HS depended on herd size and ranged from 20 per cent in herds under 10 buffaloes to as high as 98 per cent in herds exceeding 50 buffaloes. The trend in cattle was similar.

The disease was rare or nonexistent in cattle in North America. Kradel and associates described an explosive outbreak of haemorrhagic septicaemia in young dairy cattle in 1969 in New Jersey. It was speculated that the only HS outbreak reported in cattle for several decades in North America resulted from the injection of a contaminated vitamin preparation (Carter, 1982).

Haemorrhagic septicaemia was recognized in Japan in 1923 but no epidemics have been reported since 1954 (Carter, 1984a).

Pasteurella multocida serotype B:2 was isolated from poultry in Iran (Chandrasekaran *et al.*, 1985).

Hassan and Mustafa (1985) reported about serogroup B *Pasteurella multocida* in Africa, particularly in North Africa.

Jones *et al.* (1988) isolated *P. multocida* serotype F 3,4 from a calf in UK.

Jhonson *et al.* (1989) characterized six *P. multocida* isolates obtained from clinically sick cattle and buffalo in Philippines suspected of having HS, by Poly Acrylamide Gel Electrophoresis (PAGE).

Association of *Pasteurella multocida* in pneumonic pasteurellosis of sheep in Malaysia was studied by Chandrasekaran *et al.* (1991).

Hancock *et al.* (1991) reported pneumonic pasteurellosis due to *Pasteurella multocida* in a flock of lambs in Brazil.

Septicaemia due to *Pasteurella multocida* in two calves in Australia was reported (Hill and Johnson, 1992).

Lane *et al.* (1992) reported an outbreak of haemorrhagic septicaemia (septicaemic pasteurellosis) due to serogroup B *P. multocida* in cattle in Zimbabwe.

Outbreak of HS in Cameroon due to serogroup B *P. multocida* was reported (Martrenchar and Njanpop, 1993).

Sheikh *et al.* (1996) made observations on haemorrhagic septicaemia in Pakistan livestock and found that Pakistan showed 11 per cent incidence, 9 per cent mortality and 78 per cent case fatality rates of HS in buffalo, whereas these values were 4 per cent, 2.5 per cent and 62 per cent in cattle.

Voigts *et al.* (1997) confirmed outbreaks of HS due to *Pasteurella multocida* type B:2 in Namibia in cattle of different ages.

Outbreak of *P. multocida* septicaemia in neonatal lambs in America was reported (Watson and Davies, 2002).

Weiser *et al.* (2003) characterized *P. multocida* associated with pneumonia in bighorn sheep in USA.

2.3.2 Prevalence in India

Sengupta and Verma (1951) described an outbreak of swine pasteurellosis at the government piggery, Babugarh, during the year 1948 in which 29 out of 156 pigs succumbed. The strain of *Pasteurella* isolated though not pathogenic to piglets was not serotyped.

Manjrekar (1954) reported about pasteurellosis in sheep due to *Pasteurella multocida* in Bombay.

A strain of *P. multocida* of low virulence was accidently isolated from cases of paraplegia in cattle in India (Dhanda and Nilakantan, 1961).

Murthy and Adlakha (1962) reported the isolation of *P. multocida* from lung samples in cases of swine fever from different parts of Uttar Pradesh.

About 20 cases of swine pasteurellosis in five districts of Andhra Pradesh was reported by Venktanarayana (1962).

Murthy and Kaushik (1965) described an outbreak of acute septicaemic form of swine pasteurellosis due to *Pasteurella multocida* type B. This strain was pathogenic to pigs and also to buffalo, goats, rabbits, mice and guinea pigs.

Pavri and Apte (1967) isolated *Pasteurella multocida* from a fatal disease that affected horses and donkeys. Detailed description of the disease condition encountered in India was given in the report.

Palit and Rao (1969) conducted studies on the incidence of carriers and natural immunity in ovines. Eleven (5.56 per cent) out of 198 healthy sheep were found to be carriers of *Pasteurella multocida* in their nasopharynx. Serum specimens of 198 healthy sheep were examined for natural immunity by plate agglutination test employing antigens prepared from three ovine strains R_1, R_2 and R_4. Seven (3.4 per cent), 15 (7.6 per cent) and 6 (3.03 per cent) possessed natural immunity against R_1, R_2 and R_4 strains respectively. Two (1.01 per cent) sheep were naturally immune against both R_1 and R_2 strains.

Vig and Kalra (1969) carried out studies to investigate the outbreaks of primary pasteurellosis of acute septicaemic form in swine, which occurred in the Punjab state during the years 1961 and 1962. Pasteurella organisms were isolated from the heart blood and internal organs of the affected pigs. The isolates were found to be pathogenic to mice, rabbits and pigs.

Gupta and Kumar (1973) reported about the strains of Asian HS serotype, which were recovered from poultry.

Isolation of *Pasteurella multocida* from udder secretion of a crossbred cow in Assam was reported by Chakraborty *et al.* (1978).

The increasing occurrence of HS during the past four and- a- half decades was responsible for 46 to 55 per cent of the total bovine mortality and with the progressive control of Rinderpest, HS accounted for the largest proportion of reported bovine mortality (Khera, 1979).

Sharma and Boro (1980) isolated *Pasteurella multocida* from sporadic outbreaks of bovine pasteurellosis in Assam.

Haemorrhagic septicaemia outbreak due to *Pasteurella multocida* serotype B:2 in pigs in India had been reported (Verma, 1988).

Dutta *et al.* (1990) conducted epidemiological studies on the occurrence of HS in India. Mortality and morbidity-wise HS was placed, respectively at first and second position as compared to other four epizootic diseases, namely FMD, Rinder pest, Anthrax and Black quarter. Estimation of overall state wise relative risks due to HS was also conducted. Relative risk was highest for Manipur (18.57) and lowest for Dadra Nagar Haveli (0.03). State wise classification of high risk and medium risk areas were also done. Andaman, Lakshadweep and Mizoram had the disease free status.

Outbreak of pneumonia in sheep in India due to *Pasteurella multocida* was reported (Umesh, 1994).

Occurrence of F:3, A:1 and A:3 serotypes in cattle and A:3 and B:2 serotypes in buffalo was reported (Kumar *et al.*, 1996).

Kanwar *et al.* (1998) isolated *Pasteurella multocida* from twelve cases out of three hundred and seventy four lungs of goat showing pneumonic lesions, from slaughterhouses of Himachal Pradesh, Chandigarh, Delhi, Rajasthan, UP, Bihar and West Bengal.

Tomer (2000) reported the isolation of *Pasteurella multocida* serotype B:2 from outbreaks of HS in different districts of Haryana (1997, 1998, 1999) and Delhi State (1999).

2.4 CARRIER STATUS

Singh (1948) noted the occurrence of healthy carriers of *Pasteurella multocida* in apparently normal cattle and buffaloes.

Bain (1957) stated that there was more speculation than accurate knowledge on the pathogenesis of HS and that the problem could be partly solved by determining whether or not healthy cattle and buffaloes were carriers of *Pasteurella multocida* type I (Roberts).

Smith (1959) could isolate *Pasteurella multocida* from two out of seventy one students examined at Royal Veterinary College, London.

Gupta (1962) found that detection of carrier animals was related to the recent incidence of HS. During an outbreak he found that 7.5 per cent of clinically normal incontact animals are carriers, whereas forty days later he was unable to detect any carriers in the same herd.

Examination of nasopharynx of three hundred apparently healthy cattle carcasses at slaughter was carried out by Wijewanta and Karunaratne (1968) and found that fifteen per cent carried *P. multocida* in nasal and pharyngeal regions. All strains were pathogenic to mice and when examined by mouse protection test, were found to be neutralized by sera prepared against virulent HS causing strain of *P. multocida* type I (Roberts).

Studies on incidence of carriers and natural immunity of ovine pasteurellosis was conducted by Palit and Rao (1969) and found that eleven (5.56 per cent) out of 198 healthy sheep were carriers of *Pasteurella multocida* in their nasopharynx. Biochemical and fermentative characters of these strains were studied and only one out of the seven carrier strains was found typeable while others could not be typed.

Healthy carrier cattle in association with outbreaks of HS were documented by Mustafa *et al.* (1978). Carrier rate was found to be 44.4 per cent of the population in comparison with 3.89 per cent, 5.5 per cent and nil in herds unassociated with the disease. Healthy carrier rate was always higher in calves less than two years old than in adult cattle. It was found to be very low in herds with no recent history of HS. None of the carrier animals out of 44.4 per cent succumbed to disease during the period of observation, which extended 10 days after the outbreak had completely subsided.

An epizootiological study of HS in buffaloes and cattle in Srilanka were conducted by De Alwis and Vipulasiri (1980). It was found that 48.4 per cent of the buffalo herds and 38.5 per cent of the cattle herds had experienced epizootics during the period covered by the survey. Mortality due to HS was significantly higher in buffaloes than in cattle ($P<0.001$). Nearly two thirds of HS losses in buffalo and four fifth in cattle were under 2 years of age.

De Alwis and Sumanadasa (1982) reported that adult cattle and buffalo population in the high incidence HS enzootic areas were found to be immune to HS where as only calves born after previous outbreaks were found susceptible.

A progressive diminution in the percentage of carriers *viz.*, from 22 per cent in herds one week after an outbreak to 1.9 per cent in herds six weeks later was observed by Hiramune and De Alwis (1982). These findings led to the conclusion that the carrier state as evidenced by the presence of organism in the nasopharynx was a transient one.

When animals in the same herds were exposed to HS and examined repeatedly, different animals showed up as carriers on different days (De Alwis *et al.*, 1986b). It had also been found that during the post exposure period a large percentage of animals developed high levels of antibody.

Investigations on slaughtered carrier animal have shown that during periods when organism was not detectable in the nasopharynx, it persisted in the tonsils (De Alwis *et al.*, 1986a, Wijewardana *et al.*, 1986a).

Wijewardana *et al.* (1986a) compared cultural, biochemical and serological properties and pathogenicity of a wide range of isolates from healthy carriers with those of isolates from out breaks of HS.

2.5 ISOLATION OF *P. multocida* USING SELECTIVE MEDIA

Selective media intended for isolation of *Pasteurella multocida* from contaminated sources of a variety of animal species was developed incorporating esculin, cobaltchloride and crystal violet to nutrient agar base (Das, 1958).

Morris (1958) developed selective media intended for isolation of *P. multocida* from contaminated sources of a variety of animal species containing five per cent peptic blood digest, potassium tellurite, neomycin, tyrothricin and actidione.

Namioka and Murata (1961) described a solid medium, yeast proteose cystine agar, to demonstrate the colony morphology of *Pasteurella multocida*.

Burrows and Gillet (1966) suggested that the nutritional requirement of *P. multocida* was more exacting at 37°C than at a lower temperature.

Nutrient agar containing five per cent ovine or bovine blood was used for the isolation of *Pasteurella* (Carter, 1967), but it was not satisfactory for the identification of colonial variants. He also found that tryptic soy blood agar

containing five per cent bovine or ovine blood and brucella agar containing two per cent haemolysed rabbit serum improved the growth of *Pasteurella.*

De Alwis (1973) employed a simple, inert transport medium that contained disodium phosphate, thioglycollic acid, 0.4 per cent agar and methylene blue, which helped to isolate *Pasteurella* even several hours after the death of the animal.

Carter (1981) reported that *P. multocida* grew best at a temperature of 37°C aerobically or anaerobically, with five per cent CO_2 on bovine or ovine blood agar.

A selective medium incorporating clindamycin, gentamicin, potassium tellurite and amphotericin-B in brain heart infusion agar, with five per cent equine blood (CGT medium) was used for isolation of *P. multocida* (Knight *et al.*, 1983).

Smith and Baskerville (1983) developed a selective medium intended for isolation of *P. multocida* and *Bordetella bronchiseptica*, specifically from nasal specimens of swine. The medium, 8 HPG contained 2 per cent yeast extract, ferric citrate 75 mg/l, lactose 10 g/l, phenol red 25 mg/l, chloral hydrate 600 mg/l, bacitracin 5000 units/l, gentamicin 30 µg/l, polymyxin 200 µg/l, and mycostatin 50 units/l, prepared in nutrient agar base. The medium could be used for isolation of *P. multocida* from other species since it supported growth of strains associated with HS (serogroups B and E).

Dejong and Borst (1985) described a selective medium for the isolation of *P. multocida* and *Bordetella bronchiseptica*, which contained tryptose soy agar, five per cent defibrinated ovine blood, gentamicin sulphate, potassium tellurite, amphotericin-B and bacitracin. Toxigenic strains of *P. multocida* were also isolated from nasal swabs using this medium.

Wijewardana *et al.* (1986a) described the casein sucrose yeast agar (CSY agar) with or without five per cent bovine blood for the isolation of *Pasteurella.*

The methods for sampling and isolation of toxigenic *Pasteurella multocida* from the nasal cavity of pigs were compared by Chanter *et al.* (1989).

Modified KB medium composed of tryptic soy agar with five per cent peptic ovine blood, 0.05 μg per ml of kanamycin and 2.5 μg per ml of bacitracin was used by Kawamoto *et al.* (1990) for the isolation of *P. multocida* from rabbits.

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

Detection of NAD dependent *Pasteurellaceae* from the respiratory tract of slaughterhouse pigs was done by Moller (1993).

Moore *et al.* (1994) described a new selective medium, the *Pasteurella multocida* selective agar (PMSA) and *Pasteurella multocida* selective broth (PMSB) containing gentamicin, potassium tellurite and amphotericin-B.

Transport enrichment medium (TEM) developed by Warner (1996) inhibited the growth of other bacterial and fungal contaminants and improved chance of isolating *Pasteurella multocida* from diseased animals. The medium consisted of brain heart infusion agar with amikacin, gentamicin, potassium tellurite and amphotericin-B.

Lee *et al.* (1999) developed a new selective medium containing polymyxin B, crystal violet, thallous acetate, bacitracin and cycloheximide in ten per cent ovine blood dextrose starch agar, which gave a high rate of isolation of *P. multocida* from chicken alimentary tract.

2.6 BIOTYPING

The fermentation pattern of the bacterial cultures with specific sugars help in the differentiation of isolates into different subspecies, commonly referred to as biotyping.

Schneider as early as 1948, classified *P. multocida* into subgroups based on the variation in acid production from xylose, arabinose, maltose, trehalose, sorbitol, mannitol and dulcitol.

Murthy and Kaushik (1965) reported about the sugar fermentation pattern of a strain of *P. multocida* serotype B isolate from acute swine pasteurellosis. The strain produced acid but no gas from maltose, sucrose, glucose, xylose, galactose, sorbitol, fructose, arabinose and mannitol. The strain did not ferment lactose, dulcitol, raffinose, rhamnose, salicin and trehalose.

Palit and Rao (1969) subjected 17 strains of *Pasteurella multocida* from healthy carriers and clinical cases to different biochemical tests. All the 17 strains fermented glucose, galactose and fructose. None of the strains fermented lactose, maltose and glycerol.

Shigidi and Mustafa (1979) conducted biochemical studies of 42 strains of *Pasteurella multocida* isolated from different outbreaks of haemorrhagic septicaemia and also from healthy cattle in various parts of the Sudan, based on the fermentation of xylose, glucose, fructose, galactose, mannose, sucrose and sorbitol, with acid production. None of the strains fermented rhamnose, lactose, trehalose, raffinose, dulcitol or salicin.

Chandrasekaran *et al.* (1981) conducted biochemical studies of *Pasteurella multocida* isolated from cattle and buffaloes in Malaysia. None of the strains fermented rhamnose, raffinose, starch, inulin, adonitol, dulcitol, inositol, salicin, aesculin, lactose, maltose, arabinose or dextrin.

Madsen *et al.* (1985) conducted phenotypic characterization of *Pasteurella* species isolated from lungs of calves with pneumonia. Many *P. multocida* like isolates had a fermentation pattern different from what is generally accepted for *P. multocida sensu stricto*. Phenotypical characters of the 50 strains examined were compared to *P. multocida sensu stricto*. Seven strains were diagnosed as *P. multocida*, although differences were noted with respect to fermentation of xylose, lactose and trehalose. *Pasteurella multocida* like strains formed a group, which was tentatively, designated taxon 13.

Wijewardana *et al.* (1986a) compared biochemical properties of a wide range of *Pasteurella multocida* isolates from healthy carriers, with those of isolates from outbreaks of HS based on fermentation of glucose, sucrose, sorbitol and mannitol with production of acid only. None of them fermented lactose, trehalose, or salicin.

Pasteurella haemolytica can be readily distinguished from *P. multocida* based on the inability of the former to produce indole, and their ability to ferment maltose, dextrin and after several days inositol (Adlam,1989).

Biochemical analysis of 29 porcine isolates of *P. multocida* was conducted by Buttenschon and Rosendal (1990). All the 29 strains showed uniform biochemical reactivity.

Bisgaard *et al.* (1991) described the taxonomic relationship of 131 strains of *Pasteurella multocida* by an extended phenotypic characterization. De-carboxylation of ornithine, production of acid from mannitol and indole production were used for the subspecies classification.

Mohan *et al.* (1994) studied the phenotypic characteristics of *Pasteurella multocida* isolates from Zimbabwe and reported that all isolates, irrespective of their origin fermented glucose and sucrose but did not ferment arabinose, dulcitol, inositol and salicin.

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

Blackall *et al.* (1997) recognized seven different biovars among *Pasteurella multocida* isolates, of which biovar 3, previously identified as *Pasteurella multocida* subsp *multocida* was the most common.

Blackall *et al.* (2000) studied biochemical profiles of thirty-eight field isolates of *Pasteurella multocida* of porcine origin. The field isolates differed in their ability to ferment arabinose, dulcitol and lactose. Based on the differences the isolates could be identified as either *Pasteurella multocida* subspecies *multocida* (31 isolates) or *Pasteurella multocida* subspecies *gallicida* (seven isolates). The *Pasteurella multocida* subspecies *multocida* isolates were identified as biovar 3 (did not ferment arabinose, dulcitol and lactose) and biovar 12 (fermented lactose but not arabinose or dulcitol).

Positive reactions to indole and ornithine decarboxylase have been described as the most useful biochemical indicators in identification of *Pasteurella multocida* (OIE, 2000)

Dziva *et al.* (2001) studied biochemical profiles of eighty-one isolates of *Pasteurella multocida* from a variety of diseases in animals in Zimbabwe. Based on the differences in fermentation patterns of sugars like dulcitol, mannitol, sorbitol and trehalose over 80 per cent of isolates were assigned to taxa *Pasteurella multocida* subspecies *multocida* and *Pasteurella multocida* subspecies *septica*. Remaining isolates could not be assigned to the known taxa, and were designated as unassigned biotypes. *Pasteurella multocida* subspecies *multocida* isolates fermented mannitol and sorbitol but not dulcitol and trehalose, while *Pasteurella multocida* subspecies *septica* isolates fermented trehalose and mannitol but not dulcitol and sorbitol.

2.7 ANTIBIOGRAM

Clonal heritability of conserved characteristics resulted in the acquisition of resistance to antibiotics by some clones of bacteria, while others remained susceptible (Rajalakhsmi, 2001).

Antimicrobial susceptibility of *Pasteurellae* isolated from sheep was studied by Smith (1961).

Murthy and Kaushik (1965) performed the antimicrobial sensitivity pattern analysis of *P. multocida* serotype B isolates from acute swine pasteurellosis and found that it was highly sensitive to oxytetracycline and chlortetracycline and slightly sensitive to chloramphenicol, dihydrostreptomycin and penicillin and resistant to bacitracin and triple sulpha.

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

Chakraborty *et al.* (1978) conducted antibiotic sensitivity test of the *Pasteurella multocida* isolated from cases of clinical mastitis. The isolate was sensitive to terramycin and chloramphenicol only and resistant to penicillin, streptomycin, ampicillin, kanamycin, gentamicin, bacitracin and sulpha drugs.

Antimicrobial susceptibility patterns of *Pasteurellae* isolated from sheep was studied by Biberstein and Kirkham (1979); Aghomo and Ojo (1983).

Antimicrobial resistance among *Pasteurella* species recovered from cattle of Missouri and Iowa State with bovine respiratory disease complex was studied by Fales *et al.* (1982). Most of the *Pasteurella multocida* isolates did not show marked antimicrobial resistance to nine of the fifteen drugs tested. Fifty eight per cent of the *Pasteurella multocida* isolates (84/145) were resistant to streptomycin and 88 per cent (126/144) of them were resistant to three combined sulphonamides.

De Alwis (1984) reported that a collection of *Pasteurella multocida* strains from cases of haemorrhagic septicaemia from Malaysia, Indonesia, Thailand, Burma, India and Sri Lanka, were sensitive to penicillin, ampicillin, streptomycin, tetracycline, chloramphenicol, erythromycin, neomycin, sulphadiazine and sulpha- trimethoprim combination.

Haghour *et al.* (1987) observed that bovine strains of *Pasteurella multocida* were in general more resistant to antibiotics than ovine strains and this observation reflects on the more frequent use of antibiotics in bovines for treating respiratory disease.

Wilson (1990) stated that the clones of pathogenic pasteurellae had antibiotic resistance that fluctuated and mediated through transferable plasmids, while more permanent resistance was mediated by chromosomal changes.

Cote *et al.* (1991) evaluated twenty-nine field isolates of porcine *Pasteurella multocida* for their susceptibility to ten antimicrobial agents. All the isolates were susceptible to ampicillin, penicillin, gentamicin, kanamycin, spectinomycin, tetracycline, erythromycin, sulfonamide and streptomycin and resistant to clindamycin. Resistance to sulfonamides and streptomycin was observed in seven isolates, which contained R plasmids thereby conferring relationship between presence of plasmids and antibiotic resistance.

Abeynayake *et al.* (1993) reported antibiotic resistance to streptomycin and sulphonamides by *Pasteurella multocida* causing haemorrhagic septicaemia.

Rammanath and Gopal (1993) studied the sensitivity of *Pasteurella multocida* isolates of duck origin. The organisms were sensitive to chloramphenicol, chlortetracycline, oxytetracycline, co-trimoxazole, nalidixic acid, gentamicin, nitrofurantoin, streptomycin, kanamycin and neomycin and to a

lesser extent against polymyxin-B, penicillin G, amoxycillin, cloxacillin, lincomycin and vancomycin.

Pasteurella multocida isolated from pneumonic ovine lungs showed sensitivity to chloramphenicol, ampicillin and showed resistance to streptomycin and lincomycin. This pattern appeared to be common among all the three hundred and thirty isolates studied (Diker *et al.*, 1994).

Diallo *et al.* (1995) studied antimicrobial resistance patterns of forty-five avian strains of *Pasteurella multocida*. All the strains were resistant to streptomycin, lincomycin and trimethoprim, only one strain was resistant to tetracycline. All the strains were susceptible to ampicillin, penicillin, gentamicin, erythromycin, trimethoprim, nitrofurantoin and sulfanilamide.

Balakrishnan (1998) observed four different antibiotic sensitivity patterns of *Pasteurella multocida* and the isolates were found to be sensitive to oxytetracycline, pefloxacin and streptomycin, but resistant to furazolidone, metronidazole and nalidixic acid.

De Rosa *et al.* (2000) conducted antibiotic susceptibility analysis of *Pasteurella multocida* isolated from nasal and transtracheal swabs from cattle with clinical signs of bovine respiratory disease. Ninety per cent of isolates were inhibited by ampicillin, erythromycin, spectinomycin and trimetroprim-sulfamethoxazole. Antibiotic susceptibility profiles of few paired isolates were different.

2.8 SEROTYPING

Early workers developed many systems for serotyping of *P. multocida,* based on agglutination tests, which are not in use today. These systems failed to gain wide acceptance since they could not type capsulated isolates (Rimler and Rhoades, 1989).

2.8.I Serum Protection Typing

Roberts (1947) developed an immunological typing system based upon passive protection of mice by serum against live organisms. Four types, designated as I, II, III, IV were recognized among 37 cultures. Type V was distinguished later by Hudson (1954). Isolates of *P. multocida* causing HS in cattle and buffalo were grouped in Type 1.

Antigenic characterization of *P. multocida* was accomplished by capsular serogrouping and somatic serotyping.

2.8.2 Capsular Serogrouping

The system most commonly employed for the specific capsular serogrouping was based on passive haemagglutination of erythrocytes by capsule antigen (Carter, 1955). Five serogroups A, B, D, E and F have been reported in the Carter system (Carter, 1967).

Roberts type I, II and V serotypes were equated with Carter's Serogroup B, A and D, respectively. Carter observed that isolates of subtypes of A contained hyaluronic acid and suggested that they may be equated to Robert's types III and IV (Carter, 1963).

Rimler (1978) developed a co-agglutination procedure for the recognition of serogroup B and E causing HS.

De Alwis and Carter (1980) described immunodiffusion test for the identification of capsular types B and E.

Capsular serotype was identified rapidly by counter immuno electrophoresis (Carter and Chengappa, 1981b). This technique was also used to identify free capsular antigen in the tissues of infected rabbits and it could be used to identify capsular antigen in the tissues of animals that die due to HS.

Bain *et al.* (1982) used mouse protection test for the identification of B:2 strains from haemorrhagic septicaemia cases. This test involved determining whether or not specific rabbit antisera would protect mice against intraperitoneal challenge with the strain being examined.

Wijewardana *et al.* (1982) described an agar gel precipitin test in which antigen consisted of a saline extract of the strain being examined and the antibody was provided by rabbit antisera prepared from inoculation of killed serotype B or serotype E *Pasteurella multocida.*

Wijewardana *et al.* (1986a) carried out simplified capsular typing of *Pasteurella multocida* isolates by employing acid treated cells and an agglutinin absorption procedure and indirect haemagglutination test was also used for capsular typing by a modification of the method of Carter (1955).

Townsend *et al.* (2001) developed a multiplex PCR assay, using serogroup specific primers, as a rapid alternative to the conventional capsular serotyping system. The serogroup specific primers used in this assay were designed following identification, sequence determination and analysis of the capsular biosynthetic loci of each capsular serogroup. The multiplex capsular PCR assay was highly specific and its results, with an exception of those for some serogroup F strains, correlated well with conventional serotyping results.

Davies *et al.* (2004) characterized one hundred and fifty three bovine *Pasteurella multocida* strains, recovered primarily from cases of pneumonia and mastitis in England and Wales over an 11year period, by capsular PCR typing using the primers targeting specific capsular serogroups. All of the strains were of capsular type A, with an exception of a single capsular type F isolate.

2.8.3 Somatic Serotyping

Roberts (1947) identified several different immunotypes of *Pasteurella multocida* including the one that caused most outbreaks of HS by passive mouse

protection tests. He designated the variety as type I, and this designation was used for many years.

Namioka and Murata (1961) developed a somatic serotyping system based on tube agglutination tests. Eleven serotypes (1 through 11) were recognized by this system.

Heddleston *et al.* (1972) developed Heddleston system based upon gel diffusion precipitin tests employing heat extracted antigen and antipasteurella sera prepared in chicken. Sixteen serotypes (1 through 16) were recognized in this system. Two haemorrhagic septicaemia capsular types of Carter, *viz.*, B and E, were found to be somatic type 2.

Shigidi and Mustafa (1978) conducted serological studies on forty-two strains of *Pasteurella multocida* isolated from outbreaks of HS and from healthy cattle in various parts of Sudan. Indirect haemagglutination test, gel diffusion precipitin test and tube agglutination test were used to determine somatic serotypes of the strains.

Comparisons between Namioka and Heddleston serotyping systems was made by Brogden and Packer (1979). They observed that cultures, which represented a single serotype in a particular typing system, represented more than one serotype in the other system.

Carter and Chengappa (1981a) opined that systems of Carter and Heddleston may be combined and used to designate serotype, so that a serotype would be designated by its capsular type, followed by its somatic type as determined by the agar gel precipitin test. By this system two HS serotypes were designated B:2 and E:2. The B:2 is equivalent to Namioka's 6:B and the E:2 to 6:E.

Wijewardana *et al.* (1986a) compared serological properties of a wide range of *Pasteurella multocida* isolates from healthy carriers with those of

isolates from outbreaks of HS. All the isolates from the carrier animals gave positive reactions of somatic type 6. Only 14 of 21 isolates tested from abattoir animals showed positive reactions of somatic type 6.

2.9 MOLECULAR TECHNIQUES

2.9.1 Detection and Identification of *P. multocida* by Specific PCR Assays

Since the development of polymerase chain reaction (PCR) in 1985, the basic principle of *in vitro* nucleic acid amplification has had extensive applications in all aspects of fundamental and applied clinical science (Rapley *et al.*, 1992).

Kasten *et al.* (1995) described the use of oligonucleotide primers constructed to amplify the *Psl* gene encoding the P6 like protein of *Pasteurella multocida.*

Brickell *et al.* (1998) developed a PCR assay to detect *Pasteurella multocida* serotype B:2, the causal agent of HS in Asia. Nucleotide sequence of 16S rRNA–23S rRNA PCR product unique to B:2 strains was determined and primers designed from this sequence were found to specifically amplify DNA from *P. multocida* serotype B:2. This PCR assay demonstrated reasonably specific amplification of serogroup B isolates with HS significance. Amplification was also observed with one of two serogroup E *P. multocida* isolates analysed.

Townsend *et al.* (1998a) developed a *Pasteurella multocida* specific PCR (PM-PCR) that identified all subspecies of *P. multocida viz.*, subsp. *multocida,* subsp. *gallicida* and subsp. *septica,* through specific amplification of an approximately 460 bp DNA fragment within the KMT1 gene. Genomic substractive hybridization of closely related *P. multocida* isolates has generated clones useful in distinguishing HS causing type B strains from other *P. multocida* serotypes. Oligonucleotide primer pair KTT72 and KTSP61 designed from the

sequence of the clone 6b specifically amplified a DNA fragment from types B:2, B:5 and B: 2, 5 *P. multocida.*

Townsend *et al.* (1998a) developed HSB–PCR for HS causing serogroup B *P. multocida.* Serogroup B cultures with the predominant somatic antigen being either serotypes 2 or 5 are specifically identified by the amplification of a 590 bp fragment using KTSP61 and KTT72 primers.

Lee *et al.* (1999) developed a modification of *Pasteurella multocida* specific PCR for detection of *P. multocida* from chicken alimentary tract. The PCR assay demonstrated a sensitivity of less than ten organisms.

Boyce *et al.* (2000) determined the nucleotide sequence and genetic organization of *P. multocida* M1404 (B:2) capsule locus. The initial identification of the capsule biosynthetic locus of *P. multocida* M1404 (B:2) was achieved by PCR amplification of a small section of the coding region of the ABC transporter gene. The oligonucleotide primer pairs BAP446 and BAP448 produced a 350 bp fragment and sequence analysis confirmed that the amplified fragment contained the corresponding region of *P. multocida* M1404 (B:2) CexA homologue (designated CexA for capsule export).

A specific and sensitive PCR assay was used for detection of *Pasteurella multocida* from the tonsils of slaughtered pigs in Vietnam by Townsend *et al.* (2000). Out of the 36-tonsil swab specimen, *P. multocida* was detected in 16 samples by PM-PCR and all samples were negative for serogroup B by HS-B PCR (Townsend *et al.*, 1998a). Multiplex PCR assay with both the primer sets was also performed.

Blackall and Miflin (2001) developed a PCR assay using the primers derived from 23S ribosomal RNA gene sequence of *P. multocida*. The PCR assay correctly identified all 144 isolates of *P. multocida* of avian and porcine origin.

Shivshankara *et al.* (2001) studied the efficacy of PCR in identification and differentiation of *P. multocida* isolates using primer pairs KTSP61 and KTT72 for amplification of the unique sequence in *P. multocida* serotypes B:2.

A multiplex PCR assay was introduced as a rapid alternative to the conventional capsular serotyping system by Townsend *et al.* (2001). The capsular serogroup specific primers used in this assay were designed following identification, sequence determination and analysis of capsular biosynthetic loci of each capsular serogroup. The multiplex PCR will clarify the distinction between closely related serogroup A and F and constitutes a rapid assay for the definitive classification of *P. multocida* capsular types.

Rocke *et al.* (2002) developed a serotype specific PCR assay for the detection and identification of *P. multocida* serotype 1. This assay was successful in distinguishing serotype 1 from the other 15 serotypes, with the exception of serotype 14.

Anupama *et al.* (2003) evaluated polymerase chain reaction for the identification of virulent *Pasteurella multocida* and compared with that of mice inoculation studies, using serotype B:2 specific primers IPFWD & IPREV developed by Brickell *et al.* (1998). Oligos amplified three isolates that killed mice whereas two isolates, which had undergone several passages, did not show amplification and were nonpathogenic to mice.

Using the capsule specific primers (CAP A, CAP B, CAP D, CAP E and CAP F) designed by Townsend *et al.* (2001), Davies *et al.* (2003) determined capsular types of hundred avian *P. multocida* isolates by capsular PCR typing. Isolates that were negative for all five capsular types were confirmed as *P. multocida* with a *P. multocida* specific primer set, KMTIT7 and KMTISP6.

Davies *et al.* (2004) characterised 153 bovine *P. multocida* strains recovered primarily from cases of pneumonia and mastitis in England and Wales over an 11-year period, by capsular PCR typing. All the strains were of capsular type A, with the exception of a single capsular type F isolate.

Dutta *et al.* (2004) reported about PCR based detection of *Pasteurella multocida* isolates from buffalo with symptoms of HS by species specific (PM-PCR) and type specific (HS-B) PCR using genomic DNA. Out of the 32 isolates recovered from buffalo, 31 isolates were confirmed as type–B *Pasteurella multocida* by conventional serotyping and HS-B PCR assay. The primers used were same as those developed by Townsend *et al.* (1998a) and concluded that PCR based diagnosis was very effective and sensitive.

Gautam *et al.* (2004) developed a PCR assay targeting the hyaC-hyaD gene, to identify strains of *P. multocida* belonging to serogroup A. A set of serogroup specific PCR primers amplified a 564 bp product from genomic DNA. This method detected as low as 10 ng of bacterial DNA and had a specificity of 100 per cent for *P. multocida* serogroup A. A nested PCR method yielded a single 374 bp product.

Kapoor *et al.* (2004) detected *Pasteurella multocida* by species specific (PM-PCR) primers developed by Townsend *et al.* (1998a). Eleven isolates of *Pasteurellae* recovered from 470 samples cultured for bacterial isolation from catlle, buffalo, sheep, goat and rabbit were confirmed as *Pasteurella multocida* by PM-PCR. Out of eleven, six isolates were serotype A:1 and and one was serotype A:3. Serotype B: 2 could not be isolated from any of the samples.

2.9.2 PCR Detection of Toxigenic *P. multocida*

Pijoan *et al.* (1984) reported that PCR band amplified directly from nasal swabs for detection of toxigenic *P. multocida* was often faint due to small numbers of the organisms recovered in nasal swabs.

Nagai *et al.* (1994) used primers constructed from the sequence of the *tox* A gene, encoding the dermonecrotic toxin implicated in progressive atrophic rhinitis, to detect toxigenic strains of *P. multocida* by PCR.

Kamp *et al.* (1996) developed a specific and sensitive PCR assay suitable for large-scale detection of toxigenic *P. multocida* in nasal and tonsillar swab specimens from pigs.

Lichtensteiger *et al.* (1996) studied the feasibility of using PCR for accurate and rapid detection of toxigenic *P. multocida* from swabs. The PCR protocol using oligonucleotide primers TA-1 and TA-2, amplified 846-nucleotide segment of the *tox* A gene. The protocol was specific for toxigenic *P. multocida* and could detect fewer than 100 bacteria.

Techniques for detection of toxigenic *Pasteurella multocida* strains from pigs were evaluated by Amigot *et al.* (1998). Detection of toxin was carried out using fetal lung feline cell lines, commercial ELISA kit, and polymerase chain reaction using primers described by Lichtensteiger *et al.* (1996). Faint PCR products of expected size were observed from samples that were negative by both ELISA and cell culture. It was suggested that these bands were the result of either low numbers of positive cells not detectable by other methods, falsely amplified or contamination with positive DNA.

Calsamiglia *et al.* (1999) reported about the improved sensitivity of nested PCR assay in comparison to conventional PCR for detection of *Mycoplasma hyopneumoniae* from nasal swabs. Based on this work Choi and Chae (2001) developed a nested PCR assay for enhanced detection of toxigenic *P. multocida* directly from nasal swabs using nested forward primer TA-3 and reverse primer TA-2 which amplified a 690 bp fragment from within the 846 bp region.

2.9.3 Repetitive Extragenic Palindromic PCR (REP-PCR) Finger Printing

Repetitive extragenic palindromic sequences were the first family of highly conserved, repetitive DNA sequences to be identified, dispersed throughout prokaryotic genome (Higgins *et al.*, 1982, Lupski and Weinstock, 1992). These elements have been reported to be present throughout the eubacteriaceae kingdom, although REP elements are more commonly found in Gram-negative enteric bacteria and related species (Versalovic *et al.*, 1991).

Analysis of the distribution of repetitive extragenic sequences in prokaryotic genomes forms the basis of a novel PCR based DNA finger printing technique known as REP-PCR. It had been shown to be a highly discriminatory finger printing method that not only differentiates related strains but also has the potential to identify virulence associated determinants (Versalovic *et al.*, 1991, Go *et al.*, 1995).

Townsend *et al.* (1997b) performed REP-PCR on 38 *P. multocida* strains. They observed a high degree of homogeneity of REP-PCR fingerprints in HS causing strains of *P. multocida*, providing support for the existence of a disease associated REP profile that was distinct from isolates implicated in other pasteurellosis and that served as a novel method for the identification of strains, regardless of the serotype.

Gunawardana *et al.* (2000) characterized *Pasteurella multocida* isolates by REP-PCR and pulsed field gel electrophoresis (PFGE). They opined that REP-PCR was a competent alternative to the more labour intensive PFGE system for strain identification and epidemiological studies of avian *Pasteurella multocida*, due to ease and rapidity of REP-PCR, while maintaining a high level of differentiation.

Seventeen isolates of *P. multocida* from swine, subjected to REP-PCR, revealed nine distinct profiles. The amplified products ranged from 350 bp to 4.4

kb. Heterogeneity was observed among fragments between 300 and 900 bp (Townsend *et al.*, 2000)

Amonsin *et al.* (2002) used REP-PCR and Amplified fragment length polymorphism (AFLP) to characterize 43 field isolates and four attenuated vaccine strains of *P. multocida* recovered from multiple avian species. They concluded that REP-PCR and AFLP techniques could be used for rapid finger printing of *P. multocida* isolates and to establish their genetic relatedness.

2.9.4 Plasmid Profile Analysis

Guerry *et al.* (1973) opined that plasmid deoxyribonucleic acid (DNA) ranging from $5x10^6$ to 65 x 10^6 Daltons may be isolated from chromosomal DNA, by the preferential precipitation of the high molecular weight chromosomal DNA in the presence of sodium lauryl sulphate and high concentration of NaCl.

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

Antibiotic resistance plasmids had been associated with *P. multocida* that cause fowl cholera in turkeys and fatal pneumonia in feedlot cattle. Berman and Hirsh (1978) isolated two non-transmissible R plasmids from a turkey strain. The plasmids had molecular weights of 4.4 and 3.44 mega Daltons and coded for resistance to tetracycline, streptomycin and sulphonamides.

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 renatured to form an insoluble clot, leaving plasmid DNA in the supernatant. Large and small plasmid DNAs had been extracted by this method.

A representative strain of *P. multocida* from an outbreak of pneumonia in feedlot cattle examined by Silver *et al.* (1979) contained three distinct plasmids. Tetracycline resistance was associated with a 3.0 MDa plasmid, while streptomycin and sulphonamide resistance was associated with a 2.7 MDa plasmid. Function of the third plasmid was unknown.

Hirsh *et al.* (1981) described a turkey fowl cholera strain of *P. multocida* capable of conjugal transfer of a R plasmid. The strain was shown to possess the ability to transfer streptomycin and sulphadiazine resistance to *P. multocida* and to *E. coli* by conjugation. Genes necessary for transfer of resistance were associated with a 28.5 MDa plasmid and resistance genes were associated with a second plasmid of 7.2 MDa.

Kado and Liu (1981) observed that covalently closed circular DNA was released from the cells under conditions that denatured chromosomal DNA, *viz.*, using alkaline SDS (pH 12.6) at an elevated temperature of 65°C. Proteins and cell debris were removed by extraction with phenol chloroform. The centrifuged supernatant, devoid of chromosomal DNA and proteins, were used directly for electrophoretic analysis.

Hirsh *et al.* (1985) studied the resistance plasmids of 58 strains of *P. multocida* serotype 3 isolated from turkeys. Forty-one isolates contained plasmid DNA, of which 7 isolates were found to encode resistance to tetracycline, streptomycin and sulfonamides or to streptomycin and sulfonamides. The R plasmids were 2 to 10 MDa and non-conjugal. Evidence suggested that R plasmids were not widely dispersed among serotype 3 *P. multocida*.

Percentage of plasmid bearing *P. multocida* varied greatly, ranging from 70 per cent of 58 turkey strains analysed (Hirsh *et al.*, 1985) to zero per cent of 295 strains analysed from wild birds (Hirsh *et al.*, 1990). The reason for this difference could be related to the type of host (wild or domestic bird), incidence of carriage or even methods employed to detect plasmids.

Haghour *et al.* (1987) found plasmids in 35 of 163 strains of *P. multocida* from different animal species. The plasmids ranged from 1.3 to 28.8 MDa but antimicrobial resistance did not seem to be associated with these plasmids. Studies with bovine and porcine strains by Schwarz *et al.* (1989) revealed that 47 per cent of the strains carried plasmids.

Hirsh *et al.* (1989), and Price *et al.* (1993) identified many phenotypically cryptic plasmids in *P. multocida* isolated from avian host.

Hirsh *et al.* (1989) identified a large conjugative R plasmid that was capable of transferring multiple antibiotic resistance to *E. coli* and *P. multocida. P. multocida* has been shown to harbour plasmids from 1.3 kb (Diallo *et al.*, 1995) to 100 kb (Hirsh *et al.*, 1989) in size. However majority of plasmids identified were between 2 and 6 kb in size.

Lee *et al.* (1990) characterized ten temperature sensitive mutants of Clemson university (Cu) vaccine strain of *P. multocida.* Plasmid DNA was not detected in either Cu or the mutant strains.

Cote *et al.* (1991) studied the prevalence of R plasmids in *P. multocida* from swine. The R plasmids belonged to two groups, one of 5.6 kb and the other of 5.9 kb.

Price *et al.* (1993) characterized native plasmids of *P. multocida* of avian origin that could serve as a tool for molecular work on pathogenesis of fowl cholera. A simple plasmid screening revealed that only 24 per cent of the strains carried plasmid. Twelve out of fourteen strains carried small plasmids of similar size (3.4–3.8 kb).

Diallo *et al.* (1995) examined 45 strains of avian *P. multocida* for plasmids and for the correlation between presence of plasmids and resistance to antimicrobial agents. Twenty strains yielded no plasmid, seven strains contained

a single plasmid of 1.3 Kbp and 18 contained two plasmids of 2.4 and 7.5 Kbp. No correlation was found between plasmid content and resistance to antimicrobial agents. Three strain that lacked plasmids were highly virulent for mice where as six strains containing plasmids were not.

Pande and Singh (1997) reported the presence of plasmids in *P. multocida*. One plasmid was reported in B:2 serotype (P-52 strain) and three plasmids in poultry isolates of *P. multocida*. Plasmids from various field isolates were identified and correlated with the pathogenicity in mice.

On examination of twelve *P. multocida* isolates of Indian origin Shivsankara *et al.* (2000) found that, two did not possess any plasmid, seven showed single plasmid and three showed two plasmids each. They could not establish any correlation between plasmid carriage and virulence, as all the isolates were pathogenic for mice. One plasmid was reported in B:2 serotype (P-52 strain) isolated from buffalo and an isolate from sheep whereas an isolate recovered from goat exhibited two plasmids. Plasmid profile helped in the characterization of isolates.

Rubies *et al.* (2002) demonstrated plasmids in 81 out of the 218 isolates of *P. multocida* from swine. The plasmid containing isolates were assigned to six plasmid profiles. Each profile consisted of a combination of different sized plasmids. They showed that plasmid profile proved to be a good epidemiological tool for identifying different strains of *P. multocida* belonging to the same serotype.

2.10 DNA SEQUENCING

Since the introduction of polymerase chain reaction, various methods of sequencing PCR generated fragments directly have been described. These methods are mainly based on the enzymatic sequencing method of Sanger, employing chain terminating dideoxy nucleotides (Sanger *et al.*, 1977) or the

chemical cleavage method, the Maxam-Gilbert sequencing method (Maxam and Gilbert, 1977).

Townsend *et al.* (1996) used type–B *P. multocida* isolates in a modified subtractive hybridization technique with the specific aim of cloning unique DNA sequences specific to isolates of type B:2. The size of the product was 956 bp and was assigned a GenBank accession No. AF 016260.

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 Sigma-Aldrich, Bangalore Genei and Sisco Research Laboratories Private Limited (SRL). Sterile swabs and ready-made media were procured from Hi-media Laboratories Private Limited, Mumbai, unless otherwise mentioned.

3.1 ISOLATION OF *Pasteurella multocida*

3.1.1 Biomaterials Collected

Nasal swabs from apparently healthy and clinically ill ruminants, pharyngeal swabs and lung samples showing pneumonic lesions from slaughtered animals and blood samples and blood smears from animals suspected for haemorrhagic septicaemia (HS) formed the biomaterials for isolation trials and detection of DNA of organisms by polymerase chain reaction (PCR).

Biomaterials were collected at random, from apparently healthy and clinically ill ruminants in Ollukkara Block area, Thrissur district, from a total of 260 animals representing approximately 1 per cent of total ruminant population. This included 187 Nasal swabs, 17 pharyngeal swabs, 28 lung samples and 28 blood samples and blood smears. Nasal swabs were collected at random from apparently healthy ruminants maintained at livestock farm, sheep and goat farm, College of Veterinary and Animal Sciences, Mannuthy, from clinically ill ruminants presented at veterinary hospitals, Ollukkara block area and from animals killed at local slaughter house, Thrissur district. Pharyngeal swabs and lung samples showing pneumonic lesions were collected at random from animals killed at local slaughterhouse, Thrissur district. Blood samples and blood smears were obtained from animals clinically suspected with HS, brought to veterinary hospitals in Ollukkara block area.

A total of 27 samples of blood and blood smears received from areas in Thrissur district other than Ollukkara Block and total of 22 samples of blood and blood smears from various areas in Palakkad District, brought to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, for the diagnosis of HS, were also included in the study.

3.1.2 Method of Collection of Biomaterials

3.1.2.1 Nasal and Pharyngeal 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 ruminants 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 pharyngeal swabs from animals killed at local slaughterhouse were collected by swabbing the area with sterile swabs and brought to the laboratory preserved over ice.

3.1.2.2 Lung Samples

Representative portion of lung samples showing pneumonic lesions were collected at random from animals killed at local slaughterhouse and carried to laboratory preserved over ice.

3.1.2.3 Blood Samples

Sterile vials containing 0.5 to 1 ml defibrinated ovine or bovine blood were used for collection.

Blood samples were collected from jugular vein of animals clinically suspected with HS. One to two drops of blood were added into the sterile vials containing 0.5 to 1ml of defibrinated ovine or bovine blood.

3.2 REPORTS ON OCCURRENCE OF HAEMORRHAGIC SEPTICAEMIA OUTBREAKS

Reports on occurrence of haemorrhagic septicaemia (HS) outbreaks for the past five years were collected from data provided by Department of Animal Husbandry, Kerala state.

3.3 MEDIA USED FOR ISOLATION OF *Pasteurella multocida*

3.3.1 Materials

3.3.1.1 Blood Agar

Brain heart infusion agar (BHIA) supplemented with 5-10 per cent sterile ovine or bovine blood was used for isolation.

3.3.1.2 Clindamycin Gentamicin Potassium Tellurite Medium (CGT Medium)

Brain heart infusion agar base was prepared by autoclaving at 121°C for 15 min, at 15 lb pressure. After cooling, added sterile defibrinated equine blood at 5-10 per cent level and also supplemented with clindamycin hydrochloride 5 mg/l (Sigma), gentamicin sulphate 0.75 mg/l (Hi-media laboratories), potassium tellurite 2.5 mg/l (Hi-media laboratories) and amphotericin-B at 5 mg/l (Hi-media laboratories).

3.3.1.3 Pasteurella multocida Selective Agar (PMSA)

Brain heart infusion agar served as the base of the formulation. After autoclaving, base medium was added with gentamicin sulphate 0.75 mg/l (Hi-media laboratories), potassium tellurite 2.5mg/l (Hi-media laboratories), amphotericin-B at 5 mg/l (Hi-media laboratories) and sterile defibrinated ovine blood at 5 per cent level. The pH was adjusted to 10 with sterile 1 N NaOH (40 ml/litre).

3.3.2 Method of Isolation

3.3.2.1 Nasal and Pharyngeal Swabs

Swabs collected were streaked onto the selective media (CGT media or PMSA) and blood agar and incubated at 37°C overnight in a candle jar.

3.3.2.2 Lung Samples

Lung samples showing pneumonic lesions were streaked onto the selective media and blood agar and incubated at 37°C overnight in a candle jar.

3.3.2.3 Blood Samples

Blood samples obtained from animals suspected of haemorrhagic septicaemia and collected in defibrinated ovine/bovine blood were streaked onto blood agar plates and incubated at 37°C for 24 – 48 h in a candle jar.

Colonies suggestive of *Pasteurella multocida* (round, flat, mucoid in consistency and sticky in nature) were stained by Gram's method to study the morphological features.

3.4 IDENTIFICATION

The bacterial isolates were identified based on morphology, cultural characteristics, Gram's reaction, tests for catalase and oxidase, oxidative or fermentative utilization of glucose, growth on Mac Conkey's agar, haemolysis on blood agar, 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, production of acid or gas from the carbohydrates (glucose, galactose, inositol, lactose, maltose, mannitol, mannose, salicin, sucrose, dulcitol, sorbitol, trehalose, xylose, and arabinose) as described by Barrow and Feltham (1993).

3.4.1 Biotyping

3.4.1.1 Materials

Sugars such as arabinose, dulcitol, sorbitol, trehalose and xylose were prepared at one per cent concentration in Andrade's peptone water.

3.4.1.2 Method

Sugar solution prepared at one per cent concentration in peptone water was sterilized under steam successively for three days and checked for sterility by overnight incubation at 37°C and stored at 4°C until usage. Three milliliters of each of sugar solution was taken in five ml tubes and inoculated with the culture under study. The tubes were incubated at 37°C for minimum of seven days and examined daily for acid production as indicated by a colour change.

3.4.2 Antibiogram

3.4.2.1 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 (Hi-media Laboratories Private Limited, Mumbai, India).

1. Ampicillin (A) - 10 µg
2. Chloramphenicol (C) - 3 µg
3. Cloxacillin (Cx) - 5 µg
4. Co-trimoxazole (Co)- 25µg
5. Furazolidone (Fr)- 100µg
6. Gentamicin (G)- 30 µg
7. Metronidazole (Mt) – 5 µg
8. Nitrofurantoin (Nf)-300 µg
9. Penicillin G (P) -10 Units

10. Pefloxacin (Pf) - 5 µg
11. Streptomycin (S) -10 µg
12. Tetracycline (T)- 10 µg

3.4.2.2 Method

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

3.4.3 Pathogenicity Testing of Isolates

3.4.3.1 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.

3.4.3.2 Method

Each mouse was inoculated intraperitoneally with 0.1 ml of inoculum containing approximately 3 x 10^8 organisms per ml in sterile normal saline. A control mouse was injected with 0.1 ml of sterile saline. A total of six mice were used for each isolate. All the animals were observed for signs of infection. Heart blood smears collected from dead mice were stained with Leishman's stain. Re-isolation of *P. multocida* from heart blood, lung, liver and spleen of the dead mice was carried out.

3.5 STORAGE OF ISOLATES

3.5.1 Materials

3.5.1.1 Defibrinated Blood

Sterile defibrinated ovine / bovine blood in one-millilitre aliquots, taken in two millilitre vials were used for storage of isolates at -70°C.

3.5.2 Method

Pure cultures before preservation were grown in blood agar and incubated at 37°C for 24 h in a candle jar. A single colony from pure cultures of *P. multocida* was added to three milliliters of brain heart infusion broth (BHIB) and incubated at 37°C for four hours. A drop of this broth was added to the sterile defibrinated blood and incubated for six hours at 37°C. The vials were then labelled and stored at -70°C. The isolates were revived once a month. The vial stored at -70°C was thawed and streaked on ovine/ bovine blood agar and incubated at 37°C overnight.

3.6 POLYMERASE CHAIN REACTION FOR DETECTION OF *P. multocida*

3.6.1 Buffers and Reagents for PCR

3.6.1.1 Phosphate Buffered Saline (PBS) Stock Solution (10x)

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

The pH was adjusted to 7.4 by 1 N NaOH and sterilized by autoclaving at 121°C for 15 min at 15 lb pressure and stored at 4°C. The stock solution was diluted to 1x before use.

3.6.1.2 Primers

3.6.1.2a Primers for Pasteurella multocida Specific (PM–Specific) PCR

Species-specific primers, to detect *Pasteurella multocida* designed by Townsend *et al.* (1998a) were used. The sequences of the primers were as follows:

KMT1SP6 5'- GCT GTA AAC GAA CTC GCC AC- 3'

KMT1T7 5'-ATC CGC TAT TTA CCC AGT GG - 3'

The primers were custom synthesized by M/s Bangalore Genei (INDIA).

3.6.1.2b Primers for Type B Specific (HS-B Specific) PCR

Specific primers, to detect *Pasteurella multocida* type-B (TypeB-specific) designed by Townsend *et al.* (1998a) were used. The sequences of the primers were as follows:

KTSP61 5'- ATC CGC TAA CAC ACT CTG - 3'

KTT72 5'- AGG CTC GTT TGG ATT ATG AAG - 3'

The primers were custom synthesized by M/s Bangalore Genei (INDIA).

3.6.1.2c Primers for Multiplex PCR

Two sets of primers each (those for PM-PCR and HS-B PCR) were used in the present study and were custom synthesized as above.

3.6.1.2d Primers for Nested PCR

Two primers were used in the present study. Forward and reverse primers were designed using primer 3 software (NCBI) and were custom synthesized as above. The sequences of the primers were as follows:

Forward primer 5'- TGT GGC AAA GAA AAG CAC AG - 3'

Reverse primer 5'- AAC CGC TCT GTC GTT AAT GG - 3'

3.6.1.2e Primers for Repetitive Extragenic Palindromic Sequence PCR (REP-PCR)

A primer set designed by Gunawardana *et al.* (2000) was used for the molecular typing of *Pasteurella multocida.*

The sequence of the two primers were:

REP 1 5'- NNNN CGN CGN CAT CNG GC- 3' 18 mer

REP 2 5'- NCG NCT TAT CNG GCC TAC - 3' 18 mer

3.6.1.3 PCR reaction buffer (10x)

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

3.6.1.4 Taq DNA polymerase

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

3.6.1.5 Deoxy ribonucleotide triphosphate

Deoxy ribonucleotide triphosphate (dNTP) mix

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

3.6.1.6 Magnesium chloride

Magnesium chloride with strength of 25 mM

3.6.1.7 DNA molecular size markers

3.6.1.7a 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

3.6.1.7b pUC 19 DNA/*Msp I* digest

With fragments of 501, 404, 331, 242, 190, 147, 111, 67 and 34 basepairs

3.6.1.7c λDNA/*Hind III* digest

With fragments of 23.13, 9.41, 6.55, 4.36, 2.32 and 2.02 kilo basepairs

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

3.6.1.8 Reference Strains of Pasteurella multocida

Reference strain (P-52) obtained from Indian Veterinary Research Institute, Izatnagar, and two duck isolates of *Pasteurella multocida* (DP1& DP2) belonging to serotype A maintained in the Department of Microbiology were employed in this study.

3.6.1.9 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 PM-Specific PCR.

(i) *Leptospira icterohaemorrhagiae*
(ii) *Escherichia coli*
(iii) *Staphylococcus aureus*
(iv) *Pseudomonas aeruginosa*

3.6.2 Method

3.6.2.1 Preparation of samples for PCR analysis

3.6.2.1a Reference strain of Pasteurella multocida

The reference strain of *Pasteurella multocida* was grown overnight in blood agar plates at 37°C, in a candle jar 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 x g for 10 min, the supernatant was discarded, washed the pellet twice with sterile PBS (3.6.1.1) 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.

3.6.2.1b Other bacterial strains

To determine the specificity of primer pairs KMT1SP6 and KMT1T7 they were tested against the DNA from other bacterial strains (3.6.1.9). The template DNA from other bacterial strains was prepared as described for the reference strain of *Pasteurella multocida.*

3.6.2.1c 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 (3.6.1.1) 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 (3.6.1.1) 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.

3.6.2.1d Nasal and pharyngeal swabs and lung samples

Nasal and pharyngeal 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 (3.6.1.1) 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 pharyngeal swabs directly streaked onto the selective medium (CGT or PMSA) and blood agar were examined after overnight incubation for colonies suggestive of *Pasteurella multocida* and prepared for PCR analysis in the same way as described for reference strain of *Pasteurella multocida.*

3.6.2.2 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.

3.6.2.3 Setting up of PCR (test proper)

3.6.2.3a Pasteurella multocida species specific PCR (PM-PCR)

The PCR reaction was carried out with *Pasteurella multocida* specific primer pair, KMT1T7–KMT1SP6 as per the method described by Townsend *et al.* (1998a).

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 (10x)	25µl
Forward primer	10µl
Reverse 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:

	Primer KMT1SP6 and KMT1T7	
	First cycle	Next 29 cycles
Denaturation	95°C for 4 min	95°C for 45 sec
Annealing	55°C for 45 sec	55°C for 45 sec
Extension	72°C for 45 sec	72°C for 45 sec
		Final extension of 72°C for 6 min
Total number of cycles	30	

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

3.6.2.3b Type B specific PCR (HS-B Specific PCR)

Polymerase Chain Reaction was carried with Type B specific primer pair KTSP61-KTT72 as described by Townsend *et al.* (1998a).

Polymerase Chain Reaction was performed in a total volume of 25µl reaction and preparation of mastermix was same as described above.

The programme of amplification followed was:

	Primer KTSP61 and KTT72	
	First cycle	Next 29 cycles
Denaturation	95°C for 4 min	95°C for 1 min.
Annealing	55°C for 45 sec	55°C for 1 min.
Extension	72°C for 45 sec	72°C for 1 min.
		Final extension of 72°C for 9 min.
Total number of cycles	30	

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

3.6.2.3c Multiplex PCR

Polymerase Chain Reaction (PCR) was carried out with two sets of primers i.e., PM-specific and Type B-specific primer pairs simultaneously, as per method described by Townsend *et al.* (2000).

PCR was performed in a total volume of 25µl reaction mixture as described above.

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

Reagents	Quantity
PCR reaction buffer	25µl
Forward primer (PM-specific)	10µl
Reverse primer (PM-specific)	10µl
Forward primer (HS-B specific)	10 µl
Reverse primer (HS-B specific)	10 µl
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 were as follows:

	Primer KMT1SP6 and KMT1T7; Primer KTSP61 and KTT72	
	First cycle	Next 29 cycles
Denaturation	95°C for 4 min	95°C for 1 min
Annealing	55°C for 45 sec	55°C for 1 min
Extension	72°C for 45 sec	72°C for 1 min
		Final extension of 72°C for 9 min
Total number of cycles	30	

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

3.6.2.3d Nested PCR

Polymerase Chain Reaction (PCR) was carried out with upstream and down stream primers in a total volume of 25 µl reaction mixture described as above for PM-PCR.

Preparation of 200 µl master mix was as follows:

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

Template DNA was prepared by diluting the PM-PCR product with distilled water. One in five dilution of the product was used as template DNA. From the clinical samples, found negative on amplification with primer pairs KMT1SP6 and KMT1T7, 71 samples were selected at random for reamplification using nested PCR primers. These included 30 nasal swabs, 26 lung samples and 15 blood samples and blood smears.

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 were as follows:

	Forward and Reverse Primer designed by Primer 3 software	
	First cycle	Next 29 cycles
Denaturation	95°C for 4 min	95°C for 45 sec
Annealing	55°C for 45 sec	55°C for 45 sec
Extension	72°C for 45 sec	72°C for 45 sec
		Final extension of 72°C for 6 min
Total number of cycles	30	

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

3.6.2.3e Repetitive Extragenic Palindromic Sequence PCR (REP-PCR)

The PCR reaction was carried out with primer pairs REP1 and REP2 as per methods described by Gunawardana *et al.* (2000).

A total volume of 25 µl reaction 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
$MgCl_2$	2.5 mM
Taq polymerase	one unit

Preparation of 200 µl master mix was as follows:

Reagents	Quantity
PCR reaction buffer (10x)	25µl
Forward primer	10µl
Reverse primer	10µl
dNTP mix	20µl
$MgCl_2$	25µl
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. PCR amplification was carried out in an automated thermal cycler (Eppendorf, Master Cycler, Germany).

The programme of amplification were as follows:

	Primers REP1 and REP2	
	First cycle	Next 30 cycles
Denaturation	94°C for 5 min	94°C for 1min
Annealing	41°C for 2 min	41°C for 2 min
Extension	72°C for 2 min	72°C for 2 min
		Final extension of 72°C for 5 min
Total number of cycles	30	

3.7 DETECTION OF PCR PRODUCTS

3.7.1 Materials for Submarine Agarose Gel Electrophoresis

3.7.1.1 0.5 M EDTA (pH 8.0)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 70 ml of triple distilled water. The pH was adjusted to 8.0 with 1 N NaOH. The volume was made upto 100 ml, filtered, autoclaved and stored at room temperature.

3.7.1.2 TBE (Tris Borate EDTA) buffer (10x) pH 8.2

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

Triple distilled water to make 1 litre. Autoclaved and stored at room temperature. The stock solution was adjusted to 1x before use.

3.7.1.3 Agarose Gel (1.5 percent)

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

3.7.1.4 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.

3.7.1.5 Gel loading buffer (6x)

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

Stored at 4 °C.

3.7.2 Method

The amplified PCR products of PM- PCR, HS-B PCR, Mutiplex PCR and Nested PCR were detected by electrophoresis in 1.5 per cent agarose gel in TBE buffer (1x). Agarose was dissolved in TBE buffer (1x) 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 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 (1x) till it covered the top of the gel completely.

Five microlitres of amplified product was mixed with one microlitre of 6x gel loading buffer (3.7.1.5) and samples were loaded into respective slots carefully. The pBR 322/ *Alu I*digest was used as DNA molecular size marker. 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.

Amplified products of REP-PCR were analysed on two per cent agarose gels in TBE 1x by submarine gel electrophoresis as described above. Standard molecular weight markers, λ DNA */Hind III* digest (3.6.1.7c) and pUC 19 DNA/ *MspI* digest (3.6.1.7b) was used as DNA molecular size markers to ascertain the size of the DNA fragments.

The gel was visualized under UV transilluminator (Hoefer, USA) and results were documented in a gel documentation system (Bio-rad laboratories, USA).

3.8 PLASMID PROFILE OF *P. multocida* ISOLATES

3.8.1 Isolation of Plasmid DNA

3.8.1.1 Materials

3.8.1.1a Luria Bertani Broth

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

3.8.1.1b 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.

3.8.1.1c 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

3.8.1.1d 1N NaOH

NaOH	4 g
Triple distilled water to	100 ml

Stored at room temperature

3.8.1.1e 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 and stored at room temperature

3.8.1.1f TE buffer pH (7.8)

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

Distilled water to 100 ml, autoclaved and stored at room temperature

3.8.1.1g 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

3.8.1.1h Phenol: Chloroform: Isoamyl alcohol (25:24:1)

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

3.8.1.1i Chloroform: Isoamyl alcohol (24: 1)

Chloroform 24 ml
Isoamyl alcohol 1 ml

Mixed and stored in amber coloured bottles

3.8.1.1j Ethanol 70 percent

Ethanol 70 ml

Distilled water 30 ml

Mixed and stored in amber coloured bottles

3.8.1.1k Ribonuclease A

Ribonuclease A 10 mg

Distilled water 2 ml

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

3.8.1.2 Method

Pure culture of *P.multocida* 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 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 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 1x TBE buffer. Approximately 20 µl of plasmid DNA was mixed with 6x 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)

3.9 SEQUENCING OF HS-B PCR AMPLIFIED PRODUCT

Amplified product of HS–B PCR of approximately 590 bp was sequenced directly by dideoxy chain termination method using ABI PRISM Model 310 version 3.4.1. The primers KTSP61 and KTT72 were used as sequencing primers. Sequencing was carried out at School of Biotechnology, Madurai Kamraj University, Madurai, Tamilnadu. Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) network provided by National Centre for Biotechnology Information (NCBI).

Results

4. RESULTS

4.1 ISOLATION OF *Pasteurella multocida*

Biomaterials like nasal swabs from apparently healthy and clinically ill ruminants, pharyngeal swabs and lung samples showing pneumonic lesions from slaughtered animals and blood samples from animals suspected for haemorrhagic septicaemia (HS) formed the specimens for isolation studies.

Total number of samples collected from different sources and processed for the isolation of *Pasteurella multocida* were 309. These included 260 samples from cattle, buffalo, sheep and goat in Ollukkara Block area, Thrissur district, covering approximately one per cent of total ruminant population and 49 samples brought to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, for the diagnosis of HS, from areas in Thrissur district other than Ollukkara Block and from different areas in Palakkad district. The details of the samples collected are summarized in table 1.

A reference strain (P52) of *Pasteurella multocida* obtained from IVRI, Izatnagar was used for comparison in isolation studies. The selective media such as CGT medium and PMSA used in the present study supported the growth of pure cultures of reference strain, but failed to suppress the overgrowth of contaminant flora present in the samples taken from sites such as nasal and pharyngeal region and lung. *Pasteurella multocida* could not be isolated from any of the nasal and pharyngeal swabs and lung samples cultured on the selective media and blood agar.

Blood samples were streaked onto five per cent ovine or bovine blood agar and incubated at 37°C for 18-24 h in a candle jar for isolation of *P. multocida*. *Pasteurella multocida* could not be isolated from any of the 55 samples received from Ollukkara block and other areas in Thrissur district. Of the

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

Type of sample	Number of samples collected	Species from which collected	Place of sample collection
Nasal swabs	187	Cattle, buffalo, sheep and goat	I. Ollukkara block a. University Livestock farm b. University Sheep and Goat farm c. University Veterinary hospital II Slaughter house, Kuriachira
Pharyngeal swabs	17	Cattle and buffalo	Slaughter house, Kuriachira
Lung samples	28	Cattle	Slaughter house, Kuriachira
Blood samples and Blood smears	28	Cattle, buffalo and goat	a. University Veterinary hospital b. Veterinary hospitals Ollukkara Block area
Blood samples and Blood smears	27	Cattle and goat	Veterinary hospitals in Thrissur district other than Ollukkara block area
Blood samples and Blood smears	22	Cattle, buffalo and goat	Veterinary hospitals in Palakkad district
Total number of samples	309		

22 samples received from veterinary hospitals in Palakkad district, 5 samples yielded growth in pure form. Pure cultures revealed mucoid, convex, greyish-white and non-haemolytic colonies.

The isolates obtained were designated as

BP1 & BP2 from cattle (Palakkad District)

BuP1 & BuP2 from buffalo (Palakkad District)

GP1 from Goat (Palakkad District)

4.2 REPORTS ON OCCURRENCE OF HAEMORRHAGIC SEPTICAEMIA

To understand the district wise and month wise distribution of outbreak of HS for the past five years in Kerala state, data were collected from Department of Animal Husbandry, the details of which are presented in table 2.

Outbreaks and deaths in bovine due to HS for the past five years are noticeably reported from districts like Kollam, Thrissur, Ernakulam and Idukki. From the data it is clearly evident that incidence of the disease in the above mentioned districts are mainly seen in months like January, May, June, July, August, September and December. Districts like Palakkad, Kozhikode, Thiruvananthapuram, Kasargod, Kannur, Alapuzha and kottayam showed a less number of outbreaks and deaths in bovine due to HS for the past five years.

4.3 IDENTIFICATION OF THE ORGANISM

4.3.1 First Stage

All the five isolates and the reference strain P52, were Gram-negative, non-motile, and coccobacillary. They grew aerobically and anaerobically, did not grow on Mac Conkey's agar and were non-haemolytic on blood agar. All were catalase and oxidase positive and fermented glucose (Table 3).

Table 2. District wise and month wise distribution of outbreaks of HS from January 1999 to May 2004

District	Month and Year	Species	Outbreak	Affected	Death
Thrissur	July 1999 and May 2002	Bovine	2	26	11
Ernakulam	September 1999 and August 2001	Bovine	1	126	24
Palakkad	July 2002	Bovine	1	2	-
Idukki	December 2002	Bovine	2	27	3
Kozhikode	October 1999	Bovine	1	1	-
Kollam	May 2000, January 2001 and June 2002	Bovine	5	58	7
Thiruvananthapuram	May 2000	Bovine	1	2	-
Kasargod	July 2001	Bovine	-	-	1
Kannur	July 2002	Bovine	1	1	1
Alapuzha	December 2002 and April 2004	Bovine	2	2	1
Kottayam	February 2000 and January 2004	Bovine	1	1	-

Table 3. First stage biochemical tests of isolates

Tests	Isolates					
	P52	BP1	BP2	BuP1	BuP2	GP1
Gram's reaction	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli
Presence of capsule	+	+	+	+	+	+
Motility	-	-	-	-	-	-
Growth in air	+	+	+	+	+	+
Growth anaerobically	+	+	+	+	+	+
Growth on Mac Conkey's agar	-	-	-	-	-	-
Haemolysis on blood agar	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Oxidation/ Fermentation of Glucose (O/F)	F	F	F	F	F	F

4.3.2 Second Stage

In the second stage biochemical tests, all the isolates tested were indole positive, methyl red and Voges-Proskauer negative, urease negative, did not produce H_2S, reduced nitrate, gelatin liquefaction negative, beta-galactosidase activity negative, ornithine decarboxylase positive and citrate utilization negative.

With regard to the fermentation of the sugars all isolates fermented galactose, glucose, maltose and sucrose. Inositol was fermented by isolates BP1, BP2, GP1 and P52. Only BuP1 and BuP2 fermented lactose. Mannitol was utilized by all isolates except BuP1 and BuP2. Mannose was not fermented by BuP2 and salicin was not fermented by GP1 (Table 4). Results from the first and second stage biochemical tests revealed that the isolates were *Pasteurella multocida*.

4.4 BIOTYPING

Fermentation of sugars like arabinose, dulcitol, sorbitol, trehalose and xylose by the isolates were studied to biotype them. Based on the criteria followed by Mutters *et al.* (1985) one biotype was observed among the five field isolates. All the isolates and reference strain P52 did not ferment dulcitol, arabinose and trehalose but did ferment sorbitol and xylose and hence were biotyped as *Pasteurella multocida* subsp. *multocida* (Table 5).

4.5 ANTIBIOGRAM

Antibiogram of *Pasteurella multocida* isolates indicating susceptibility and resistance to various antibiotics or antibacterial agents is presented in table 6.

All the isolates were uniformly sensitive to chloramphenicol, cloxacillin, furazolidone, gentamicin, nitrofurantoin, penicillin, pefloxacin, streptomycin, tetracycline and co-trimoxazole. The isolate BP1 was resistant to ampicillin but all the other isolates were sensitive to ampicillin. All the isolates were resistant to

Table 4. Second stage biochemical tests of isolates

Tests	Isolates					
	P52	BP1	BP2	BuP1	BuP2	GP1
Indole Production	+	+	+	+	+	+
Methyl Red Test	–	–	–	–	–	–
Voges-Proskauer Test	–	–	–	–	–	–
Urease	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+
Citrate Utilization	–	–	–	–	–	–
Gelatin Liquefaction	–	–	–	–	–	–
Beta-Galactosidase activity	–	–	–	–	–	–
Lysine decarboxylase	–	–	–	–	–	–
Ornithine decarboxylase	+	+	+	+	+	+
Sugar fermentation						
Glucose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Inositol	+	+	+	-	-	+
Lactose	-	-	-	+	+	-
Maltose	+	+	+	+	+	+
Mannitol	+	+	+	-	-	+
Mannose	+	+	+	+	-	+
Salicin	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+

Table 5. Biotyping of isolates

Sugars	Isolates					
	P52	BP1	BP2	BuP1	Bup2	GP1
Arabinose	–	–	–	–	–	–
Dulcitol	–	–	–	–	–	–
Sorbitol	+	+	+	+	+	+
Trehalose	–	–	–	–	–	–
Xylose	+	+	+	+	+	+

Table 6. Antibiogram of isolates

Antibiotics/ Antibacterials	Isolates					
	P52	BP1	BP2	BuP1	BuP2	GP1
Ampicillin	S	R	S	S	S	S
Chloramphenicol	S	S	S	S	S	S
Cloxacillin	S	S	S	S	S	S
Furazolidone	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S
Metronidazole	R	R	R	R	R	R
Nitrofurantoin	S	S	S	S	S	S
Penicillin	S	S	S	S	S	S
Pefloxacin	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S
Co-trimoxazole	S	S	S	S	S	S

S -Sensitive

R -Resistant

metronidazole. All the isolates except BP1 showed similar antibiotic sensitivity pattern.

4.6 PATHOGENICITY TESTING IN MICE

All the isolates of *Pasteurella multocida* and P52 were able to kill the weaned mice. A concentration of 0.3 x 10^8 organisms per 0.1 ml was able to kill mice within 24 h, when injected by intraperitoneal route.

The gross lesions observed in the internal organs of dead mice were petechiae in the pericardium and congestion of lung, liver and spleen. Heart blood smears and impression smears, from spleen and liver collected from dead mice, stained with Leishman's stain revealed the presence of bipolar shaped organisms (Fig. 1). Re-isolation of *P. multocida* was done from the heart blood, lung, liver and spleen on ovine or bovine blood agar.

4.7. STORAGE OF ISOLATES

The *P. multocida* isolates, stored at –70^{0}C in defibrinated ovine or bovine blood were revived once in a month. All the isolates stored in this manner could be revived in blood agar and were found to be pure. Isolates were found to be viable for the period of observation of six months.

4.8 POLYMERASE CHAIN REACTION FOR DETECTION OF *P. multocida*

4.8.1 *Pasteurella multocida* species specific PCR (PM-PCR)

All the five isolates of *P. multocida* obtained from cattle, buffalo, sheep and goat and the standard reference strain P52, when subjected to specific amplification by PM-PCR, were found to be PM-PCR positive. Agarose gel (1.5 percent) electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (pBR 322 DNA/*Alu* Idigest) in 1x TBE buffer. Analysis of the electrophoresed gel under UV transilluminator revealed the presence of a 460 base pair (bp) band in all the five isolates as well

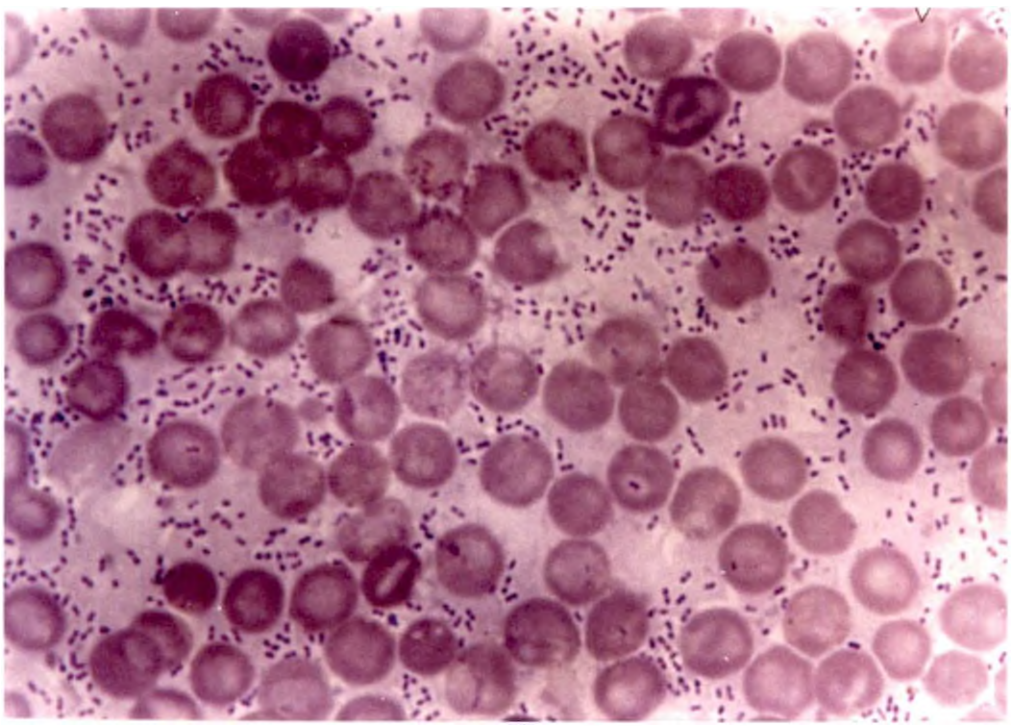

Fig 1. Mouse heart blood smear showing bipolar stained organism (Leishman's stain 1000 X)

as in the reference strain P52 (Fig. 2). In the negative control, amplification product was not detected. Similar types of results were obtained with all type of preparations such as bacterial culture lysate, DNA extracts prepared from blood samples and blood smears.

4.8.1.1 Specificity of the Primers

No amplification product was detected when primers KMT1SP6 and KMT1T7 were used to amplify the DNA prepared from *Leptospira icterohaemorrhagiae, Escherichia coli, Staphylococcus aureus and Psuedomonas aeruginosa.*

Hence the primer pairs KMT1SP6 and KMT1T7 were selected for the amplification of *P. multocida* DNA from clinical samples.

4.9.1.2 Amplification of Pasteurella multocida DNA from Clinical Samples

4.9.1.2a Blood Samples and Blood smears

The blood samples and blood smears from clinical cases were processed and amplified with primers KMT1SP6 and KMT1T7. Presence of *Pasteurella multocida* DNA in clinical samples was observed by the amplification of 460 bp fragment, although the intensity of band obtained was less compared to those derived from template DNA prepared from bacterial cultures. In the negative control kept along with samples no amplification product was detected (Fig. 3). Total number of samples tested and results are presented in table 7.

4.9.1.2b Nasal and Pharyngeal Swabs and Lung samples

Nasal swabs collected from apparently healthy and clinically ill ruminants, pharyngeal swabs and lung samples collected from animals killed at local slaughterhouse were processed and amplified with primers KMT1SP6 and KMT1T7. Presence of *Pasteurella multocida* DNA in clinical sample was observed by the amplification of 460 bp fragment, although the intensity of band

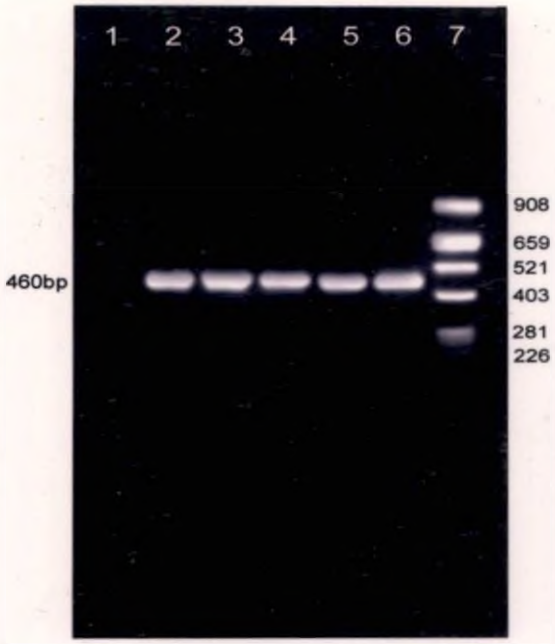

Fig 2. Detection of *Pasteurella multocida* by PM-PCR

Lane 1 Negative control
Lane 2 BP1
Lane 3 BP2
Lane 4 BuP1
Lane 5 GP1
Lane 6 P52
Lane 7 pBR 322 DNA *AluI*/ Digest

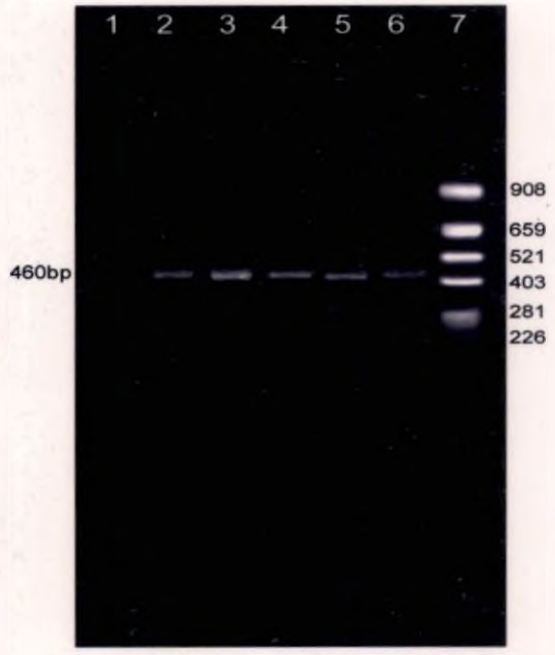

Fig 3. Detection of *Pasteurella multocida* by PM-PCR of clinical samples

Lane 1 Negative control
Lane 2 Nasal swab
Lane 3 Blood smear
Lane 4 to 6 Blood samples
Lane 7 pBR 322 DNA *AluI*/ Digest

Table 7. Results of clinical samples tested by Polymerase Chain Reaction

Area	Sample	Polymerase chain reaction		
		Number tested	Number positive	Per cent positive
Ollukkara Block	a. Blood samples	19	1	5.26
	b. Blood smears	9	1	11.1
Ollukkara block and Slaughter house, Kuriachira	a. Nasal swabs	187	1	0.53
	b. Pharyngeal swabs	17	Nil	--
	c. Lung samples	28	Nil	--
Areas in Thrissur district other than Ollukkara block	a. Blood samples	15	2	13.33
	b. Blood smears	12	2	16.67
Palakkad district	a. Blood samples	15	5	33.33
	b. Blood smears	7	2	28.57

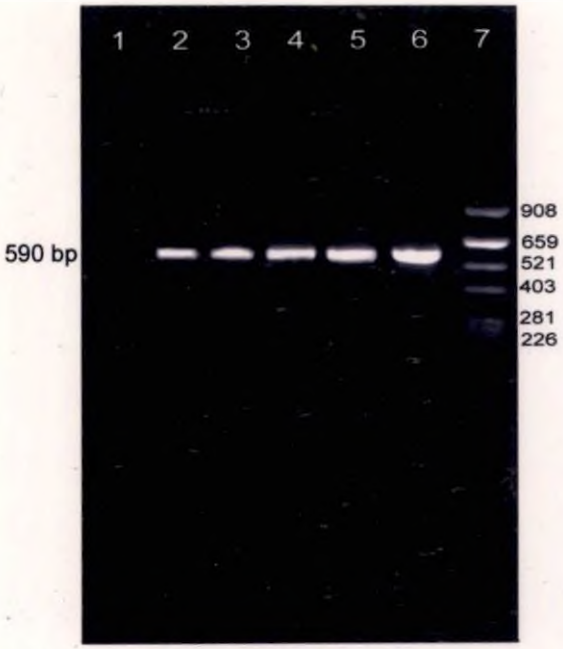

Fig 4. Detection of *Pasteurella multocida* by HS-B PCR

Lane 1 Negative control
Lane 2 BP1
Lane 3 BP2
Lane 4 BuP1
Lane 5 BuP2
Lane 6 P52
Lane 7 pBR 322 DNA *AluI*/ Digest

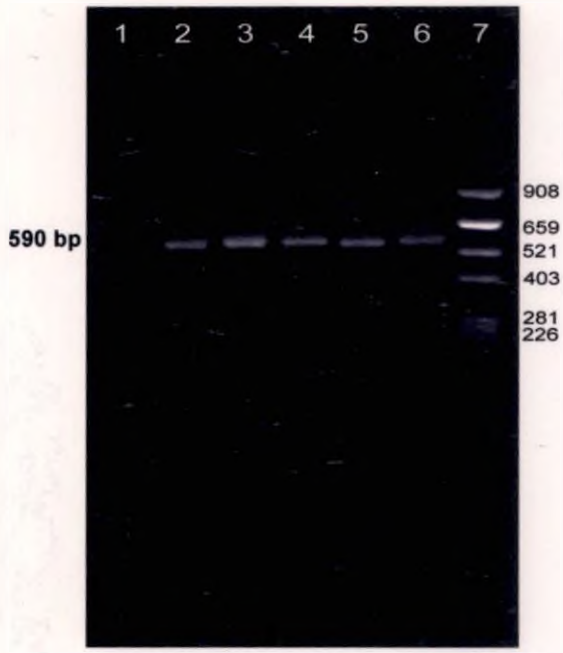

Fig 5. Detection of *Pasteurella multocida* by HS-B PCR of clinical samples

Lane 1 Negative control
Lane 2 to 6 Blood samples
Lane 7 pBR 322 DNA *AluI*/ Digest

obtained was less compared to those derived from template DNA prepared from bacterial cultures. In the negative control kept along with samples no amplification product was detected (Fig. 3). Total number of samples tested and results are presented in table 7.

Only one sample was found to be positive by PM-PCR among the 187 nasal swabs tested. None of the pharyngeal swabs and lung samples from bovines tested was found to be positive by PM-PCR.

4.9.2 Type–B Specific PCR (HS–B Specific PCR)

Blood samples, blood smears and nasal swab that were found to be positive by *Pasteurella multocida* species specific PCR (PM-PCR) were subjected to amplification by the Type-B specific primer pairs (KTSP61and KTT72). Presence of *Pasteurella multocida* type-B specific DNA in the clinical samples was observed by the amplification of a product of approximately 590 bp size.

Reference strain P52 and all the positive isolates including BP1, BP2, BuP1, BuP2 and GP1 were amplified with primer pairs KTSP61 and KTT72, giving an amplified product of approximately 590 bp, indicating that all were HS causing Type–B isolates of *Pasteurella multocida* (Fig. 4).

All the 13 blood samples and blood smears found positive by PM-PCR were specifically amplified by Type–B specific (HS-B specific) primer pairs. (Fig. 5).

4.9.3 Multiplex PCR

Clinical samples that were found to be positive by PM-PCR and Type-B specific (HS-B) PCR were subjected to multiplex PCR, using two sets of primer pairs KMT1SP6 and KMT1T7 and KTSP61 and KTT72 respectively.

Multiplex PCR assay of reference strain P52 and the positive isolates belonging to serotype B showed two bands corresponding to 460 bp and 590 bp, while the isolate of *Pasteurella multocida* from ducks belonging to serotype A gave a single band only corresponding to 460 bp (Fig. 6).

Clinical samples like blood samples and blood smears that were found to be positive by PM-PCR and HS-B PCR showed two bands each, corresponding to 460 bp and 590 bp respectively (Fig. 7).

4.9.4 Nested PCR

When the PCR product (460 bp) amplified by using primer pairs KMT1SP6 and KMT1T7 was used for reamplification with nested PCR primer pair designed using Primer 3 software, a product of 214 bp size was observed. The 14 samples found positive by PM-PCR when subjected to nested PCR, gave an amplified product of size 214 bp (Fig. 8).

Since most of the clinical samples like nasal swabs, pharyngeal swabs and lung samples tested by PM-PCR gave a negative result; an attempt was made to detect *P. multocida* DNA in the negative clinical samples by nested PCR, which is considered to be a more sensitive method. Presence of *Pasteurella multocida* DNA could be detected in the samples as indicated by the amplification of a 214 bp fragment. The negative control kept along with the samples did not give any amplification. (Fig. 9). The details of samples tested and the results are presented in table 8.

4.9. 5 Repetitive Extragenic Palindromic Sequence PCR (REP-PCR)

Positive isolates of *Pasteurella multocida* and reference strain P52, which were found to be positive by PM-PCR, were subjected to REP-PCR, using the

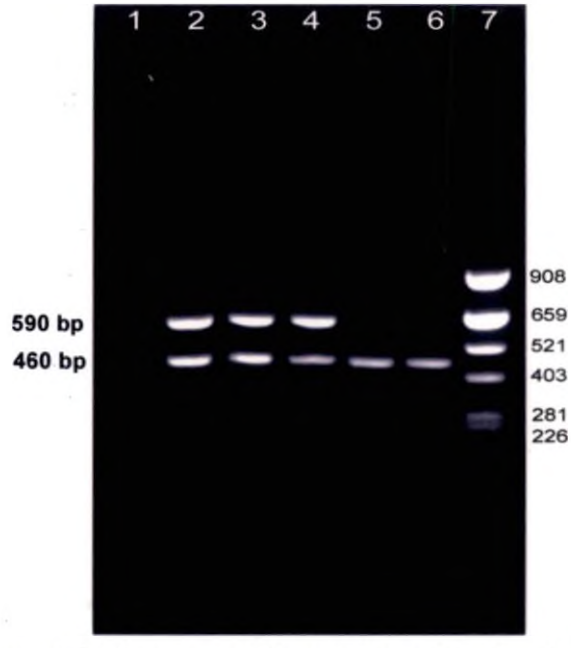

Fig 6. Detection of *Pasteurella multocida* by Multiplex PCR

Lane 1 Negative control
Lane 2 BP1
Lane 3 BP2
Lane 4 P52
Lane 5 DP1(Duck isolate)
Lane 6 DP2(Duck isolate)
Lane 7 pBR 322 DNA *AluI*/ Digest

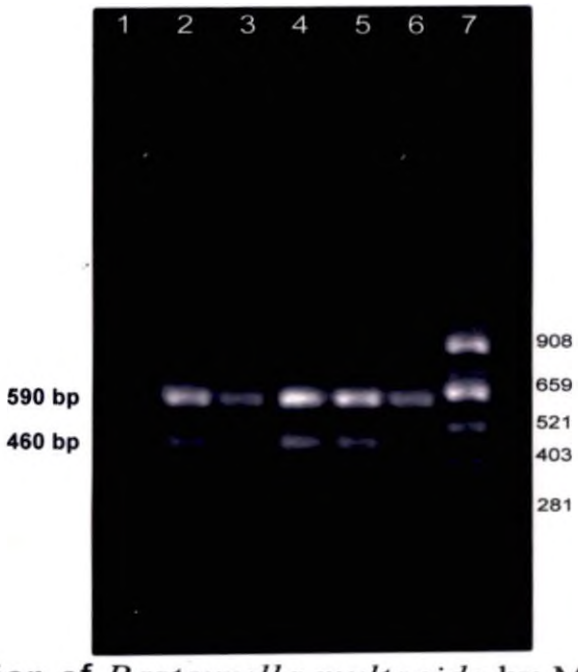

Fig 7. Detection of *Pasteurella multocida* by Multiplex PCR of clinical samples

Lane 1 Negative control
Lane 2 to 6 Blood samples
Lane 7 pBR 322 DNA *AluI*/ Digest

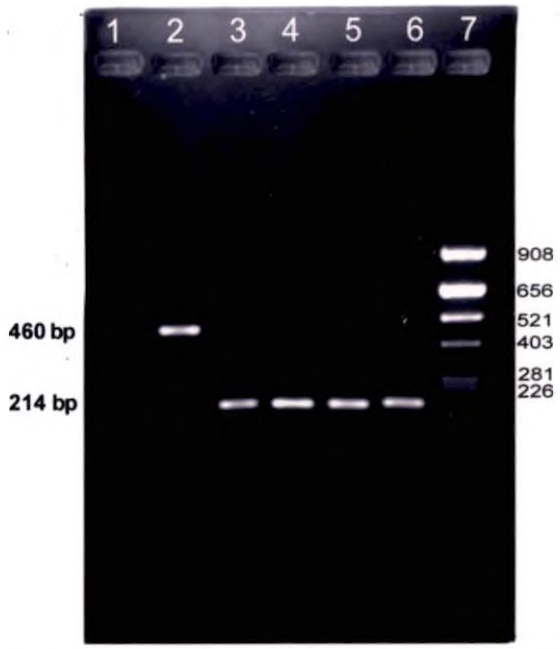

Fig 8. Detection of *Pasteurella multocida* by Nested PCR

Lane 1 Negative control
Lane 2 P 52 PM PCR amplified product
Lane 3 BP1
Lane 4 BP2
Lane 5 BuP1
Lane 6 GP1
Lane 7 pBR 322 DNA *AluI*/ Digest

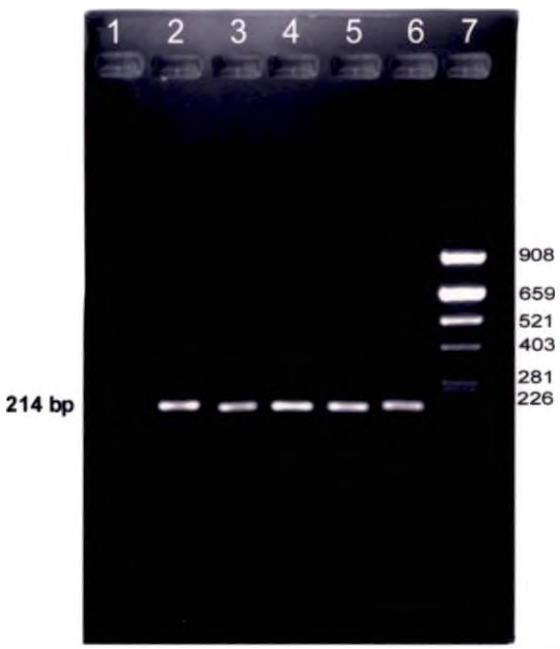

Fig 9. Detection of *Pasteurella multocida* by Nested PCR of clinical samples

Lane 1 Negative control
Lane 2 Nasal swab
Lane 3 Pharyngeal swab
Lane 4 Lung sample
Lane 5 to 6 Blood sample
Lane 7 pBR 322 DNA *AluI*/ Digest

Table 8. Results of Nested PCR using PM-PCR product

Samples		Number of samples tested	Number Positive	Number Negative
Positive samples	1. Total positive samples	14	14	Nil
	a. Positive isolates	5	5	Nil
	b. Blood samples and blood smears	8	8	Nil
	c. Nasal swab	1	1	Nil
Negative samples	1.Total nasal swabs	30	20	10
	a. Bovine	19	14	5
	b. Sheep	10	5	5
	c. Buffalo	1	1	Nil
	2. Lung samples	26	24	2
	3. Total blood samples and blood smears	15	9	6
	a. Bovine	13	7	6
	b. Goat	2	2	Nil

primer pairs REP-1 and REP-2. Analysis of banding patterns of REP fragments indicated a total of 7 bands ranging in size from 4 kbp to 242 bp. The profiles of REP-PCR of all the isolates and reference strain appeared to be identical (Fig. 10).

4.10 PLASMID PROFILE OF *Pasteurella multocida* ISOLATES

The plasmid profiles of the positive isolates of *Pasteurella multocida* have been analysed. Among the five isolates only two isolates (BP2 and BuP1) harboured a single plasmid of size 5.22 kbp (Fig. 11).

4.11 SEQUENCING OF HS-B PCR AMPLIFIED PRODUCT

The HS-B PCR product with an approximate molecular size of 590 bp was sequenced by Sanger's dideoxy chain termination method.

The amplified product was checked by electrophoresis for the presence of any non-specific bands or oligonucleotide primers. Dideoxy chain termination method of sequencing produced the sequence of 520 bp and is shown in the Fig. 12.

Sequence similarity searches were performed with Basic Local Alignment Search Tool (BLAST) network provided by National Centre for Biotechnology Information (NCBI). The sequence had 99 percent identity with *Pasteurella multocida* unknown protein 1 and protein 2 gene (Accession No AF016260). The result of alignment is shown in Fig. 13.

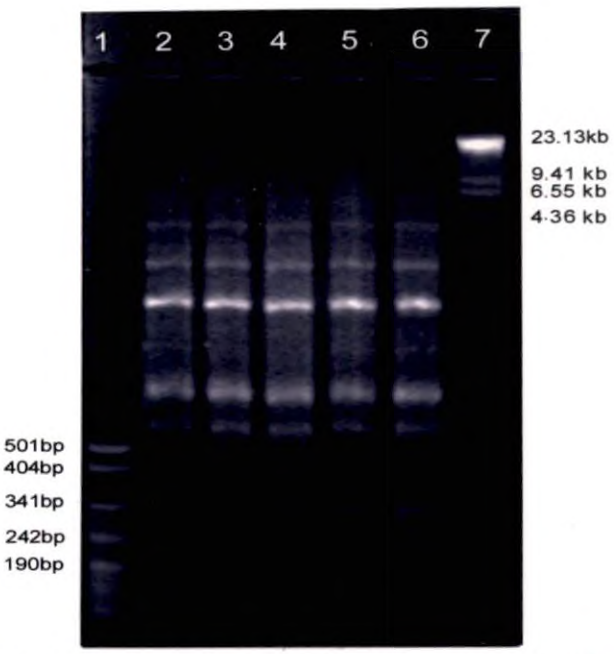

Fig 10. REP-PCR profiles of *Pasteurella multocida*

Lane 1 pUC 19 DNA *Msp I*/ Digest
Lane 2 BP1
Lane 3 BP2
Lane 4 BuP1
Lane 5 BuP2
Lane 6 P 52
Lane 7 Lambda DNA *Hind III*/ Digest

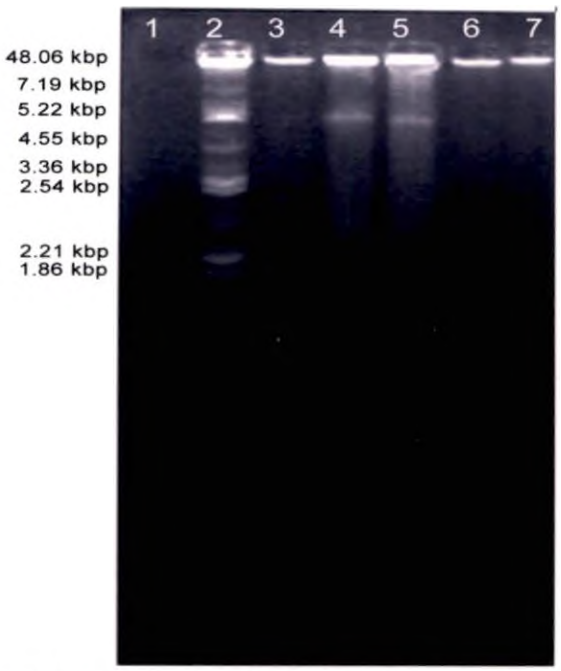

Fig 11. Plasmid profiles of *Pasteurella multocida*

Lane 2 *E coli* V 517
Lane 3 BP1
Lane 4 BP2
Lane 5 BuP1
Lane 6 BuP2
Lane 7 GP1

Fig 12. **Sequence** *of HS-B PCR amplified product* **of BuP1**

NGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGCTCGCCCATACCG
CTTTGAGTATCGCTCCACAGATTTTGCACTGTCTGCAACTCAAACATCCC
CGAAGGGCTTATTTGTTGTTTGTGTATGCCTGCTGTTAAGATTTCGACAAT
TTGATAAATCCCCACATCATCTTTTCGCTGACCGTTCAACACATCAGCGA
CCACAAATATCCCCCAGCGAGCCGACACTAAACGAGCATTTGCATTTGGC
ATTTGTCCAAGCCACGCCACATAAACTGCAGGTGGATTACGCACAATGC
GACGGATAGAGCTGTCATCCCATTGCCCTGGGTGCGTTTCGACTTCTCGT
AGATAATCCCACATAACCCTTTAATTTTGGCAATTAAAGCCTCACTGGTT
TTGGCAATAATACTCAAATGAACCCCCGTGGAATGTTCACGATTCCATAC
AGAGCCTGCACTTTCCATTACCGCTGAAATTATCCGTTTCCACCGCTTCAT
CGTTTTCTGATAGCCCAAGA- 520 bp

Fig 13. Results of sequence alignment

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.9 [May-01-2004]

Query= (487 letters)

Pasteurella multocida unknown protein 1 gene, partial cds

Pasteurella multocida unknown protein 2 gene, complete cds
Length = 956

Score = 924 bits (466), Expect = 0.0
Identities = 480/482 (99%), Gaps = 2/482 (0%)
Strand = Plus / Minus

```
Query: 6   cgcccataccgctttgagtatcgctccacagattttgcactgtctgcaactcaaacatcc 65
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 720 cgcccataccgctttgagtatcgctccacagattttgcactgtctgcaactcaaacatcc 661

Query: 66  ccgaagggcttatttgttgtttgtgtatgcctgctgttaagatttcgacaatttgataaa 125
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 660 ccgaagggcttatttgttgtttgtgtatgcctgctgttaagatttcgacaatttgataaa 601

Query: 126 tccccacatcatcttttcgctgaccgttcaacacatcagcgaccacaaatatcccccagc 185
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 600 tccccacatcatcttttcgctgaccgttcaacacatcagcgaccacaaatatcccccagc 541

Query: 186 gagccgacactaaacgagcatttgcatttggcatttgtccaagccacgccacataaactg 245
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 540 gagccgacactaaacgagcatttgcatttggcatttgtccaagccacgccacataaactg 481

Query: 246 caggtggattacgcacaatgcgacggatagagctgtcatcccattgccctgggtgcgttt 305
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 480 caggtggattacgcacaatgcgacggatagagctgtcatcccattgccctgggtgcgttt 421

Query: 306 cgacttctcgtagataatcccacataaccctttaattttggcaattaaagcctcactggt 365
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 420 cgacttctcgtagataatcccacataaccctttaattttggcaattaaagcctcactggt 361

Query: 366 tttggcaataatactcaaatgaaccccgtggaatgttcacgattccatacagagcctgc 425
           |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 360 tttggcaataatactcaaatgaaccccgt-gaatgttcacgattccatacagagcctgc 302

Query: 426 actttccattaccgctgaaattatccgtttccaccgcttcatcgttttctgatagcccaa 485
           |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 301 actttccattaccgctg-aattatccgtttccaccgcttcatcgttttctgatagcccaa 243
```

```
Query: 486 ga 487
           ||
Sbjct: 242 ga 241
```

Pasteurella multocida DNA fragment specific for haemorrhagic septicaemia
Length = 620

Score = 811 bits (409), Expect = 0.0
Identities = 455/467 (97%), Gaps = 3/467 (0%)
Strand = Plus / Minus

```
Query: 9   ccataccgctttgagtatcgctccacagattttgcactgtctgcaactcaaacatccccg 68
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 543 ccataccgctttgagtatcgctccacagattttgcactgtctgcaactcaaacatccccg 484

Query: 69  aagggcttatttgttgtttgtgtatgcctgctgttaagatttcgacaatttgataaatcc 128
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 483 aagggcttatttgttgtttgtgtatgcctgctgttaagatttcgacaatttgataaatcc 424

Query: 129 ccacatcatcttttcgctgaccgttcaacacatcagcgaccacaaatatcccccagcgag 188
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 423 ccacatcatcttttcgctgaccgttcaacacatcagcgaccacaaatatcccccagcgag 364

Query: 189 ccgacactaaacgagcatttgcatttggcatttgtccaagccacgccacataaactgcag 248
           |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct: 363 ccgacactaaacgagcatttgcatttggcatttgtccaagccacggcacataaactgcag 304

Query: 249 gtggattacgcacaatgcgacggatagagctgtcatcccattgccctgggtgcgtttcga 308
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 303 gtggattacgcacaatgcgacggatagagctgtcatcccattgccctgggtgcgtttcga 244

Query: 309 cttctcgtagataatcccacataacccttaattttggcaattaaagcctcactggtttt 368
           |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 243 cttctcgtagataaccccacataacccttaattttggcaattaaagcctcactggtttt 184

Query: 369 ggcaataatactcaaat-gaaccccccgtggaatgttcacgattccatacagagcctgcac 427
           |||||||||||||||| || ||||||| ||||| |||||.||||||||||||||||||
Sbjct: 183 ggcaataatactcaaatggavccccccgt-gaatg-tcacggttccatacagagcctgcac 126

Query: 428 tttccattaccgctgaaattatccgtttccaccgcttcatcgttttc 474
           |||||||||||||| |||||||||| ||||||| ||||||| |||||
Sbjct: 125 tttccattaccgcttgaattatccgtgtccaccggttcatcggtttc 79
```

Lambda	K	H
1.37	0.711	1.31

Gapped

Lambda	K	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3

Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 8,106,124
Number of Sequences: 1365814
Number of extensions: 8106124
Number of successful extensions: 643
Number of sequences better than 1000.0: 140
Number of HSP's better than 1000.0 without gapping: 140
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 345
Number of HSP's gapped (non-prelim): 298
length of query: 976
length of database: 776,449,540
effective HSP length: 20
effective length of query: 467
effective length of database: 772,882,080
effective search space: 360935931360
effective search space used: 360935931360
T: 0
A: 0
X1: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: 12 (24.3 bits)
S2: 15 (30.2 bits)

Discussion

5. DISCUSSION

Haemorrhagic septicaemia (HS) is an acute septicaemic disease primarily affecting cattle and buffalo, caused by two specific serotypes of *Pasteurella multocida viz.*, B: 2 and E: 2. In India, HS is caused by serotype B: 2 (De Alwis, 1992). *Pasteurella multocida* affects and causes great economic losses in a wide spectrum of hosts, including domestic ruminants like sheep and goat besides cattle and buffalo. In this country, confirmatory diagnosis of the disease depends mostly on identification and characterization of *Pasteurella multocida* by conventional culture methods. Since conventional methods are time consuming and less sensitive, they have been replaced by modern molecular biology techniques, which are based on the genetic information of the organism. Nucleic acid based recognition methods allow rapid detection and presumptive identification of organisms directly from clinical specimens and thus help to overcome limitations faced in the identification and characterisation of bacteria.

5.1 ISOLATION OF *Pasteurella multocida*

Isolation of *P. multocida* was attempted from nasal swabs from apparently healthy and clinically ill ruminants, pharyngeal swabs and lung samples showing pneumonic lesions from slaughtered animals and blood samples collected from animals suspected of HS.

Primary isolation of organisms from nasal swabs, pharyngeal swabs and lung samples were attempted by using selective media like CGT or PMSA and blood agar. All the media were equally used for primary isolation. Knight *et al.* (1983) used CGT medium containing clindamycin, gentamicin, potassium tellurite and amphotericin-B, in five per cent equine blood agar, which allowed unimpaired growth of almost all strains of *P. multocida* and *P. pneumotropica,*

while inhibiting other bacteria that might be encountered in upper respiratory tract. Eventhough the medium was practically useful in the culture of specimens from upper respiratory tract of animals, it was not much selective. Moore *et al.* (1994) used selective enrichment procedure using media like PMSA and *Pasteurella multocida* selective broth (PMSB) to isolate *P. multocida* from environmental samples contaminated with other bacteria.

The presence of healthy animals carrying the agent of HS in their nasopharynx is well documented (Carter and De Alwis, 1989). Hence attempts were made to isolate *P. multocida* from nasopharynx. The percentage of carriers varied greatly from 1 per cent to over 40 per cent (Singh, 1948; Mustafa *et al.*, 1978 and Wijewantha and Karunaratne, 1968). Mustafa *et al.* (1978) detected 44 per cent animal carriers in a herd with recent history of HS but less than one per cent in herds with no history of disease. De Alwis (1982) found a progressive diminution in the percentage of carriers detected, from 22 per cent of herds one week after outbreak to 1.9 per cent, six weeks later. These findings led to the conclusion that, carrier state as evidenced by the presence of organism in the nasopharynx, was only a transient one. Carter and De Alwis (1989) also reported about the presence of latent carriers, which harboured organisms in tonsils, in which case recovery of organisms from nasopharynx might not be possible always.

Kanwar *et al.* (1998) reported about involvement of *P. multocida* as a causative organism in pneumonic lesions encountered in lung of animals killed at local slaughterhouse. In this study also attempts were made to isolate *P. multocida* from lung samples showing pneumonic lesions.

Selective media used in the present study were supporting the growth of pure cultures of reference strain of *P. multocida,* but were not effective in suppressing overgrowth of contaminant bacteria. *Pasteurella multocida* could not be isolated from any of the samples like nasal swabs, pharyngeal swabs and lung,

cultured in selective media and blood agar. Townsend *et al.* (1998a) have also reported similar finding that isolation of *P. multocida* could prove difficult during field surveys of carrier status, when samples were taken from a contaminated site on the animal such as nose or throat, despite extensive subculturing. Amigot *et al.* (1998) have also reported that attempts to isolate *P. multocida* from nasal swabs could be frustrating. Attempts for direct culture of *P. multocida* was satisfactory only if the material was fresh and free from contaminants or postmortem invaders that would otherwise overgrow any *Pasteurella* present (OIE, 2000).

Primary isolation of *P. multocida* from blood samples taken from animals suspected for HS was done in five per cent ovine and bovine blood agar. Both the types of blood agar were equally useful for primary isolation of *P. multocida.* Rimler and Rhoades (1989) have suggested the use of bovine, equine or ovine blood in the media for isolation of *P. multocida.*

The blood agar plates, streaked with suspected material were incubated at 37^0C with mild CO_2 tension. These conditions were found to be ideal for the growth of *P. multocida.* These findings are in agreement with the observations of Carter (1981) and Rajalakshmi (2001).

5.2 REPORTS ON OCCURRENCE OF HAEMORRHAGIC SEPTICAEMIA OUTBREAKS

Haemorrhagic septicaemia usually commenses in wet, humid weather and an increased incidence is recorded during wet seasons. Systematic epidemiological studies revealed that the outbreaks of HS do occur at all times of the year, but those occurring during wet seasons tend to spread (De Alwis, 1992).

From the data provided by Department of Animal husbandry it is evident that incidence of the disease are mainly seen in months like January, May, June, July, August, September and December in the districts where significant reports

of outbreaks and death due to HS occurred. This is in agreement with the epidemiological studies on occurrence of HS in India conducted by Dutta *et al.* (1990). They found that incidence of HS increased from March to August and highest seasonal index was recorded in August, followed by July and September.

It is evident from the data that a less number of outbreaks and deaths in bovine due to HS were reported for the past five years from Palakkad district. This is not a true reflection of laboratory findings of the present study because all the isolates of *P. multocida* obtained in this study are from suspected cases of HS from Palakkad district. Hence no correlation exists between laboratory findings and details given in the report. This may be due to under reporting of the cases of HS.

5.3 IDENTIFICATION

All the isolates and reference strain produced colonies that were mucoid, convex, greyish-white and non-haemolytic. The highly mucoid nature of colonies is suggestive of the high amount of capsular material. Similar observations have been made by Carter (1967).

All the five isolates from cattle, buffalo, goat and the reference strain P52 were Gram-negative, coccobacillary, non-motile and catalase and oxidase positive. None of the isolates grew on Mac Conkey's agar. These results are in agreement with those of Shigidi and Mustafa (1979) and Carter (1984a).

The second stage biochemical reactions used for characterization of *P. multocida* (Barrow and Feltham, 1993) were almost identical for the five isolates as well as P52. Variations were observed only in the fermentation of sugars. Similar findings have been reported by Heddleston (1976); Bisgaard *et al.* (1991); Madsen *et al.* (1985) and Mohan *et al.* (1994).

All the isolates uniformly fermented glucose, galactose, maltose and sucrose. Only two isolates utilized lactose. Rimler and Rhoades (1989) have identified the sugars such as glucose, mannose, galactose and fructose as those most commonly fermented by *P. multocida*.

The isolates differed in their ability to ferment, inositol, lactose, mannitol, mannose and salicin. Several workers have reported such variations in sugar fermentation (Rimler and Rhoades, 1989; Mohan *et al.*, 1994 and Dziva *et al.*, 2001). However Blackall *et al.* (2000) have reported fermentation of mannitol by all the isolates of *P. multocida* tested.

Positive reactions to indole and ornithine decarboxylase have been described as the most useful biochemical indicators in the identification of *P. multocida* (OIE, 2000). *Pasteurella haemolytica* can be readily distinguished from *P. multocida* based on the inability of the former to produce indole, and their ability to ferment maltose, dextrin and after several days inositol (Adlam, 1989).

On the basis of morphological, cultural and biochemical characteristics all the isolates were identified as *P. multocida*.

5.4 BIOTYPING

Separation of isolates of *P. multocida* into subgroups or biotypes is based upon variation in biochemical characteristics. This sub grouping has been based mostly upon reaction patterns observed with acid production from certain pentoses (such as xylose and arabinose), disaccharides (such as maltose and trehalose) and polyhydric alcohols (such as sorbitol, mannitol and dulcitol) (Rimler and Rhoades, 1989).

Mutters *et al.* (1985) reported that action on dulcitol and sorbitol was of taxonomic significance in dividing the taxon *P. multocida* into the following three subspecies. The sorbitol and dulcitol positive variety becomes subsp. *gallicida*; negative for both, subsp. *septica* and positive for sorbitol but negative for dulcitol becomes subsp. *multocida.* Based on the above criteria the five isolates of *P. multocida* and P52 could be grouped into one subsp. *viz.*, *P. multocida* subsp. *multocida.*

In several studies, investigators who used sorbitol and dulcitol in biotyping *P. multocida,* found that on an average 82 per cent of the strains were sorbitol positive and dulcitol negative (Schneider, 1948; Smith, 1959; Heddleston, 1976 and Blackall *et al.*, 1997). The results of the present study are in agreement with their observation.

5.5 ANTIBIOGRAM

Antibacterial therapy was effective against HS when treatment was initiated immediately after temperature elevation. Eventhough a variety of chemotherapeutic agents have been used in the treatment of HS, administration of sulphadimidine intravenously had been a common practice (De Alwis, 1992).

All the isolates of *P. multocida* were subjected to antibiotic sensitivity testing. They were uniformly sensitive to chloramphenicol, cloxacillin, furazolidone, gentamicin, nitrofurantoin, penicillin, pefloxacin, streptomycin, tetracycline and co-trimoxazole. All isolates except BP1 were sensitive to ampicillin. The results obtained in this study were in agreement with those of De Alwis (1984) who observed that a collection of HS strains from India, Indonesia, Malaysia, Thailand, Burma and Srilanka were sensitive to penicillin, ampicillin, streptomycin, tetracycline, chloramphenicol, sulphonamide-trimethoprim combination, erythromycin and neomycin.

With regard to sensitivity to chloramphenicol and ampicillin the results of the present study were in agreement with those of Diker *et al.* (1994) who also found that *P. multocida* isolated from pneumonic ovine lungs to be sensitive to the above drugs. However the same isolates from pneumonic ovine lungs were found to be resistant to streptomycin.

Resistance to streptomycin, triple sulpha and tetracycline were also reported among *P. multocida* isolates recovered from cattle with bovine respiratory disease complex (Fales *et al.*, 1982).

All isolates tested were resistant to metronidazole. Similar observations have been made by Balakrishnan (1998). With regard to sensitivity to pefloxacin the results of present study are in agreement with the observations of Rammanath and Gopal (1993) who have also found that *P. multocida* isolates recovered from ducks were sensitive to this drug.

5.6 PATHOGENICITY TESTING IN MICE

All the isolates were found to be pathogenic to mice by intraperitoneal route. The mice died within 24 h post-inoculation.

Shivshankara *et al.* (2000) and Gupta (1994) observed that *P. multocida* of bovine origin killed mice within 24 h post-inoculation. Rammanath and Gopal (1993) observed that pure cultures of *P. multocida* of duck origin killed mice within 18 to 24 h post-inoculation.

Petechiae in the pericardium, congestion of lung, liver and spleen were observed as gross lesions in mice, which died after experimental infection. Bipolar shaped organisms could be demonstrated in the heart blood and impression smears of spleen and liver by Leishman's staining. The organism could also be isolated from the heart blood and visceral organs.

There was substantial increase in the number of *P. multocida* organisms in the visceral organs of mice died after experimental infection. Similar observations have also been made by Balakrishnan (1998) and Rajalakshmi (2001).

5.7 STORAGE OF ISOLATES

The storage of *P. multocida* in defibrinated ovine/bovine blood at –70°C was found to be a simple and efficient method of maintaining the isolates. This could replace the conventional method of storage of isolates on blood agar slants, which is labourious, time consuming and requires sub culturing at more frequent intervals.

5.8 POLYMERASE CHAIN REACTION FOR DETECTION OF *P. multocida*

5.8.1 *Pasteurella multocida* Species Specific PCR (PM–PCR)

Amplification of all the *P. multocida* isolates by PCR using species-specific primer pairs, KMT1SP6 and KMT1T7 generated product of approximately 460 bp size. These results were in accordance with those of Townsend *et al.* (1998a), Townsend *et al.* (2000), Dutta *et al.* (2004) and Kapoor *et al.* (2004).

The specificity of the primers was tested by performing PM–PCR on template DNA prepared from non-pasteurella organisms like *Leptospira icterohaemorrhagiae, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. None of these organisms gave an amplified product. These results were in agreement with those of Townsend *et al.* (1998a), who reported that the same primer pairs amplified template DNA from all strains of *P. multocida, viz.*, three subspecies *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida*, *P. multocida* subsp. *septica*, as well as *P. canis* biovar 2, but had failed to amplify

DNA from other members of *Pasteurellaceae* family, or unrelated bacteria. In India, so far there is no report of *P. canis* biovar 2 and hence it might not interfere with the diagnosis of pasteurellosis by PM-PCR. Hunt *et al.* (2000), Dutta *et al.* (2004) and Kapoor *et al.* (2004) have also reported similar findings.

In the present study template DNA prepared from different sources were used for PCR amplification. These included boiled culture lysates prepared from positive isolates, nasal swabs, pharyngeal swabs and lung samples and DNA extracts from blood samples and blood smears. Since the time and effort needed to prepare boiled culture lysates and DNA extracts were very less, and number of samples to be screened by PM-PCR were more, boiled culture lysates and DNA extracts were routinely used as template DNA for PCR amplification.

In this study PCR was also used for rapid detection of *P. multocida* directly from clinical specimens like nasal swabs, pharyngeal swabs, and lung samples. Lichtensteiger *et al.* (1996) reported that PCR was useful in accurate and rapid detection of toxigenic *P. multocida* directly from clinical specimens like nasal swab. Choi and Chae (2001) also reported similar findings in which enhanced detection of toxigenic *P. multocida* was carried out directly from nasal swabs, using nested PCR.

In the present study PM-PCR assay used in the detection of *P. multocida* directly from clinical specimens like nasal swabs, pharyngeal swabs, and lung samples gave negative results in most of the cases except one and the band intensity in the amplified product was also very faint. This might be due to very low number of *P. multocida* recovered in these samples. Several workers have reported similar findings (Pijoan *et al.*, 1984; Amigot *et al.*, 1998 and Choi and Chae, 2001).

Performing PCR on template DNA prepared from blood smears greatly reduces the time required for a specific diagnosis. The PM-PCR assay using

template from blood smears gave clear-cut results, although the band intensity was less compared to those achieved by use of boiled culture lysates as template DNA. This technique was more useful in this study since blood smears were the only material sent by field veterinarians to the Department of Microbiology in many cases. Moreover the presence of artifacts resembling bipolar organisms frequently affected the accuracy of diagnosis based on microscopic examination of stained blood smears.

Blood samples received for the diagnosis of HS, which were found to be negative by isolation trials could also give positive amplification in PM-PCR. Thus PCR was found to be more sensitive, when compared to isolation and identification, in detection of *P. multocida* from clinical samples. Townsend *et al.* (1998a) reported PCR as a rapid and sensitive method for identification of *P. multocida*. The results of present study were in agreement with the above finding.

On the basis of observations made by Townsend *et al.* (1998a); Townsend *et al.* (2000); Dutta *et al.* (2004) and Shivshankara *et al.* (2001), and the results obtained in the present study, it could be concluded that PM-PCR offers a rapid and specific method for diagnosis of pasteurellosis. The PCR assay conducted on the template DNA prepared from blood smears represents a novel and practical means of detection of animal pasteurellosis.

Modifications to sample preparations have allowed PCR analysis to be performed on clinical specimens, dramatically reducing the time required for bacterial identification (Hunt *et al.*, 2000). Results obtained in this work also indicate such conclusions.

5.8.2 Type–B specific PCR (HS–B specific PCR)

In the present study, HS–B specific (serotype specific) PCR was performed by using KTSP61 and KTT72 primers in those samples, which were

found to be positive by PM-PCR. Amplification of *P. multocida* belonging to Type–B generated a product of approximately 590 bp size. The primers did not amplify other serotypes of *P. multocida viz.*, serotype A:1 obtained from ducks. This confirmed that the oligos were specific to only B serotypes as only DNA from that serotype had been amplified. These results were in accordance with those of Townsend *et al.* (1998a), Hunt *et al.* (2000) and Dutta *et al.* (2004).

Similar work in which serotype specific PCR developed for detection and identification of *P. multocida* serotype 1 was also reported by Rocke *et al.* (2002).

In this study PCR was performed using boiled culture lysates and boiled DNA extracts prepared from blood smears and blood samples. This technique was found to be useful for rapid diagnosis and confirmation of HS cases. The serotype of *P. multocida* responsible for the disease also could be confirmed by HS-B PCR. Template DNA from blood samples and blood smears gave clear-cut results in HS-B PCR also, although the band intensity was less compared to those achieved by use of boiled culture lysates as template DNA.

On the basis of observations made by Townsend *et al.* (1998a), Shivshankara *et al.* (2001) and Dutta *et al.* (2004), and the results obtained in the present study, it could be concluded that HS-B PCR offers a rapid and specific method for diagnosis of animal pasteurellosis. This study also confirms that Type–B PCR is having tremendous advantages to overcome problems associated with conventional isolation and serotyping, as it needs only the DNA material for detection and results can be assessed within a day after getting field samples.

5.8.3 Multiplex PCR

Multiplex PCR assay was performed using two sets of primers, *viz.*, PM–specific and Type–B specific, and these generated products with two bands of

approximately 460 bp and 590 bp size. Only serotype B *P. multocida* isolates gave two bands, while serotype A isolates gave one band corresponding to 460 bp only. These results are in accordance with those of Townsend *et al.* (2000) and Dutta *et al.* (2004).

Similar work based on the development of multiplex capsular PCR typing system as a rapid and highly specific alternative to conventional capsular serotyping had also been reported by Townsend *et al.* (2001).

From the present study it can be concluded that multiplex PCR assay could clearly distinguish between serotype B and serotype A isolates and that this PCR assay provides a highly specific alternative to conventional capsular serotyping which is time consuming and labourious and can be carried out only in highly equipped laboratories. Moreover this PCR assay could be helpful for rapid and confirmative diagnosis of HS in Indian conditions where disease remains endemic.

5.8.4 Nested PCR

Pasteurella multocida species specific primers KMT1SP6 and KTSP61 designed by Townsend *et al.* (1998a), were used in this study to detect *P. multocida* DNA in clinical samples like nasal and pharyngeal swabs, lung samples, blood samples and blood smears. But PM-PCR could not demonstrate *P. multocida* in majority of the clinical samples tested.

Improved sensitivity of nested PCR assay for enhanced detection of toxigenic *P. multocida* directly from nasal swabs had been reported by Choi and Chae (2001). Nested PCR assay using the primer pair TA-2 and TA-3 was derived from a conventional PCR previously described by Lichtensteiger *et al.* (1996). Conventional PCR using primer pair TA-1 and TA-2 amplified a 846 bp from the *tox*A gene, while nested primers amplified 690 bp fragment from within

846 bp region. While conventional PCR described by Lichtensteiger *et al.* (1996) showed a sensitivity of detection of approximately 2.1x 10^4 organisms, nested PCR showed greater sensitivity of detection of approximately 20 organisms. Calsamiglia *et al.* (1999) also reported about the increased sensitivity of nested PCR assay in comparison to conventional PCR when used for the detection of another microorganism, *Mycoplasma hyopneumoniae* from nasal swabs. Pijoan *et al.* (1984) had reported that some carrier animals harbouring *P. multocida* in their nasopharynx could be missed when screening nasal swabs by conventional PCR methodology. Based on the above observations enhanced detection of *P. multocida* in clinical samples was attempted in the present study by a nested PCR assay using primer pair designed by Primer 3 software.

Nested PCR could detect *P. multocida* DNA in both PM-PCR positive and PM-PCR negative clinical samples as indicated by the amplification of 214 bp fragment from within 460 bp region. The results of present study indicated that a high proportion of the clinical samples previously found negative by PM-PCR gave positive results in nested PCR. This finding does not correlate well with the results of PM-PCR. Moreover only 24 per cent of the samples tested negative by PM-PCR could be screened by nested PCR. With this small proportion of samples screened, it is difficult to arrive at a definite conclusion.

On the basis of observations in the present study it could be concluded that *P. multocida* could be demonstrated in clinical samples like nasal and pharyngeal swabs, lung samples, blood samples and blood smears by nested PCR, but a high sensitivity obtained needs further investigation before recommending the use of this PCR assay as a promising diagnostic tool for confirming the presence of *P. multocida* while screening nasal and pharyngeal swabs, lung samples, blood and blood smears, where conventional PM-PCR failed to detect *P. multocida.*

5.8.5 REP–PCR

Amplification of multiple *P. multocida* genomic DNA fragments by outwardly directed primers based on the Repetitive Extragenic Palindromic sequences (REP) generated complex profiles in a PCR based finger printing method known as REP-PCR. This finger printing method is shown to be highly discriminatory that differentiates strains of related bacteria. Analysis of the distribution of REP sequences in prokaryotic genome form the basis of a novel PCR based DNA finger printing technique.

REP–PCR finger prints of *P. multocida* isolates causing HS showed a high degree of homogeneity while that of *P. multocida* isolates causing fowl cholera exhibited a marked heterogeneity (Townsend *et al.*, 1997b). In the present study all the five isolates of *P. multocida* from cases of HS showed similar REP-PCR profile.

These findings were similar to those observed by Townsend *et al.* (1998b) and Gunawardana *et al.* (2000). They reported that isolates of *P. multocida* from HS (serotype B: 2) from Vietnam showed only minimal variation, with a single REP-profile for each of them. The isolates of *P. multocida* used in the present study belong to capsular serogroup B as evidenced by HS–B PCR assay.

5.9 PLASMID PROFILE OF *P. multocida* ISOLATES

The plasmid DNA from the *P. multocida* isolates were analysed on agarose gel electrophoresis. Of the five isolates only two carried plasmids. Shivsankara *et al.* (2000) demonstrated that among 12 isolates obtained from animal and avian origin, only ten showed the presence of plasmids. However, two other studies showed greater variation in the occurrence of plasmids in avian

isolates and it varied from 24 percent (Price *et al.*, 1993) to 70.7 percent (Hirsh *et al.*, 1985).

The size of plasmid detected in the present study was 5.22 kbp. Strains of *P. multocida* have been shown to harbour plasmids from 1.3 kbp (Diallo *et al.*, 1995) to approximately 100 kbp (Hirsh *et al.*, 1989) in size.

In the present study the two isolates which carried single plasmid belonged to serotype B. Shivsankara *et al.* (2000) demonstrated that isolates of serotype B: 2 recovered from buffalo and pig carried only single plasmid and 10 out of 12 isolates which carried plasmids were placed into two groups; group one containing seven isolates, each carrying a single plasmid and the remaining isolates forming group two harbouring two plasmids each.

All the isolates were susceptible to most of the antibiotics tested. Only BP1 was found to be resistant to two out of twelve antibiotics tested. But that isolate did not harbour any plasmid. No correlation between presence of plasmids and antibiotic resistance could be ascertained in the present study. Similar observations also have been made by Diallo *et al.* (1995) who found that only 55 percent of the 45 avian isolates of *P. multocida* carried plasmids, while the isolates were uniformly resistant to streptomycin, trimethoprim and lincomycin.

Pathogenicity tests conducted in mice revealed that all the isolates, even those which lacked plasmids, were pathogenic. All the isolates killed weaned mice within 24 h. This observation could not establish any correlation between presence of plasmids and virulence. These results are in accordance with the observations made by Diallo *et al.* (1995) and Shivshankara *et al.* (2000).

5.10 SEQUENCING OF HS-B PCR AMPLIFIED PRODUCT

The PCR product with an approximate molecular size of 590 bp, sequenced by Sanger's dideoxy chain termination method revealed a product of 520 bp. Townsend *et al.* (1996) have analyzed a unique sequence specific to Carter type B isolates of *Pasteurella multocida.* Sequence similarity searches performed with BLAST provided by NCBI showed a 99 per cent identity with *Pasteurella multocida* unknown protein 1 and protein 2 gene (Accession No AF016260). Results in present study are in agreement with the observations made by Townsend *et al.* (1996).

In the present study, eventhough *P. multocida* could be demonstrated in PM-PCR negative clinical samples by nested PCR, a high sensitivity obtained needs further investigation before recommending the use of this PCR assay as a promising diagnostic tool for confirming the presence of *P. multocida* while screening nasal and pharyngeal swabs, lung samples, blood samples and blood smears where conventional PM-PCR failed to detect *P. multocida.*

Summary

6. SUMMARY

Haemorrhagic septicaemia (HS) has a wide distribution in the Asian continent. Among Asian countries, India ranks HS as one of the most important bacterial diseases affecting cattle and buffalo. The disease is prevalent in almost all the states of the country. From the records maintained by the Department of Animal Husbandry, Kerala state, it is evident that there are reports on outbreaks of HS from almost all the districts of the state, even though the total number of animals affected and deaths due to HS are very less.

There are reports about the significant association of the occurrence of *Pasteurella multocida*, the organism causing HS, in the nasopharynx of apparently normal bovines and the outbreak of the disease. Hence detection of *P. multocida* in apparently healthy domestic ruminants can be considered as an indicator of the susceptibility of the herd for HS. The occurrence of *P. multocida* causing HS in apparently healthy domestic ruminants in this state has not been systematically studied so far. The present study was undertaken with a view to understand the occurrence of *P. multocida* organisms causing HS in apparently healthy and clinically ill domestic ruminants in a specified area of Thrissur district *viz.*, Ollukkara block area, examining one per cent of total ruminant population in the area. Besides the samples collected from Ollukkara block area, those brought to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy from suspected cases of HS, were also included in this study. Polymerase chain reaction (PCR) using species specific, type specific and nested primers had been employed in this study to achieve this objective.

A total of 309 samples comprising of nasal swabs, pharyngeal swabs, lung samples, blood samples and blood smears were processed for isolation of *P. multocida* and detection by PCR. This included 260 samples from Ollukkara block area and 49 samples brought to the Department of Microbiology, College

of Veterinary and Animal Sciences, Mannuthy for diagnosis of HS. *Pasteurella multocida* could not be isolated from any of the clinical samples cultured for isolation from Ollukkara block area. Five isolates could be obtained from the blood samples from Palakkad district. These were designated as BP1 & BP2 from cattle, BuP1 and BuP2 from buffalo and GP1 from goat.

The five isolates have been conventionally characterized as *P. multocida* by morphological, cultural and biochemical tests. A reference strain of *P. multocida* (P52) was used for comparison.

All the isolates were found to be pathogenic for mice. Pure cultures of all the isolates as well as the reference strain were able to kill weaned mice within 24 h post inoculation.

Based on the variation in fermentation patterns of dulcitol, sorbitol, trehalose and xylose the five isolates as well as the P52 could be biotyped as *P. multocida* subsp. *multocida.*

All the isolates were uniformly sensitive to ten out of twelve antibiotics tested. Only one isolate (BP1) was found to be resistant to ampicillin and all the isolates were uniformly resistant to metronidazole.

Storage of *P. multocida* in defibrinated ovine/bovine blood at -70° C was found to be a simple and efficient method of maintaining isolates, with a potential to replace the laborious, time-consuming conventional methods.

All the isolates obtained were confirmed as Type-B *P. multocida* using species-specific primer pair KMT1SP6 and KMT1T7 and HS-B specific primer pair KTSP61 and KTT72. Species-specific PCR (PM-PCR) of isolates generated an amplified product of size 460 bp and type specific PCR (HS-B PCR) generated a product of size 590 bp. All the isolates belonging to serotype-B, when characterized using multiplex PCR, generated two bands of size 460 bp and 590 bp respectively.

Pasteurella multocida could be detected in only 14 clinical samples (including the isolates) tested by species specific PCR (PM-PCR). This included one nasal swab and 13 blood samples and blood smears. The PM-PCR assay-using template from blood smears gave clear-cut results and this technique was useful in this study when blood smears were the only material sent by field veterinarians to the Department of Microbiology for the diagnosis of HS. The entire samples tested positive by PM-PCR were confirmed as type-B *P. multocida* by HS-B specific PCR.

A nested PCR assay using the primer pairs designed by Primer 3 software, was developed to detect *P. multocida* in clinical samples tested positive and negative by PM-PCR. When the PCR product (460 bp) of 14 samples amplified by using primer pairs KMT1SP6 and KMT1T7 was used for reamplification with nested PCR primers a product of 214 bp size was observed.

A total number of 71 clinical samples tested negative by PM-PCR were selected at random for amplification with nested PCR primer pairs. A high proportion of the clinical samples previously found negative by PM-PCR gave positive results in nested PCR. This finding does not correlate well with the results of PM-PCR. Hence a definite conclusion could not be arrived about the feasibility of using this PCR for enhanced detection of *P. multocida* from clinical samples like nasal and pharyngeal swabs, lung samples, blood samples and blood smears.

All the isolates showed a single REP-PCR profile, indicating a high level of homogeneity among them.

Among the five isolates examined, only two (BP2 and BuP1) harboured plasmids of size 5.22 kbp. Other isolates did not reveal the presence of any plasmid. Pathogenecity tests conducted in mice revealed that all the isolates, even those that lacked plasmids, were pathogenic. No correlation between presence of plasmids and virulence could be ascertained in the present study.

The HS-B PCR product with an approximate molecular size of 590 bp was sequenced by Sanger's dideoxy chain termination method. The size of the product was 520 base pairs. Sequence similarity searches were performed with Basic Local Alignment Search Tool (BLAST) network provided by National Center for Biotechnology Information (NCBI). The sequence had 99 per cent identity with *Pasteurella multocida* unknown protein 1 and protein 2 gene (Accession No AF016260).

References

REFERENCES

*Abeynayake, P., Wijewardana, T.G. and Thalagoda, S.A. 1993. Antimicrobial susceptibility of *Pasteurella multocida* isolates. *Proceedings of International Workshop on Pasteurellosis in Production Animals, August 10-13, 1992* (eds. Wijewardana, T.G. and Thalagoda, S.A.). Bali, Indonesia, pp. 193-196

Adlam, C. 1989. The structure, function and properties of cellular and extra cellular components of *Pasteurella haemolytica*. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 75-92

Aghomo, H.O. and Ojo, M.O. 1983. Antimicrobial susceptibility of *Pasteurellae* isolated from ovine lungs. *Trop. Vet.* 1: 218

Amigot, A.J., Torrelorell, M. and Pijoan, C. 1998. Evaluation of techniques for detection of toxigenic *Pasteurella multocida* strains from pigs. *J. Vet. Diagn. Invest.* 10: 169-173

Amonsin, A., Wellehan, J.F.X., Li, L.L., Laber, J. and Kapur, V. 2002. DNA fingerprinting of *Pasteurella multocida* recovered from avian sources. *J. Clin. Microbiol.* 40: 3025-3031

Anupama, M., Venkatesha, M.D., Yasmeen, N. and Sreenivasa Gowda, R.N. 2003. Evaluation of polymerase chain reaction (PCR) for identification of virulent *Pasteurella multocida* and its comparison with animal inoculation test. *Indian J. Anim. Sci.*73: 166-167

Bain, R.V.S. 1951. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Bain, R.V.S. 1957. The problem of haemorrhagic septicaemia in cattle. *Ceylon. Vet. J.* 5: 2-3

Bain, R.V.S. 1959. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Bain, R.V.S., De Alwis, M.C.L., Carter, G.R. and Gupta, B.K. 1982. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 133-134

Bain, R.V.S. and Jones, R.F. 1958. The production of dense cultures of *P. multocida. Br. Vet. J.* 114: 215-220

Balakrishnan, G. 1998. Plasmid profile of avian strains of *Pasteurella multocida.* M.V.Sc. thesis, Kerala Agricultural University, Thrissur, 110 p.

Barrow, C.I. and Feltham, R.K.A. 1993. *Cowan and Steel's Manual for Identification of Medical Bacteria.* Third edition. Cambridge University Press, Cambridge, 364 p.

*Bastianello, S.S. and Jonker, M.R. 1981. A report on the occurrence of septicaemia caused by *P. multocida* type E in cattle from South Africa. *J. S. Afr. Vet. Assoc.* 52: 99-104

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45: 493-496

Berman, S.M. and Hirsh, D.C. 1978. Partial characterization of R-plasmids from *Pasteurella multocida* isolated from turkeys. *Antimicrob. Agents Chemother.* 14: 348-352

Biberstein, E.L. and Kirkham, C. 1979. Antimicrobial susceptibility patterns of A and T types of *Pasteurella haemolytica*. *Res. Vet. Sci.* 26: 324-328

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7: 1517-1523

Bisgaard, M., Houghton, S.B., Mutters, R. and Stenzel, A. 1991. Reclassification of German, British and Dutch isolates of so called *Pasteurella multocida* obtained from pneumonic calf lungs. *Vet. Microbiol.* 26: 115-124

Blackall, P.J. and Miflin, J.K. 2000. Identification and typing of *Pasteurella multocida*: a review. *Avian Pathol.* 29:271–287

Blackall, P.J. and Miflin, J.K. 2001. Development of 23S rRNA- based PCR assay for the identification of *Pasteurella multocida*. *Lett. Appl. Microbiol.* 33: 216

Blackall, P.J., Pahoff, J.L. and Bowles, R. 1997. Phenotypic characterization of *Pasteurella multocida* isolates from Australian pigs. *Vet. Microbiol.* 57: 355-360

Blackall, P.J., Fegan, N., Pahoff, J.L., Storie, G.J., McIntosh, G.B., Cameron, R.D.A., Boyle, D.O., Frost, A.J., Bara, M.R., Marr, G. and Holder, J. 2000. The molecular epidemiology of four outbreaks of porcine pasteurellosis. *Vet. Microbiol.* 72: 111-120

Bollinger, W. 1878. Pasteurellosis. *Infectious Diseases of Animals: Diseases due to Bacteria* (eds. Stableforth, A.W. and Galloway, I.A.). Butterworth Scientific Publications, London, 421 p.

Boyce, J.D., Chung, J.Y. and Adler, B. 2000. Genetic organization of the capsule biosynthetic locus of *Pasteurella multocida* B: 2. *Vet. Microbiol.* 72: 121-134

Brogden, K.A. and Packer, R.A. 1979. Comparison of *Pasteurella multocida* serotyping systems. *Am. J. Vet. Res.* 40: 1332-1335

Brickell, S.K., Thomas, L.M., Long, K.A., Panaccio, M. and Widders, P.R. 1998. Development of a PCR test based on a gene region associated with pathogenicity of *P. multocida* serotype B:2, the causal agent of HS in Asia. *Vet. Microbiol.* 59: 295-307

*Buchanan, R.E., Holt, J.G. and Lessel, E.F. 1966. *Index bergeyana* (eds. Holt, J.G. and Lessel, E.F.). William and Wilkins Co., London, pp. 786-793

Burrill, T.J. 1883. A note on the name *Pasteurella multocida. Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 35-36

Burrows, T.W. and Gillett, W.A. 1966. Pasteurella. *Animal Microbiology* (eds. Buxton, A. and Fraser, G.). Blackwell Scientific Publication, Oxford, pp. 121-130

Buttenschon, J. and Rosendal, S. 1990. Phenotypical and genotypical characteristics of paired isolates of *Pasteurella multocida* from the lungs and kidneys of slaughtered pigs. *Vet. Microbiol.* 25: 67-75

Calsamiglia, M., Pijoan, C. and Trigo, A. 1999. Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyopneumoniae* from nasal swabs. *J. Vet. Diagn. Invest.* 11: 246-251

Carter, G.R. 1955. A haemagglutination test for the identification of serological types. *Am. J. Vet. Res.* 16: 481-484

Carter, G.R. 1961. A new serological type of *Pasteurella multocida* from central Africa. *Vet. Rec.* 73: 1052

Carter, G.R. 1963. Proposed modification of the serological classification of *P. multocida. Vet. Rec.* 75: 1264

Carter, G.R. 1967. Pasteurellosis: *Pasteurella multocida* and *Pasteurella haemolytica. Adv. Vet. Sci. Comp. Med.* 11: 321

Carter, G.R. 1972. Improved hemagglutination test for identifying Type A strains of *Pasteurella multocida. Appl. Microbiol.* 24: 162-163

Carter, G.R. 1981. Prokaryotes. *A Handbook on Habitats, Isolation and Identification of Bacteria* (eds. Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Selegal, H.G.). Springer-Verlag, NewYork, pp. 1383-1391

Carter, G.R. 1982. Whatever happened to haemorrhagic septicaemia. *J. Am. Vet. Med. Assoc.* 180: 1176- 1177

Carter, G.R. 1984a. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Carter, G.R. and Chengappa, M.M. 1981. Identification of type B *Pasteurella multocida* from cases of haemorrhagic septicaemia. *J. Clin. Microbiol.* 11: 94-96

Carter, G.R. and Chengappa, M.M. 1981a. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Carter, G.R. and Chengappa, M.M. 1981b. Identification of type B and E *Pasteurella multocida* by counterimmuno electrophoresis. *Vet. Rec.* 108: 145-146

Carter, G.R. and De Alwis, M.C.L. 1989. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 131-160

Chakraborty, A.K., Sarmah, A.K. and Boro, B.R. 1978. A case of clinical mastitis due to *Pasteurella multocida*. *Vet. Rec.* 103: 313

*Chandrasekaran, S., Muniandy, N., Kennet, L. and Mukkur, T.K.S. 1991. Protection, antibody response and isotype specificity in buffaloes immunized with conventional haemorrhagic septiceamia vaccines. *Proceedings of the FAO/APHCA Workshop on Haemorrhagic Septiceamia. February, 1991* (eds. Kennet, L. and Mukkur, T.K.S.). Kandy, Srilanka, pp. 98-100

Chandrasekaran, S., Rhoades, K.R. and Sotoodehnia, A. 1985. *Pasteurella multocida* type B: 2 isolated from poultry in Iran. *Vet. Rec.* 117: 155

Chandrasekaran, S., Yeap, P.C. and Chuink, B.H. 1981. Biochemical and serological studies of *Pasteurella multocida* isolated from cattle and buffaloes in Malaysia. *Br. Vet. J.* 137: 361

Chanter, N., Goodwin, R.F.W. and Rutter, J.M. 1989. Comparison of methods for the sampling and isolation of *Pasteurella multocida* from the nasal cavity of pigs. *Res. Vet. Sci.* 47: 355- 358

Choi, C. and Chae, C. 2001. Enhanced detection of toxigenic *Pasteurella multocida* directly from nasal swabs using nested polymerase chain reaction. *The Vet. J.* 162: 255-258

Cote, S., Harel, J., Higgins, R. and Jacques, M. 1991. Resistance to antimicrobial agents and prevalence of R plasmids in *Pasteurella multocida* from swine. *Am. J. Vet. Res.* 52: 1653 –1664

Das, M.S. 1958. Studies on *Pasteurella septica* (*Pasteurella multocida*). *J. Comp. Pathol. Therap.* 68: 288-294

Davies, R.L., MacCorquodale, R. and Caffrey, B. 2003. Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. *Vet. Microbiol.* 91: 169-182

Davies, R.L., MacCorquodale, R. and Reilly, S. 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Vet. Microbiol.* 99: 145-158

*De Alwis, M.C.L. 1973. *Haemorrhagic septicaemia.* Monograph No.57. Australian Center for International Agricultural Research (ACIAR), *Australia,* 93 p.

De Alwis, M.C.L. 1981. Mortality among cattle and buffaloes due to haemorrhagic septicaemia. *Trop. Anim. Hlth. Prod.* 13: 195-202

De Alwis, M.C.L. 1982. The immune status of buffalo calves exposed to natural infection with haemorrhagic septicaemia. *Trop. Anim. Hlth. Prod.* 14: 29-30

De Alwis, M.C.L. 1984. Haemorrhagic septicaemia in cattle and buffaloes. *Rev. Sci. Tech. Off. Int. Epiz.* 3: 707- 730

De Alwis, M.C.L. 1992. Haemorrhagic septicaemia –a general review. *Br. Vet. J.* 148: 99-112

De Alwis, M.C.L. 1999. *Haemorrhagic septicaemia.* Monograph No.57. Australian Centre for International Agricultural Research (ACIAR), *Australia,* 93 p.

De Alwis, M.C.L. and Carter, G.R. 1980. Preliminary field trials with a streptomycin dependent live vaccine against haemorrhagic septicaemia. *Vet. Rec.* 106: 435-437

De Alwis, M.C.L. and Panangala, V.S. 1974. A biochemical and serological study of strains of *Pasteurella multocida* associated with haemorrhagic septicaemia in cattle and buffaloes in srilanka. *Ceylon Vet. J.* 22: 58-65

De Alwis, M.C.L. and Sumanadasa. M.A. 1982. Naturally acquired immunity to haemorrhagic septicaemia among cattle and buffaloes in Srilanka *Trop. Anim. Hlth. Prod.* 14: 27-28

De Alwis, M.C.L. and Vipulasiri, A.A. 1980. An epizootiological study of haemorrhagic septicaemia in buffaloes and cattle in Srilanka. *Ceylon. Vet. J.* 28: 24-35

De Alwis, M.C.L., Wijewardana, T.G., Gomis, A.I.U. and Vipulasiri, A.A. 1980. Persistence of the carrier status in haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B infection) in buffaloes. *Trop. Anim. Hlth. Prod.* 22: 185-194

De Alwis, M.C.L., Wijewardana, T.G., Sivaram, A. and Vipulasiri, A.A. 1986b. The carrier and antibody status of cattle and buffaloes exposed to haemorrhagic septiceamia: investigations on survivors following natural outbreaks. *Srilanka. Vet. J.* 34: 33-42

De Alwis, M.C.L., Wijewardana, T.G. and Vipulasiri, A.A. 1986a. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

De Jong, M.F. and Borst, G.H.A. 1985. Selective medium for the isolation of *Pasteurella multocida* and *Bordetella bronchiseptica. Vet. Rec.* 116: 551

De Rosa, D.C., Mechor, G.D., Staats, J.J., Chengappa, M.M. and Shryock, T.R. 2000. Comparison of pasteurella spp simultaneously isolated from nasal and transtracheal swabs from cattle and clinical signs of bovine respiratory disease. *J. Clin. Microbiol.* 38: 327 –332

Dhanda, M.R. and Nilakantan, P.R. 1961. Isolation of *Pasteurella septica* of low virulence from cases of paraplegia in cattle in Andhra Pradesh. *Indian. Vet. J.* 38: 339

Diallo, I.S., Bensink, J.C., Frost, A.J. and Spradbrow, P.B. 1995. Molecular studies on avian strains of *Pasteurella multocida* in Australia. *Vet. Microbiol.* 46: 335-342

Diker, K.S., Akan, M. and Haziroglu, R. 1994. Antimicrobial susceptibility of *Pasteurella haemolytica* and *Pasteurella multocida* isolated from pneumonic ovine lungs. *Vet. Rec.* 134: 597-598

Dutta, T.K., Gautam, R., Singh, V.P. and Kumar, A.A. 2004. PCR based detection and analysis of *Pasteurella multocida* isolates causing haemorrhagic septicaemia in India buffaloes. *Indian. J. Anim. Sci.* 74: 577-580

Dutta, J., Rathore, B.S., Mallick, S.G., Singh, R. and Sharma, G.C. 1990. Epidemiological studies on occurrence of haemorrhagic septicaemia in India. *Indian. Vet. J.* 67: 893

Dutta, T.K., Singh, V.P. and Kumar, A.A. 2001. Rapid and specific diagnosis of animal pasteurellosis by using PCR assay. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 22: 43-46

Dziva, F., Christensen, H., Olsen, J.E. and Mohan, K. 2001. Random amplification of polymorphic DNA and phenotypic typing of Zimbabwean isolates of *Pasteurella multocida*. *Vet. Microbiol.* 82: 361-372

Fales, W.H., Selby, L.A., Webber, J.J., Hoffman, L.J., Kintner, L.D., Nelson, S.L., Miller, R.B., Thorne, J.G., McGinity, J.T. and Smith, D.K. 1982. Antimicrobial resistance among pasteurella spp recovered from Missouri and Iowa cattle with bovine respiratory disease complex. *J. Am. Vet. Med. Assoc.* 181: 477-483

Francis, B.K. and Schels, H.F. and Carter, O.R. 1980. Type E *Pasteurella multocida* associated with haemorrhagic septicaemia in Zambia. *Vet. Rec.* 107: 135

Gautam, R., Kumar, A.A., Singh, V.P., Dutta, T.K. and Shivachandra, S.B. 2004. Specific identification of *Pasteurella multocida* serogroup-A isolates by PCR assay. *Res. Vet. Sci.* 76: 179-185

Go, M.F., Chan, K.Y., Versalovic, J., Koeuth, T., Graham, D.Y. and Lupski, J.R. 1995. Cluster analysis of Helicobacter pylori genome DNA fingerprints suggests gastroduodenal disease specific associations. *Scand. J. Gastroenterol.* 30: 640-646

Gochenour, W.S. 1924. Haemorrhagic septicaemia studies: The development of a potent immunizing agent (natural aggression) by the use of highly virulent strains of haemorrhagic septicaemia organisms. *J. Am. Vet. Med. Assoc.* 65: 433- 445

Gunawardana, G.A., Townsend, K.M. and Frost, A.J. 2000. Molecular characterization of avian *Pasteurella multocida* isolates from Australia and Vietnam by REP-PCR and PFGE. *Vet. Microbiol.* 72: 97-109

Gupta, V. 1994. Studies on virulence and anti-microbial drug resistance of field isolates of *Pasteurella multocida* of animal and avian origin. M.V.Sc. thesis. Deemed University, IVRI, Izatnagar, 100 p.

Gupta, B.K. 1962. Studies on the carrier problem in haemorrhagic septicaemia. M.V.Sc thesis, Punjab University, Chandigarh, India, 78 p.

Gupta, B.K. and Kumar, S. 1973. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Guerry, P., Leblanc, D.J. and Falkow, S. 1973. General method for the isolation of plasmid deoxyribonucleic acid. *J. Bacteriol.* 116: 1064-1066

Haghour, R., Hellmann, E. and Schmidt, J. 1987. Plasmids and resistance to nine chemotherapeutic agents of *Pasteurella multocida* and *Pasteurella haemolytica*: Epidemiological aspects. *J. Vet. Med.* 34: 509-518

Hancock, R.D., Fallavena, L.C.B. and Ribeiro, L.A.O. 1991. Pneumonic pasteurellosis due to *Pasteurella multocida* in a flock of lambs in Brazil. *Vet. Rec.* 128: 154-155

Hassan, A.K. and Mustafa, A.A. 1985. Haemorrhagic septicaemia due to serogroup B *P. multocida* in North Africa. *Rev. Elev. Med. Vet. Pays. Trop.* 38: 31-33

Heddleston, K.L. 1976. Physiologic characteristics of 1268 cultures of *Pasteurella multocida*. *Am. J. Vet. Res.* 37: 745-747

Heddleston, K.L., Gallagher, J.E. and Rebers, P.A. 1972. Fowl cholera: gel diffusion precipitation test for serotyping *Pasteurella multocida* from avian species. *Avian Dis.* 16: 925-936

Heddleston, K.L., Rhoades, K.R. and Rebers, P.A. 1967. Experimental pasteurellosis- comparative studies on *Pasteurella multocida* from Asia, Africa and North America. *Am. J. Vet. Res.* 28: 1003-1012

Higgins, C.F., Ames, G.F.L., Barner, W.M., Clement, J.M. and Hofung, M. 1982. A novel intercistronic regulatory element of prokaryotic operons. *Nature.* 298: 760-762

Hill, B.D. and Johnson, R.B. 1992. *Pasteurella multocida* septicaemia in two calves. *Aust. Vet. J.* 69: 197-198

Hiramune, T. and De Alwis, M.C.L. 1982. Haemorrhagic septicaemia carrier status of cattle and buffaloes in Srilanka. *Trop. Anim. Hlth. Prod.* 14: 91-92

Hirsh, D.C., Hansen, L.M., Dorfman, L.C., Snipes, K.P., Carpenter, T.E., Hird, D.W. and McCapes, R.H. 1989. Resistance to antimicrobial agents and prevalence of R plasmids in *Pasteurella multocida* from turkeys. *Antimicrob. Agents Chemother.* 33: 670-673

Hirsh, D.C., Jessup, D.A., Snipes, K.P., Carpenter, T.E., Hird, D.W. and McCapes, R.H. 1990. Characteristics of *Pasteurella multocida* isolated from waterfowl and associated with avian species in California. *J. Wildlife Dis.* 26: 204-209

Hirsh, D.C., Martin, L.D. and Rhoades, K.R. 1981. Conjugal transfer of an R-plasmid in *Pasteurella multocida. Antimicrob. Agents Chemother.* 20: 415-417

Hirsh, D.C., Martin, L.D. and Rhoades, K.R. 1985. Resistance plasmids of *Pasteurella multocida* isolated from turkeys. *Am. J. Vet. Res.* 46: 1490-1493

Holmes, B. 1998. Actinobacillus, Pasteurella and Eikenella. *Topley and Wilson's Microbiology and Microbial infections* (eds. Balows, A. and Duerdem, B.I.). Ninth edition. Oxford University press Inc., New York, pp. 1198-1209

Hudson, 1954. *Pasteurella multocida. Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp 37-73

*Hueppe, F. 1886. Ueber die wild seuche und ihre bedeutung fur die nationalokonomie und die hygiene. *Berliner. Klinische. Wochenschrift.* 44: 753-758

Hunt, M.L., Adler, B. and Townsend, K.M. 2000. The molecular biology of *Pasteurella multocida. Vet. Microbiol.* 72: 3-25

Johnson, R.B., Dawkins, H.J.S., Spencer, T.L., Bascon, J.L.T., Almazan, M.J.P.P., Agor, E.C. and Rivera, W.C. 1993. Characterisation of *Pasteurella multocida* (Haemorhagic septicaemia) isolates from the Philippines. *Proceedings of International Workshop on Pasteurellosis in Production Animals. August 10-13, 1992* (eds. Agor, E.C. and Rivera, W.C.). Bali, Indonesia, pp. 203-208

Johnson, R.B., Dawkins, H.J.S., Spencer, T.L., Saharee, A.A., Bahaman, A.R. and Ramadani, B.E. 1989. Evaluation of bovine antibody responses to haemorrhagic septicaemia vaccine. *Res. Vet. Sci.* 47: 277-279

Jones, T.O., Rimler, R.B. and Minns, M. 1988. Isolation of *Pasteurella multocida* F –3,4 from a calf in the UK. *Vet. Rec.* 123: 354

Kado, C.I. and Liu, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* 145: 1365-1373

Kamp, E.M., Bokken, G.C., Vermeulen, T.M., Dejong, M.F., Buys, H.E., Reek, F.H. and Smits, M.A. 1996. A specific and sensitive PCR assay for large scale detection of toxigenic *Pasteurella multocida* in nasal and tonsilar swab specimens of pigs. *J. Vet. Diagn. Invest.* 8: 304-309

Kanwar, N.S., Paliwal, O.P. and Ramkumar, A.A. 1998. Pneumonic pasteurellosis in goats. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 19: 99-101

Kapoor, V., Katoch, R.C., Sharma, M., Kumar, A.A. and Verma, S. 2004. Detection of *Pasteurella multocida* by PCR. *Indian. J. Anim. Sci.* 74: 724-725

Kasali, O.B. 1972. Case of haemorrhagic septicaemia in an African buffalo (*Syncerus nanus*) in Nigeria. *Bull. Epizoot. Dis. Afr.* 20: 203-204

Kasten, R.W., Hansen, L.M., Hinojoza, J., Bieber, D., Ruchl, W.W. and Hirsh, D.C. 1995. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae. Infect. Immun.* 63: 989-993

Kawamoto, E., Sawada, T. and Maruyama, T. 1990. Prevalence and characterization of *Pasteurella multocida* in rabbits and their environment in Japan. *Jpn. J. Vet. Sci.* 52: 915-921

Khera, S. 1979. Epidemiological studies on occurrence of haemorrhagic septicaemia. *Bull. Inf. Epiz.* 91: 331

Kitt, T.H. 1893. A note on the name *Pasteurella multocida. Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp.35-36

Kitt, T. 1885. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Knight, D.P., Paine, J.E. and Speller, D.C.E. 1983. A selective medium for *Pasteurella multocida* and its use with animal and human specimens. *J. Clin. Pathol.* 36: 591-594

Kumar, A.A., Harbola, P.C., Rimler, B.R. and Kumar, P.N. 1996. Studies on *Pasteurella multocida* isolates of animal and avian origin from India. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 17: 120-124

Lane, E., Kock, N., Hill, F.W.G. and Mohan, K. 1992. An outbreak of haemorrhagic septicaemia in cattle in Zimbabwe. *Trop. Anim. Hlth. Prod.* 24: 97-102

Lee, D.M., Glisson, R.J., Wooley, R.E. and Brown, J. 1990. Characterisation of *Pasteurella multocida* mutants of low virulence. *Avian. Dis.* 34: 958-963

Lee, C.W., Wilkie, I.W., Townsend, K.M. and Frost, A.J. 1999. The demonstration of *Pasteurella multocida* in the alimentary tract of chickens after experimental oral infection. *Vet. Microbiol.* 72: 47-55

Lehmann, K.B. and Neumann, R. 1899. A note on the name *Pasteurella multocida*. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, pp. 35-36

Lichtensteiger, C.A., Steinbergen, S.M., Lee, R.M., Polson, D.D. and Vimur, E.R. 1996. Direct PCR analysis for toxigenic *Pasteurella multocida*. *J. Clin. Microbiol.* 34: 3035-3039

Lupski, J.R. and Weinstock, G.M. 1992. Short interspersed repetitive DNA sequences in prokaryotic genomes. *J. Bacteriol.* 174: 4525-4529

Madsen, E.B., Bisgaard, M., Mutters, R. and Pedersen, K.B. 1985. Characterisation of pasteurella species isolated from lungs of calves with pneumonia. *Can. J. Comp. Med.* 49: 63-67

Manjrekar, S.L. 1954. Occurrence of pasteurellosis in sheep due to *P. multocida*. *Bombay Vet. Coll. Mag.* 4: 16

Mannheim, W. 1984. Pasteurellaceae. *Bergey's Manual of Systematic Bacteriology* (eds. Kreig, N.R. and Holt, J.G.). First edition. Williams and Wilkins Co., London, pp. 559-569

Mannheim, W. and Carter, G.R. 1984. Pasteurellaceae. *Bergey's Manual of Systematic Bacteriology* (eds. Kreig, N.R. and Holt, J.G.). First edition. Williams and Wilkins Co., London, pp. 550-558

Martrenchar, A. and Njanpop, B.M. 1993. First case of an outbreak of haemorrhagic septicaemia caused by *P. multocida* serotype B: 6 in northern Cameroon. *Rev. Elev. Med: Vet. Pays. Trop.* 47: 19-20

Maxam, A.M. and Gilbert, W. 1977. A new method for sequencing DNA. *Proc. Natl. Acd. Sci. USA.* 74: 560-564

Meyers, J.A., Sanchez, D., Elwell, L.P. and Falkow, S. 1976. Simple agarose gel electrophoretic method for the identification and characterisation of plasmid deoxyribonucleic acid. *J. Bacteriol.* 127: 1529-1537

Miflin, J.K. and Blackall, P.J. 2001. Development of a 23S rRNA-based PCR assay for the identification of *Pasteurella multocida. Lett. Appl. Microbiol.* 33: 216-221

Mohan, K., Sadza, M., Madsen, M., Hill, F.W.G. and Pawandiwa, A. 1994. Phenotypic characterization of Zimbabwean isolates of *Pasteurella multocida. Vet. Microbiol.* 38: 351-357

Mohler, J.R. and Eichorn, A. 1913. Immunization against haemorrhagic septicaemia. *Am. Vet. Rev.* 42: 409-418

Moller, K., Anderson, L.V., Christensen, G. and Kilian, M. 1993. Optimalization of the detection of NAD dependent pasteurellaceae from the respiratory tract of slaughterhouse pigs. *Vet. Microbiol.* 36: 261-271

Moore, M.K., Chubbs, L.C. and Gates, R.J. 1994. A new selective enrichment procedure for isolating *Pasteurella multocida* from avian and environmental samples. *Avian Dis.* 38: 317-324

Morris, E.J. 1958. Selective media for some *Pasteurella* species. *J. Gen. Microbiol.* 19: 305-311

Murthy, D.K. and Adlakha, S.C. 1962. Primary pasteurellosis in pigs–studies on outbreaks in Punjab. *Indian. Vet. J.* 39: 406

Murthy, D.K. and Kaushik, R.K. 1965. Studies on an outbreak of acute swine pasteurellosis due to *Pasteurella multocida* type B. *Vet. Rec.* 77: 411

Mustafa, A.A., Ghalib, H.W. and Shigidi, M.T. 1978. Carrier rate of *Pasteurella multocida* in cattle associated with an outbreak of haemorrhagic septicaemia in Sudan. *Br. Vet. J.* 134: 375-378

Mutters, R.S., Ihm, P., Pohl, S., Frederiksen, W. and Mannheim, W. 1985. Reclassification of the genus *Pasteurella trevisan* 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis* and *Pasteurella langaa. Intl. J. System. Bacterial.* 35: 309-322

Nagai, S., Someno, S. and Yagihashi, T. 1994. Differentiation of toxigenic from non-toxigenic isolates of *Pasteurella multocida* by PCR. *J. Clin. Microbiol.* 32: 1004-1010

Namioka, S. and Bruner, D.W. 1963. Serological studies on *Pasteurella multocida* IV: Type distribution of the organism on the basis of their capsule and O groups. *Cornell. Vet.* 53: 41-53

Namioka, S. and Murata, M. 1961. Serological studies on *Pásteurella multocida* I: a simple method of capsular typing of the organism. *Cornell. Vet.* 51: 498-507

Namioka, S. and Murata, M. 1961a. Serological studies on *Pasteurella multocida* II: characteristics of the somatic (O) antigen of the organism. *Cornell. Vet.* 51: 507-521

Namioka, S. and Murata, M. 1961b. Serological studies on *Pasteurella multocida* III: O antigenic analysis of cultures isolated from various animals. *Cornell. Vet.* 51: 522-528

Namioka, S. and Murata, M. 1964. Serological studies on *Pasteurella multocida* V. Some epizootiological findings resulting from 'O' antigen analysis. *Cornell Vet.* 54: 520-534

Office International Des Epizooties. 2000. *Manual of Standards, Diagnostic Tests and Vaccines*. Fourth edition. Paris, France, 582 p.

Office International Des Epizooties. 2002. *International Animal Health Code.* Tenth edition. Paris, France, 603 p.

Oreste, A. and Armanni, B. 1887. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Palit, N. and Rao, C.C.P. 1969. Studies on ovine pasteurellosis, incidence of carriers and natural immunity. *Indian. Vet. J.* 46: 459-466

Pande, A. and Singh, V.P. 1997. Plasmid profile analysis of *Pasteurella multocida* isolates. *Indian Vet. J.* 74: 891

*Pasteur, L. 1880. De l'attenuation du virus du cholera des poules. *Campt. Renel. Acad. Sci.* 90: 673-696

Pavri, K.M. and Apte, W.H. 1967. Isolation of *Pasteurella multocida* from a fatal disease of horses and donkeys in India. *Vet. Rec.* 80: 437-439

*Perreau, P. 1961. Contribution a letude immunologique de *Pasteurella multocida*. *Rev. Elev. Med. Vet. Pays. Trop.* 14: 245-256

Pijoan, C., Lastra, A., Ramires, C. and Leman, A.D. 1984. Isolation of toxigenic strains of *Pasteurella multocida* from lungs of pneumonic swine. *J. Am. Vet. Med. Assoc.* 185: 522-523

Price, S.B., Freeman, M.D. and Macewen, M.W. 1993. Molecular analysis of a cryptic plasmid isolated from avian strains of *Pasteurella multocida*. *Vet. Microbiol.* 37: 31-43

Rajalakshmi, S. 2001. Nucleic acid and protein profiles of *Pasteurella multocida* of avian origin. M.V.Sc. thesis, Kerala Agricultural University, Thrissur, 115 p.

Rammanath, K.R. and Gopal, T. 1993. A note on the isolation and characterization of *Pasteurella multocida* from ducks. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 14: 32-33

*Rapley, R., Theophilus, B.D.M., Bevan, I.S. and Walker, M.R. 1992. Fundamentals of polymerase chain reaction: a future in clinical diagnostics? *Med. Lab. Sci.* 49: 119-128

Rimler, R.B. 1978. Coagglutination test for identification of *P. multocida* associated with haemorrhagic septicaemia. *J. Clin. Microbiol.* 8: 214-218

Rimler, R.B. and Rhoades, K.R. 1989. Fowl cholera. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 95-113

Roberts, R.S. 1947. An immunological study of *Pasteurella septica*. *J. Comp. Pathol.* 57: 261-278

Rocke, T.E., Smith, S.R., Miyamoto, A. and Shadduck, D.J. 2002. A serotype-specific polymerase chain reaction for identification of *Pasteurella multocida* serotype 1. *Avian. Dis.* 46: 370-377

Rosenbusch, C.T. and Merchant, I.A. 1939. A study of haemorrhagic septicaemia pasteurellae. *J. Bacteriol.* 37: 69-89

Rubies, X., Casal, J. and Pijoan, C. 2002. Plasmid and restriction endonuclease patterns in *Pasteurella multocida* isolated from a swine pyramid. *Vet. Microbiol.* 84: 69-78

Sambrook, J., Fritsch, E.T. and Maniatis, T. 1989. *Molecular Cloning A Laboratory Manual.* Second edition. Cold Spring Harbor Laboratory, New York, 868 p.

*Sanger, F., Nicklen, S. and Coulson, R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74: 5463-5467

Sarma, G. and Boro, B.R. 1980. Isolation of *Pasteurella multocida* from sporadic outbreaks of bovine pasteurellosis in Assam. *Vet. Rec.* 106: 57-58

Schneider, L. 1948. *Pasteurella multocida. Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 37-73

Schwarz. S.U., Schafer, F. and Blobel, H. 1989. Isolation and interspecies-transfer of a plasmid from *Pasteurella multocida* encoding for streptomycin resistance. *Med. Microbiol. Immunol.* 178: 121-125

Sengupta, K.C. and Verma, N.S. 1951. Outbreaks of pasteurellosis in swine. *Indian. J. Vet. Sci.* 21: 197

Sheikh, M.A., Anzam, M. and Shakoori, A.R. 1996. Observations on haemorrhagic septicaemia in Pakistan livestock. *Zentralbl. Veterinarmed B.* 43: 293-304

Shigidi, M.T.A. and Mustafa, A.A. 1979. Biochemical and serological studies on *Pasteurella multocida* isolated from cattle in Sudan. *Cornell. Vet.* 69: 77-84

Shivashankara, N., Saxena, M.K. and Singh, V.P. 2001. Rapid diagnosis of haemorrhagic septicaemia by PCR assay. *Indian Vet. J.* 78: 101-103

Shivshankara, N., Singh, V.P., Saxena, M.K. and Kumar, A.A. 2000. Plasmid profile analysis of *Pasteurella multocida. Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 21: 115-117

Singh, N. 1948. Nasal carriers in bovine pasteurellosis. *Indian. J. Vet. Sci.* 18: 77-78

Silver, R.P., Leming, B., Garon, C.F. and Hjerpe, C.A. 1979. R-plasmids in *Pasteurella multocida. Plasmid.* 2: 493-497

Smith, J.E. 1959. Studies on *Pasteurella septica.* III. Strains from human beings. *Jour. Compar. Path. Ther.* 65: 239-240

Smith, G.R. 1961. The characters of two types of *Pasteurella haemolytica* associated with different pathological conditions in sheep. *J. Pathol. Bacteriol.* 81: 431-440

Smith, I.M. and Baskerville, A.J. 1983. A selective medium for the isolation of *Pasteurella multocida* in nasal specimens from pigs. *Br. Vet. J.* 139: 476

Smith, G.R. and Phillips, J.E. 1990. Pasteurella. *Topley and Wilson's Principles of Bacteriology, Virology and Immunity* (eds. Parkar, M.T. and Duedven, B.I.). Eighth edition. Edward Arnold, Melbourne, Auckland, pp. 335-351

Tomer, P. 2000. Characterisation of *Pasteurella multocida* isolates from outbreaks of haemorrhagic septicaemia in bovines. M.V.Sc thesis, Haryana Agricultural University, Hisar, 83 p.

Topley, W.W.C. and Wilson, G.S. 1929. *The Principles of Bacteriology and Immunity.* Edward Arnold and Co., London, 488 p.

Townsend, K.M., Boyce, J.D., Chung, J.Y., Frost, A.J. and Adler, B. 2001. Genetic organization of *Pasteurella multocida* cap loci and development of a multiplex capsular PCR typing system. *J. Clin. Microbiol.* 39: 924-929

Townsend, K.M., Dawkins, H.J.S. and Papadimitriou, J.M. 1997b. REP-PCR analysis of *Pasteurella multocida* isolates that cause haemorrhagic septicaemia. *Res. Vet. Sci.* 63: 151-155

Townsend, K.M., Dawkins, H.J.S., Zeng, B.J., Watson, M.W. and Papadimitriou, J. M. 1996. Cloning a unique sequence specific to isolates of type B:2 *Pasteurella multocida. Res. Vet. Sci.* 61: 199-205

Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M. and Dawkins, H.J. 1998a. Development of PCR assays for species and type specific identification of *Pasteurella multocida* isolates. *J. Clin. Microbiol.* 36: 1096-1100

Townsend, K.M., Hanh, T.X., O'Boyle, D., Wilkie, I., Phan, T.T., Wijewardana, T.G., Trung, N.T. and Frost, A.T. 2000. PCR detection and analysis of *Pasteurella multocida* from the tonsils of slaughtered pigs in Vietnam *Vet. Microbiol.* 72: 69-78

Townsend, K.M., O'Boyle, D., Phan, T.T., Hanh, T.X., Wijewardana, T.G., Trung, N.T. and Frost, A.J. 1998b. Acute septicaemic pasteurellosis in Vietnamese pigs. *Vet. Microbiol.* 63: 205-215

Trevisan, V. 1887. Haemorrhagic septicaemia. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 130-160

Umesh, D. 1994. An outbreak of pneumonia due to *Pasteurella multocida* in sheep. *Indian Vet. J.* 71: 1163-1167

Venkatanarayana, J. 1962. *Annual Report of Disease Investigation Officer (Pigs).* Andrapradesh, Hyderabad, 56 p.

Verma, N.D. 1988. *Pasteurella multocida* B:2 in haemorrhagic septicaemia outbreak in pigs in India. *Vet. Rec.* 123: 63

*Versalovic, J., Kocuth, T. and Lupskei, J.R. 1991. Distribution of repetitive DNA sequence in eubacteria and application to finger printing of bacterial genomes. *Nucleic Acids Res.* 19: 6823-6831

Vig, R.P. and Kalra, D.S. 1969. Primary pasteurellosis in pigs–studies on outbreaks in Punjab. *Indian. Vet. J.* 46: 842-847

Voigts, A., Ngaisiue, G., Henton, M.M. and Hubschle, O.J.B. 1997. Haemorrhagic septicaemia due to *Pasteurella multocida* type B: 2 in Namibia. *Trop. Anim. Hlth. Prod.* 4: 247-248

Warner, R. 1996. *Haemorrhagic septicaemia.* Monograph No.57. Australian Centre for International Agricultural Research (ACIAR), *Australia,* 93 p.

Watson, P.J. and Davies, R.L. 2002. Outbreak of *Pasteurella multocida* septicaemia in neonatal lambs. *Vet. Rec.* 151: 420-422

Weiser, G.C., Delong, W.J., Paz, J.L., Shafi, B., Price, W.J. and Waed, A.C. 2003. Characterization of *Pasteurella multocida* associated with pneumonia in bighorn sheep. *J. Wild. Dis.* 39: 536-544

Wijewardana, T.G., De Alwis, M.C.L. and Bastiansz, H.L. 1986a. Cultural, biochemical and pathogenicity studies on strains of *Pasteurella multocida* isolated from carrier animals and outbreaks of haemorrhagic septicaemia. *Sri Lanka Vet. J.* 34: 43-57

Wijewardana, T.G., De Alwis, M.C.L. and Vipulasiri, A.A. 1982. An agar gel diffusion test for rapid identification of *Pasteurella multocida* type B. *Sri Lanka Vet. J.* 30: 12-14

Wijewanta, E.A. and Karunaratne, K.G. 1968. Studies on the occurrence of *Pasteurella multocida* in the nasopharynx of healthy cattle. *Cornell. Vet.* 58: 462-465

Wilson, P.J. 1990. *Haemophilus*, *Actinobacillus*, *Pasteurella*: Mechanisms of resistance and antibiotic therapy. Can. J. Vet. Res. 54: 73-77

Zopf, W. 1885. A note on the name *Pasteurella multocida*. *Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.). Academic Press, London, pp. 35-36

* Originals not consulted.

DETECTION OF *Pasteurella multocida* IN DOMESTIC RUMINANTS BY ISOLATION AND POLYMERASE CHAIN REACTION

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ABSTRACT

A study was undertaken to examine the occurrence of *P. multocida* organisms causing HS in apparently healthy and clinically ill domestic ruminants in a specified area of Thrissur district *viz.*, Ollukkara block, covering one per cent of total ruminant population in the area. Besides the samples collected from Ollukkara block area, those brought to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy from suspected cases of HS, were also included in the study.

A total of 309 samples comprising of nasal swabs, pharyngeal swabs, lung samples and blood samples were processed for isolation of *P. multocida* and detection by PCR. Detection of *P. multocida* by PCR was carried out using species-specific (PM-PCR), type-B specific (HS-B PCR) and nested primers.

Pasteurella multocida could not be isolated from any of the clinical samples cultured for isolation from Ollukkara block area. Five isolates could be obtained from the blood samples from Palakkad district.

Isolates obtained were characterized as *P. multocida* using standard bacteriological procedures. A reference strain P52 obtained from IVRI was used for comparison. All the isolates were found to be pathogenic for mice. Based on the fermentation patterns of dulcitol, sorbitol, trehalose and xylose all isolates as well as P52 could be biotyped as *P. multocida* subsp. *multocida.* All isolates were uniformly sensitive to ten out of twelve antibiotics tested and uniformly resistant to metronidazole.

All isolates were confirmed as type-B *P. multocida* using species-specific primer pairs, KMT1SP6 and KMT1T7 and HS-B specific primer pairs, KTSP61

and KTT72. Multiplex PCR was used to characterize all the serotype B isolates, which generated two bands of size 460 bp and 590 bp.

Only 14 clinical samples including the isolates were positive for *P. multocida* by PM-PCR. The entire samples tested positive by PM-PCR were confirmed as type-B *P. multocida* by HS-B specific PCR.

The 14 samples found positive by PM-PCR when subjected to nested PCR, gave an amplified product of size 214 bp. Since nested PCR could detect *Pasteurella multocida* DNA in a high proportion of clinical samples found previously negative by PM-PCR, a definite conclusion could not be arrived about the feasibility of using this PCR assay for enhanced detection of *P. multocida* as only a random number of negative clinical samples could be screened.

All the isolates showed a single REP-PCR profile, indicating a high level of homogeneity among them. Among the five isolates examined, only two (BP2 and BuP1) harboured plasmids.

The sequence similarity searches of HS-B PCR amplified product with BLAST network showed that the sequence had 99 per cent identity with *Pasteurella multocida* unknown protein 1 and protein 2 gene (Accession No AF016260).