ISOLATION AND CHARACTERIZATION OF *Pasteurella multocida* FROM ANIMALS AND BIRDS

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DECLARATION

I hereby declare that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF *Pasteurella multocida* FROM ANIMALS AND BIRDS"** 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 **"ISOLATION AND CHARACTERIZATION OF *Pasteurella multocida* FROM ANIMALS AND BIRDS "** is a record of research work done independently by **Ambili K.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to her.

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EXTERNAL EXAMINER

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Dedicated to my parents

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Introduction

1. INTRODUCTION

Pasteurella multocida (*P. multocida*) is the most prevalent species of the genus *Pasteurella* causing a wide variety of infections in many domestic and wild animals and birds. It is a primary or, more frequently, a secondary pathogen of cattle, swine, sheep, goats, and other animals. As a secondary invader, it is often involved in pneumonia of cattle (shipping fever) and in enzootic pneumonia of swine. It is responsible for a variety of sporadic infections in many animals, including abortion, encephalitis and meningitis. It produces severe mastitis in cattle and sheep and toxin-producing strains are involved in atrophic rhinitis, an economically important disease of swine. In birds it produces fowl cholera. This agent is responsible for annual losses of several hundred million dollars to animal production world wide.

Laboratory detection of *P. multocida* depends on the isolation and identification of suspect bacterial colonies by microscopy and biochemical tests. Isolation of *P. multocida* becomes difficult when samples are taken from contaminated sites on the animal, such as the nose or throat (Townsend *et al*., 1998). The detection of the organism involved in a particular disease process is one of the most important aspects of a disease diagnosis. Early diagnosis of pasteurellosis allows development of suitable treatment and effective implementation of prevention and control strategies. The use of molecular methods like *P. multocida* specific polymerase chain reaction (PCR) will help to overcome this problem to a great extent. Townsend *et al*. (1998) developed a *Pasteurella multocida* specific PCR (PM-PCR); useful for the identification of *P. multocida*, from bacterial cultures without extraction and purification of genomic DNA.

The species *P. multocida* is divided into three subspecies: *P. multocida* subsp. *multocida, P. multocida* subsp. *gallicida,* and *P. multocida* subsp. *septica ,*

mainly on the basis of ability to ferment dulcitol and sorbitol (Mutters *et al.,* 1985). This species also has been subgrouped on the basis of capsular serogroup. Five capsular serogroups, designated A, B, D, E, and F (Carter, 1967) providing some correlation with disease manifestation (type A with pneumonic pasteurellosis in cattle, type A and F with fowl cholera, type D with atrophic rhinitis in pigs, and types B and E with haemorrhagic septicemia) and 16 somatic serotypes (designated 1 through 16) (Heddleston *et al.*, 1972) of *P. multocida* have been described.

Difficulties are experienced in the preparation of antisera against specific serogroups which makes current *P. multocida* serotyping procedures impractical for most laboratories in different countries. A multiplex PCR assay was introduced as a rapid alternative to the conventional capsular serotyping system by Townsend *et al*. (2001). An epidemiological study will reveal the homogeneity or heterogeneity between the isolates which in turn help in choosing the vaccine strains. Identification and strain differentiation of the organism forms the basis of an epidemiological study. Both capsular and somatic serotyping has proved to be very useful for detection and identification of this bacterium, but it provides insufficient information for epidemiological studies to distinguish among different strains of the same serotype. Also the conventional methods used for strain differentiation in the epidemiological studies like biotyping, OMP profile and phage typing are time-consuming and often produce ambiguous results (Saxena *et al*., 2006).

One of the most widely used PCR based method for epidemiological studies is Repetitive extragenic palindromic PCR (REP-PCR). The ease and rapidity of REP-PCR while maintaining a high level of differentiation, supports the use of REP-PCR as a competent alternative to the more labour-intensive PFGE system for strain identification and epidemiological studies of avian *P. multocida* (Gunarwardana *et al.,* 2000).

Another important and highly reproducible technique is genotypic characterization like restriction endonuclease analysis (REA). It is a valuable component of bacterial epidemiologic studies, particularly in investigations of outbreaks of pasteurellosis. This method is a highly reproducible technique that is not influenced by the inconsistent expression of phenotypic traits that limit the sensitivity and specifcity of conventional typing methods (Snipes *et al*., 1989).

The present study was undertaken to give an insight into the frequency of occurrence of *P.multocida* as an etiological agent for respiratory infection in animals and birds. Data on the molecular characterization will help in identifying homogeneity/heterogeneity between the isolates; this in turn will help to formulate more efficient vaccines against Hemorrhagic septicaemia (HS) and avian pasteurellosis. Objectives of the present study include,

(1) Isolation of *P. multocida* from apparently healthy/ clinically ill animals and birds

(2) Detection of *P. multocida* from the biomaterials by polymerase chain reaction (PCR)

(3) Pathogenicity test of the isolates obtained

(4) Determination of homogeneity /heterogeneity between the isolates, using molecular techniques.

Review of Literature

2. REVIEW OF LITERATURE

Pasteurella multocida is a Gram-negative bacterial species isolated from a variety of wild and domesticated animals and birds. The bacterium is associated with a variety of specific diseases of domestic animals, such as pneumonic pasteurellosis in cattle, fowl cholera in poultry, atrophic rhinitis in pigs, and hemorrhagic septicemia in cattle and buffalo (Ogunnariwo *et al*., 2001).

2.1 HISTORY

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

In birds the disease was first studied by Chabert (1782) and Maillet (1836) named the disease as fowl cholera.

Pasteur (1880) isolated the organism that caused fowl cholera and grew pure cultures in chicken broth.

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

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

Webster (1924) reproduced snuffles in rabbits by intranasal inoculation of *P.multocida* in *Pasteurella* free rabbits.

First description of fowl cholera in turkeys was made by DeVolt and Davis (1932).

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

Flatt and Dungworth (1971) reported that *P.multocida, Bordetella bronchiseptica* and *Staphylococcus aureus* were commonly recovered from pneumonic lungs. But as *B. bronchiseptica* and *S. aureus* were also isolated from normal lungs, they probably had little role in the etiology of the disease.

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 haemorrhagic septicaemia 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.

2.2 PREVALENCE

2.2.1 Prevalence Outside India

Mustafa *et al.* (1978) reported that HS was wide spread in Sudan. Outbreaks have been noted to occur at the beginning of rains, just before the lush season.

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.

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

De Alwis (1981) analyzed the data on mortality in cattle and buffaloe in Sri Lanka due to haemorrhagic septicaemia. Mortality in buffalo was higher than for cattle (45.2 and 15.8 per cent respectively).

The disease was rare or nonexistent in cattle in North America. The only HS outbreak reported in cattle for several decades in North America resulted from the injection of a contaminated vitamin preparation (Carter, 1982).

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

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

Carpenter *et al*.(1989) recovered 49 isolates of *P. multocida* from turkeys in 11 of the outbreaks from California.

Hancock *et al*. (1991) reported an outbreak of pneumonic pasteurellosis in sheep in Brazil.

Johnson *et al.* (1993) characterized six *P. multocida* isolates obtained from clinically sick cattle and buffalo in Philippines suspected of having HS, by Poly Acrylamide Gel Electrophoresis (PAGE).

Prevalence of fowl cholera in psittacine birds had been reported by Morishita *et al.* (1996 a and b).

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

Prevalence of pasteurellosis in free ranging chicks and ducks had been reported from Tanzania (Muhairwa *et al.,* 2001).

Davies *et al*. (2003 b) recovered strains of *P. multocida* from cases of ovine pneumonia, from the vaginal tracts of healthy ewes and cases of neonatal septicaemia from UK.

2.2.2 Prevalence in India

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

Vig and Kalra (1969) carried out studies to investigate the outbreaks of pasteurellosis of acute septicaemic form in swine, which occurred in the Punjab state during the years 1961 and 1962.

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

Haemorrhagic septicaemia outbreak due to *P. multocida* serotype B:2 in pigs in Shillong, 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 foot and mouth disease, 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 per cent) and lowest for Dadra Nagar Haveli (0.03 per cent). State wise classification of high risk and medium risk areas was also done. Andaman, Lakshadweep and Mizoram had the disease free status.

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 *P. multocida* from twelve cases out of three hundred and seventy four caprine lungs showing pneumonic lesions, from slaughterhouses of Himachal Pradesh, Chandigarh, Delhi, Rajasthan, Uttar Pradesh, Bihar and West Bengal.

Tomer (2000) reported the isolation of *P. multocida* serotype B:2 from outbreaks of HS in different districts of Haryana and Delhi States.

An outbreak of fowl cholera in Japanese quails in Tirupathi, resulting in high mortality (33.7 per cent) was reported by Srilatha *et al.* (2003).

2.3 ISOLATION OF *P. multocida*

Enriched media like five per cent bovine blood agar (Dorsey, 1963), six per cent bovine blood agar (Curtis and Ollerhead, 1981), ten per cent ovine blood agar (Dwivedi and Sodhi, 1989), media containing five per cent sterile serum or blood (Rimler and Rhoades, 1989) and dextrose starch agar containing five per cent chicken serum (Rhoades and Rimler, 1991), were found to be useful for the isolation of *P. multocida.*

Carter (1981) opined that *P. multocida* grew best at a temperature of 35-37°C in air or air plus 5 per cent carbon dioxide.

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

Jericho and Carter (1985) isolated *P multocida* from 61 per cent of deep nasal swabs from 790 calves in 12 herds.

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

Chanter *et al*. (1989) assessed the efficacy of detecting toxegenic *Pasteurella multocida* from nasal swabs of slaughtered and live pigs. Individual sows from one of the infected herds were repeatedly swabbed to find out the best method of isolating the toxigenic *P. multocida*.

A medium containing kanamycin and bacitracin in tryptic soya agar supplemented with 5 per cent ovine blood was used by Kawamoto *et al.* (1990).

Zhao *et al*. (1992) isolated *P. multocida* from swine herds by plating onto 5 per cent sheep blood agar and incubating at 37°C overnight.

Christensen *et al.* (1998) used bovine blood agar for the isolation of *P. multocida* from avian species.

Loubinoux *et al*. (1999) isolated 56 strains of *P. multocida* from gingival swabs of dogs on ovine blood agar and chocolate agar plates, incubated at 37°C in an atmosphere of 5 per cent carbon dioxide.

Mehrotra and Bhargava (1999) found five per cent ovine blood agar useful for the isolation of *P. multocida* from cattle, sheep and goats.

Muhairwa *et al*. (2000) reported the isolation of *P.multocida* from cloacal mucosa of apparently healthy domestic poultry.

Rajalakshmi (2001); Antony (2004) and Karunakaran (2004) used blood agar plates for the primary isolation of *P. multocida*, suspected material were streaked on blood agar incubated and incubated at 37°C with mild CO_2 tension.

2.4 IDENTIFICATION OF *P. multocida*

Mutters *et al.* (1989) observed that the colonies of *P. multocida* on blood agar were circular, low convex and regular with an entire margin. The surface of the colony might be greyish or yellowish. A colonial diameter of 1.5-2.0 mm was observed after 24 h at 37°C.

Rimler and Rhoades (1989) had identified the sugars such as glucose, mannose, galactose and fructose as those most commonly fermented by *P. multocida*.

Kawamoto *et al.* (1990) identified *P.multocida* isolates from rabbits based on the following criteria: Gram negative rod; negative motility; nogrowth on

MacConkey agar; positive activity of catalase and oxidase; no hydrolysis of urea and fermentation of sugars.

Bisgaard *et al*. (1991) reported that the 24 *P.multocida* isolates of their study differed in tests like oxidase, fermentation of arabinose, xylose, fructose, lactose and trehalose.

The second stage biochemical reactions used for characterization of *P. multocida* were reported by Barrow and Feltham, 1993.

Mohan *et al.* (1994) reported that all the isolates of *P. multocida* in their study gave positive reactions for catalase, oxidase, indole, nitrate, acid in glucose and sucrose but negative in tests for arginine, lysine, methyl red and Voges Proskauer reactions.

Pasteurella multocida produces catalase, oxidase, ornithine decarboxylase and indole, but does not produce urease, lysine decarboxylase, beta-galactosidase, or arginine dihydrolase. Nitrate is reduced, and methyl red and Voges–Proskauer tests are negative. (OIE, 2008).

2.5 BIOTYPING

Murthy and Kaushik (1965) found that a strain of *P. multocida* serotype B isolate from acute swine pasteurellosis 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.

Heddleston (1976) reported that all (100 per cent) of the 1268 cultures of *P.multocida* from various hosts fermented glucose, fructose and sucrose. No cultures fermented inositol, inulin, rhamnose and salicin.

All of the 43 strains of *P. multocida* isolated from different outbreaks of haemorrhagic septicaemia and also from healthy cattle in various parts of Sudan,

fermented xylose, glucose, fructose, galactose, mannose, sucrose and sorbitol, with acid production. None of the strains fermented rhamnose, lactose, trehalose, raffinose, dulcitol or salicin. (Shigidi and Mustafa, 1979).

Chandrasekaran *et al*. (1981) reported that none of the strains of *P. multocida* isolated from cattle and buffaloe in Malaysia in their study 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. 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.

Based on the fermentation patterns of dulcitol and sorbitol *P. multocida* could be divided into three subspecies for epidemiological purposes. The sorbitol and dulcitol positive variety became *P. multocida* subsp. *gallicida*; those strains negative for both became *P. multocida* subsp. *septica* and those positive for sorbitol but negative for dulcitol were *P. multocida* subsp. *multocida* (Mutters *et.al*.,1985)

Wijewardana *et al*. (1986) studied a total of 40 isolates of *P. multocida* from clinically normal buffaloe and cattle. All the isolates fermented glucose, sucrose, sorbitol and mannitol with production of acid only. None of them fermented lactose, trehalose, or salicin.

Based on variation in biochemical characteristics like 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) *P. multocida* could be grouped into subgroups or biotypes (Rimler and Rhoades, 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 *P. 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 60 Zimbabwean *P. multocida* isolates from different host species and serotyped a number of representative strains. All isolates, irrespective of their origin fermented glucose and sucrose but did not ferment arabinose, dulcitol, inositol and salicin.

Blackall *et al.* (1995) observed that 22 isolates of their study fermented glucose, mannitol and sucrose but not dulcitol or lactose. They identified 4 distinct biochemical types, termed as biovars, based on the differences in their ability to ferment maltose, sorbitol, trehalose and xylose.

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

Blackall *et al*. (2000) studied biochemical profiles of thirty-eight field isolates of *P. 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 *P. multocida* subspecies *multocida* (31 isolates) or *P. multocida* subspecies *gallicida* (seven isolates). The *P. 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).

Dziva *et al.* (2001) characterized eighty-one isolates of *P. 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 *P. multocida* subspecies *multocida* and *P. multocida* subspecies *septica.* Remaining isolates could not be assigned to the known taxa and were designated as unassigned biotypes. *P. multocida* subspecies *multocida* isolates fermented mannitol and sorbitol but not dulcitol and trehalose, while *P. multocida* subspecies *septica* isolates fermented trehalose and mannitol but not dulcitol and sorbitol.

Muhairwa *et al.* (2001) carried out extensive phenotypic characterization which revealed phenotypic similarities between two isolates from chicken and duck strains.

Antony (2004) reported that all the *P. multocida* isolates of the study fermented glucose, galactose, mannose and sucrose but could not utilize lactose. Inositol could be fermented by only three isolates.

2.6 ANTIBIOGRAM

Murthy and Kaushik (1965) found that *P. multocida* serotype B isolates from acute swine pasteurellosis were highly sensitive to oxytetracycline and chlortetracycline, 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.

Donahue and Olson (1972) determined the invtro antibiotic sensitivities of 181 turkey isolates of *P. multocida* and found that most of them were resistant to sulphadiazine.

Fales *et al*. (1982) studied antimicrobial resistance among *Pasteurella* species recovered from cattle, of Missouri and Iowa State, with respiratory disease complex. Most of the *P. multocida* isolates did not show marked antimicrobial resistance to nine of the fifteen drugs tested. Fifty eight per cent of the *P. 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 *P. 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.

Prescott and Yielding (1990) reported that *P. multocida* isolate from pig was highly susceptible to norfloxacin, enrofloxacin and ciprofloxacin.

Pasteurella multocida isolates from ducks in Karnataka were found to be sensitive to chloramphenicol, chlortetracycline, oxytetracycline, co-trimoxazole, nalidixic acid, gentamicin, streptomycin, kanamycin and neomycin (Rammanath and Gopal, 1993).

Diker *et al*. (1994) studied antimicrobial susceptibility patterns of 81 isolates of *P. multocida* obtained from pneumonic ovine lungs. Isolates were sensitive to chloramphenicol, ampicillin and showed resistance to streptomycin and lincomycin.

Diallo *et al.* (1995) studied antimicrobial resistance patterns of 45 avian strains of *P. 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, nitrofurantoin and sulfanilamide.

Balakrishnan (1998) found that antibiotic sensitivity patterns could help in differentiating strains of *P. multocida* and the isolates were found to be sensitive to oxytetracycline, pefloxacin and streptomycin, but resistant to furazolidone, metronidazole and nalidixic acid.

Study conducted by Antony (2004) revealed that all the duck isolates and fowl isolates of *P. multocida* used were found to be sensitive to enrofloxacin, pefloxacin and chloramphenicol.

Karunakaran (2004) reported that all the isolates of the study were uniformly sensitive to chloramphenicol, cloxacillin, furazolidone, gentamicin, nitrofurantoin, penicillin, pefloxacin, streptomycin, tetracycline and co-trimoxazole.

Zahoor *et al*. (2006) observed antimicrobial susceptibility results with different patterns showing, variable responses to different antimicrobial agents. All the six isolates from Pakistan were found to be susceptible to chloramphenicol and ciprofolxacin and resistant to cephradine and streptomycin. Trimethoprim Sulpha, neomycin and nitrofurantoin gave variable results for the isolates.

2.7 PATHOGENICITY STUDIES IN MICE

A group of adult albino mice were inoculated subcutaneously with 0.5 ml of a 24 h broth culture of *P. multocida*. The organism was recovered from dead mice by inoculation of heart blood on to blood agar plates and serum broth (Sarma and Boro, 1980).

Verma (1988) observed that *P. multocida* B:2 isolates when injected subcutaneously in various numbers ranging from 2.88×10^5 to 3.45×10^9 colony forming units (CFU) were highly pathogenic to mice.

Ramnath and Gopal (1993) examined pathogenicity of *P. multocida* isolates by injecting 0.1ml of 18 h broth culture subcutaneously to Swiss mice and the inoculated mice died within 18 to 24 h post infection. They observed characteristic haemorrhagic respiratory, gastrointestinal and cardiac lesions of pasteurellosis on post mortem.

Kumar *et al*. (1996) observed that all the 43 isolates from different animals and birds killed mice within 24 to 48 h and reisolated the organisms were from their blood.

The 18 h broth culture of pure colonies of *P. multocida* was pathogenic to Swiss Albino mice which died in 24h after inoculation of 0.2ml intraperitonially and the impression smears of mice visceral organs revealed bipolar organisms and pure cultures of *P. multocida* could be isolated from mice (Sastry and Rajeswari, 2000).

Shivashankara *et al.* (2000) found that when 0.1 ml broth culture of *P. multocida* was inoculated by intraperitonial route, 70 to 100 per cent of the mice died for all the isolates except one in which mortality was only 50 per cent.

Goto *et al* (2001) reported that all mice died within 24 h when challenged with 3.9×10^2 colony forming units of pure colonies of *P. multocida* isolated from quail and severe bacteraemia was observed in blood of mice.

Anupama *et al*. (2003) reported that when 0.2ml of 18 h old broth cultures of *P. multocida* were inoculated subcutaneously to adult mice, they within 24 h.

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).

2.8.1 Serum Protection Typing

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

2.8.2 Capsular Serogrouping

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

The system most commonly used for specific capsular serogrouping is based on passive haemagglutination of erythrocytes by capsular antigen (Carter, 1955).

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 might be equated to Robert's types III and IV (Carter, 1963).

Five serogroups A, B, D, E and F have been reported in the Carter system (Carter, 1967).

Carter (1972) described an improved, indirect haemagglutination test for the recognition of type A strains of *P. multocida*.

Staphylococcal hyaluronidase inhibition test for the identification of mucoid type *P.multocida* and acriflavin test for the recognition of type D provided simple procedures for the identification of these two varieties (Carter and Rundell, 1975).

Bain *et al*. (1982) used mouse protection test for the identification of B:2 strains . This test involved determining whether or not specific rabbit antisera

would protect mice against intraperitoneal challenge with the strain being examined.

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

Buttenschon and Rosendal (1990) carried out hyaluronidase sensitivity and acriflavin flocculation tests for capsular seotyping of *P. multocida* isolates from lungs and kidneys of slaughtered pigs. All the strains were found to be capsular type A.

Townsend *et al*. (2001) developed a multiplex polymerase chain reaction (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*. (2003 a) reported that PCR based capsular typing was a reliable and rapid method for typing of large numbers of *P.multocida* isolates.

2.8.3 Somatic Serotyping

Roberts (1947) identified several different immunotypes of *P.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.

Comparison 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.

Shigidi and Mustafa (1979) used indirect haemagglutination test, gel diffusion precipitin test and tube agglutination test to determine somatic serotypes of the 42 strains of *P. multocida* isolated from outbreaks of HS and from healthy cattle in various parts of Sudan. Of these 38 were identified as type B and 5 as type E.

Carter and Chengappa (1981) opined that systems of Carter and Heddleston might 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.* (1986) carried out somatic serotyping of a wide range of *P. multocida* isolates from healthy carriers and from outbreaks of HS. All the isolates from the carrier animals gave positive reactions of somatic type six. Only 14 of 21 isolates tested from abattoir animals showed positive reactions of somatic type 6.

Majority of the 520 isolates of *P. multocida* collected from turkeys and wild birds in California were classified as serotypes 3, 4 and capsular type A (Snipes *et al.*, 1990).

Rammanath and Gopal (1993) found that the isolate of *P. multocida* causing fowl cholera outbreak in ducks was belonging to serotype A:1, sharing somatic antigen 14 to a minor extent.

All eight isolates of *P. multocida* from fowl cholera outbreaks in chicken and ducks from Zimbabwe were serotyped as A:3 at the FAO *Pasteurella* Reference Labortory at Peradeniya, Sri Lanka (Mohan *et al.*, 1994).

Kumar *et al*. (1996) used Carter-Heddleston system for typing of 43 isolates of animal and avian origin. They reported the isolation of A:1,A:3,F:3,F:3,4 from cattle and A:3 from buffaloe for the first time in India.

Serotyping of 73 avian *P. multocida* isolates from Australia and 22 from Vietnam revealed that majority of them belonged to capsular type A, with predominant somatic serovars 1,3,4 and 3,4 (Gunawardana *et al*., 2000).

2.9 MOLECULAR TECHNIQUES

2.9.1 Detection and Identification of *P. multocida* by Specific Polymerase Chain Reaction Assays

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.

Lichtensteiger *et al.* (1996) studied the feasibility of using PCR for accurate and rapid detection of toxigenic *P. multocida* from swabs.

Amigot *et al.* (1998) detected dermonecrotic gene (*Tox* A) of toxigenic *P. multocida* strains from pigs, using PCR. Detection of toxin was also carried out

using fetal lung feline cell lines and commercial Enzyme linked immunosorbent assay kit.

Townsend *et al*. (1998) developed a *P. multocida* specific PCR (PM-PCR). It was useful for the identification of *P. multocida*, from bacterial cultures without extraction and purification of genomic DNA, 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 could specifically identify *P. multocida* types B:2, B:5 and B: 2, 5.

Polymerase chain reaction amplification with the primer pair KTSP61-KTT72 specifically produced a product of approximately 590 bp from HS-causing serogroup B isolates of *P. multocida* with the predominant somatic antigen being either 2 or 5 (Townsend *et al*., 1998).

Calsamiglia *et al.* (1999) reported the improved sensitivity of nested PCR assay in comparison to conventional PCR for detection of *Mycoplasma hyopneumoniae* from nasal swabs.

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) *hex*A homologue (designated *cex*A for capsule export).

Hunt *et al.* (2000) opined that DNA hybridization and nucleic acid amplification had allowed bacterial detection from clinical specimens, dramatically reducing the time required for bacterial identification.

Townsend *et al*. (2000) detected *P. multocida* using PM-PCR from tonsil swab samples collected from healthy swine prior to slaughter.

Choi and Chae (2001) developed a nested PCR assay for enhanced detection of toxigenic *P. multocida* directly from nasal swabs.

Shivshankara *et al*. (2001) opined that PCR technique could be implemented in regional laboratories in India to rapidly confirm the field outbreaks of HS.

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

Multiplex PCR analysis of 158 porcine isolates of *P.multocida* resulted in the amplification of a single band representing sergroup specific regions of the biosynthetic loci in all the isolates except for two. Seventy five per cent of the isolates were of capsular type A, 23 per cent were capsular serotype D, 1 per cent were of capsular type F and 1 per cent were untypeable. (Davies *et al.*, 2003 a).

Dutta *et al.* (2004) tested a total of 32 *P. 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, expect one, all the isolates were confirmed as type–B *P. multocida* by conventional serotyping and HS-B PCR assay. They concluded that it was very imperative to adopt latest techniques like PCR for quick diagnosis since the conventional isolation and serotyping facilities in India were inadequate.

Use of template DNA prepared from blood smears and blood samples for performing PM-PCR greatly reduced the time required for a specific diagnosis (Antony, 2004 and Karunakaran, 2004).

Karunakaran (2004) used PM-PCR assay in the detection of *P. multocida* directly from clinical specimens like nasal and pharyngeal swabs and lung samples and reported that band intensity in the amplified product was less.

Study conducted by Jaglic *et al*. (2005) showed that among 107 isolates identified as *P. multocida*, three capsular serogroups A, D, and F were found by capsular PCR typing. Isolates belonging to the serogroup A had the highest incidence (n = 74) and they were the most common in each of the host (pigs, calves, rabbits, chickens, cat, manul and human). Twenty five isolates were classified as members of the serogroup D and they were found in pigs, calves, and rabbits. The serogroup F isolates (n = 8) were found in rabbits only.

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

Townsend *et al.* (1997) observed a high degree of homogeneity among 38 isolates of *P multocida* of B and E serogroups in REP-PCR profiles. They opined that since REP-PCR profiles of other *P.multocida* serotypes were highly variable, this technique could be suitable for molecular finger printing of isolates from fowl cholera or atrophic rhinitis.

Townsend *et al.* (2000) characterized *P.multocida* from the tonsils of slaughtered pigs in Vietnam by REP-PCR and biotyping, which revealed 9 distinct REP profiles and 7 biotypes among the 16 PM-PCR positive isolates. They identified some correlation between the isolates from a previous Australian outbreak of acute swine pasteurellosis and those isolated from fowl cholera outbreaks in Vietnamese poultry, thus indicating the transmission of the organism between pigs and poultry.

Repetitive Extragenic Palindromic elements are present in a wide range of enteric bacteria and are distributed widely across the genome. So the amplification of *P. multocida* genomic DNA fragments by outwardly directed primers based on the REP sequences generated complex profiles with multiplicity of bands (Blackall and Miflin, 2000).

Gunawardana *et al*. (2000) analyzed 95 isolates of *P. multocida* by Pulsed Field Gel Electrophoresis (PFGE). REP-PCR profiles of representative isolates from PFGE classifications yielded 21 profiles, with most of the subgroups in accordance with PFGE analysis. They suggested that the ease and rapidity of REP-PCR while maintaining a high level of differentiation, supported the use of REP-PCR as a competent alternative to the more labour intensive PFGE system for strain identification and epidemiological studies of avian *P. multocida*.

Amonsin *et al.* (2002) used REP-RCR and Amplified Fragment Length Polymorphism (AFLP) to characterize 43 field isolates and 4 attenuated vaccine strains of *P.multocida* recovered from multiple avian species. They found that both REP-PCR and AFLP assays were rapid and reproducible, with high indices of discrimination.

Chen *et al*. (2002) were able to distinguish between *P.multocida subsp multocida* and *P.multocida subsp septica* with REP-PCR. According to their study REP-PCR could detect differences in the entire genomic DNA by virtue of the various locations of the repetitive extragenic sequences.

Saxena *et al*. (2006) characterized 67 *P.multocida* isolates from different animal species using REP-PCR and observed that somatic and antigenic typing of the isolates did not reveal any correlation with REP-PCR profiles.

Karunakaran *et al.* (2009) characterized five isolates of *P. multocida* obtained from cases of HS using (REP-PCR). All the isolates obtained were confirmed as serotype B *P. multocida* using serotype-specific (HS-B) PCR. The five isolates gave a similar a similar REP-PCR profile, indicating a high degree of homogeneity among them.

2.9.3 Isolation of Genomic DNA from Bacterial Cultures

Harel *et al.* (1990) used lysozyme and sodium dodecyl sulphate (SDS) on bacterial suspension, followed by elimination of protein using proteinase K. Pure DNA was extracted from the supernatants using phenol: chloroform extractions.

Kim and Nagaraja (1990) carried out phenol: chloroform extraction procedures for the separation of genomic DNA from *P. multocida* isolated from turkeys. They used Triton X-100 instead of SDS. Ribonuclease (RNase) was added to eliminate ribonucleic acid.

Carpenter *et al.* (1991) lysed bacterial cells with SDS, proteins and other cellular debris were removed by digestion with proteinase K, precipitated with

hexadecyltrimethyl ammonium bromide (CTAB) and DNA was precipitated with isopropanol .

Wilson *et al.* (1992) used lysozyme and SDS for the lysis of frozen bacterial pellets. This was followed by Ribonuclease (RNase) treatment and proteins were removed by addition of proteinase K. The DNA was extracted by phenol: chloroform treatment and precipitated with ethanol.

Zhao *et al.* (1992) suspended the cell extract of *P. multocida* in Tris-EDTA sucrose containing lysozyme. Proteinase K, sarkosyl and ammonium acetate were added sequentially and proteinase K was inactivated by phenyl methyl sulphonyl fluoride. The DNA was precipitated with cold absolute ethanol.

Christensen *et al.* (1993) introduced a simple technique for isolation of genomic DNA from bacterial cultures. In this method, bacterial cells were lysed with SDS at 56°C for 30 min. The cell proteins were precipitated by addition of 7.5 M ammonium acetate. The DNA was extracted by phenol: chloroform treatment and precipitated with isopropanol. This technique was adopted by several workers (Dziva *et al.*, 2001 and Muhairwa *et al.*, 2001).

Morishita *et al.* (1996 a) removed proteins and other cellular debris by digestion with proteinase K and precipitation with CTAB and DNA was precipitated with isopropanol. The concentration of DNA was determined using a DNA flurometer.

Dutta *et al.* (2003) used SDS and proteinase K to lyse bacterial cells and digest the proteins. The cell wall debris, polysaccharides and denatured proteins were eliminated by precipitation with 5 M NaCl and CTAB.

2.9.4 Restriction Endonuclease Analysis

Restriction endonuclease analysis (REA) has proved to be a valuable component of epidemiologic studies, particularly in investigations of outbreaks of pasteurellosis. This method is a highly reproducible technique that is not

influenced by inconsistent expression of phenotypic traits that limit the sensitivity and specificity of conventional typing methods (Snipes *et al*., 1989).

Kim and Nagaraja (1990) documented the existence of genotypic differences among *P. multocida* strains of turkey origin, belonging to the same serotype using restriction endonucleases *Hind* III, *EcoR*1 and *Bgl* II.

Snipes *et al.* (1990) got eight different restriction patterns using restriction enzyme *Sma*1 among 55 strains of *P. multocida* serotype 3, 4 recovered from turkey.

A study conducted by Christiansen *et al.* (1992) on the outbreaks of fowl cholera in turkeys on three premises revealed that strains of the organism were found to be enzootic on two premises. Their findings were based on REA of chromosomal DNA using *Sma* 1 and *Xho*1, serology and plasmid profiles.

Wilson *et al.* (1992) recognized a unique DNA fingerprint profile for each of the 16 reference somatic serotypes of *P. multocida*, with *Hha*I restriction endonuclease. They also found that all 13 serogroup E isolates had identical DNA fingerprint profiles when *Hha*I endonuclease was used.

Zhao *et al.* (1992) characterized 164 clinical isolates of *Pasteurella multocida* recovered from two swine herds in Minnesota by restriction endonuclease analysis and rRNA gene restriction fragment length patterns. Four different REA patterns were observed among the 156 serotype A strains isolated from herds A and B. The two most common REA types (1 and 2) represented 92 per cent of the strains analyzed, while REA types 3 and 4 were observed only in lung samples and accounted for 8 per cent of the isolates. They concluded that these genomic fingerprinting techniques were highly discriminatory and that capsular serotyping in combination with REA or ribotyping was an appropriate technique for epidemiological studies of *P. multocida* of swine origin.

Wilson *et.al* (1993) compared the DNA fingerprint profiles and serotypes of 63 avian *P. multocida* field isolates, 13 attenuated vaccine isolates (propagated from vaccines manufactured by five companies), and 16 somatic reference strains.They concluded that problems encountered in the *P. multocida* serotyping system did not affect DNA fingerprinting. Deoxyribonucleic acid fingerprinting demonstrated similarities or differences in isolates that could not be distinguished by serotyping, and it permitted precise identification of *P. multocida*.

Gardner *et al*. (1994) evaluated the use of REA, ribotyping, and plasmid analysis for fingerprinting of *P. multocida* strains from the nasal cavities of pigs and to use the techniques to elucidate the epidemiology of progressive atrophic rhinitis outbreaks in Australian swine. They concluded that there was evidence of genomic heterogeneity among strains of the same phenotype isolated from different herds and also variation within herds. Whole cell REA with *Sma*I provided a reliable method for further discrimination among different phenotypes of *P. multocida* isolated from the nasal cavities of swine and for the investigation of outbreaks of progressive atrophic rhinitis. The data obtained indicated that the REA provided better discrimination of isolates than did ribotyping and avoided the need to use a radioactive probe.

All the 314 isolates of *P. multocida*, serotype A:1, isolated from wild birds in North America showed a single REA profile with *Hha* I. However, *Hpa* II generated three profiles in the same isolates. (Wilson *et al.*, 1995).

Christensen *et al.* (1998) proved that two outbreaks of fowl cholera in the avifauna of Denmark were caused by the same clone of *P. multocida* sub sp. *multocida*, using enzymes *Hpa* II and *Hha* I.

According to Blackall *et al.* (2000) molecular typing methods of REA and ribotyping were superior to biochemical characterization for epidemiological investigation of porcine pasteurellosis.

Restriction enzymes *Hpa* II (C/CGG) and *Hha* I (GCG/C) cut the genome of a bacterium such as *P. multocida*, which had a relatively low G + C content, less frequently than an enzyme whose recognition sequence contained only adenine (A) and thymine (T). (Blackall and Miflin, 2000).

Rubies *et al.* (2002) used REA and plasmid analysis to study the epidemiology of *P.multocida* in a swine pyramid structure. The enzyme *Hpa*II was used to cleave the extracted DNA. They got 17 REA patterns from 218 isolates, with one clearly predominant pattern. They concluded that REA and plasmid analysis were good epidemiological tools for identifying different strains of *P.multocida* with same phenotype.

Eleven *P. multocida* strains of different serotypes were characterized by REA using 11 different restriction endonucleases out of which unique restriction enzyme profiles were generated by digestion with *Hpa* II and *Hha* I and *Hpa*II gave a finer discrimination than *Hha*I (Dutta *et al.,* 2003).

Pedersen *et al*. (2003) distinguished a total of ten REA patterns among 68 isolates of eiders and gulls. Isolates that were indistinguishable with PFGE were also indistinguishable with REA, and those that were identical in PFGE were also identical with REA. However, a few isolates that were identical with REA could be differentiated with PFGE. Thus, REA was less discriminatory than PGFE.

Twenty seven avian isolates of *P. multocida* serogroup A, were analysed by REA using *Hha* I and *Hpa* II. Each of these enzymes yielded three different profiles and were equally discriminatory (Antony, 2004).

Munir *et al.* (2006) used the REA technique for the comparison of field isolates and vaccinal strain of *P. multocida* using *Hha-I* enzyme. Data obtained in their study provided clear evidence that eight isolates of *P. multocida* used were serologically different but genotypically were the same, with the restriction enzyme used.

Shivachandra *et al.* (2006) typed *P.multocida* from different avian species belonging to different geographical regions of India by REA using *Hha* I and *Hpa* II and AFLP. The study indicated that REA using *Hpa* II was a simple and resource efficient method.

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 and reagents used were of molecular biology grade, obtained from M/s Sigma-Aldrich, Bangalore Genei and Sisco Research Laboratories (SRL) Private Limited, unless otherwise mentioned. Sterile swabs were procured from Hi-media Laboratories Private Limited, Mumbai.

3.1 ISOLATION OF *Pasteurella multocida*

3.1.1 Materials

3.1.1.1 Collection of Samples

The samples were collected from apparently healthy animals and birds, cases of field outbreaks, birds and animals (ailing/dead) brought to the Veterinary Microbiology and Pathology departments for disease diagnosis and post mortem examination.

3.1.1.2 Media for Isolation of Pasteurella

Brain heart infusion agar (BHIA) or Tryptone Soya Agar (TSA) supplemented with sterile defibrinated ovine/bovine blood at five per cent level was used for isolation.

3.1.1.3 Reference Strains of Pasteurella multocida

Reference strain (P_{52}) obtained from Indian Veterinary Research Institute, Izatnagar, and the isolate of *Pasteurella multocida* (DP1- obtained from duck) belonging to serotype A and maintained in the Department of Veterinary Microbiology were employed in this study. The purity of reference strains were checked as per Barrow and Feltham (1993).

3.1.2 Method

3.1.2.1 Isolation of *Pasteurella multocida*

Samples of heart blood, lung, liver and spleen collected from the dead animals and sacrificed/dead birds suspected to be suffering from pasteurellosis were streaked on to blood agar plates and incubated at 37°C for 24 - 48 h in a candle jar. Heart blood smears and impression smears from liver, spleen and lungs were also prepared.

3.1.2.2 Nasal and Pharyngeal Swabs

Swabs collected were streaked onto blood agar and incubated at 37°C overnight in a candle jar.

3.1.2.3 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.

3.2 IDENTIFICATION

The bacterial isolates were identified based on morphology, cultural characteristics, biochemical characteristics and sugar fermentation tests as described by Barrow and Feltham (1993). The blood and tissue impression smears prepared were stained by Leishman's staining technique and examined under oil immersion objective of the microscope for the presence of bipolar organisms.

3.2.1 Biotyping

3.2.1.1 Method

The discs of the sugars to be tested such as arabinose, dulcitol, sorbitol, trehalose and xylose were added to five milliliters of Andrade's peptone water taken in a test tube and it was 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.

3.2.2 Antibiogram

3.2.2.1 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 (mcg) or international units (IU) per disc, were used (Hi-media Laboratories Private Limited, Mumbai, India).

1. Ampicillin (10 mcg)
2. Amoxycillin (10mcg)
3. Penicillin G (10 units)
4. Enrofloxacin (10mcg)
5. Cephotaxime (10mcg)
6. Gentamicin (30mcg)
7. Streptomycin(10mcg)
8. Tetracycline(30mcg)
9. Metronidazole (5mcg)
10. Nitrofurantoin (300mcg)
11. Chloramphenicol (30mcg)
12. Co-trimoxazole (25mcg)
13. Sulphadiazine (100mcg)
14. Erythromycin (10mcg)

15. Norfloxacin (10mcg)

3.2.2.2 Method

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

3.2.3 Pathogenicity Testing of Isolates

3.2.3.1 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.

3.2.3.2 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. 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.

3.3 STORAGE OF ISOLATES

3.3.1 Materials

3.3.1.1 Defibrinated Blood

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

3.3.2 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.

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

3.4.1 Buffers and Reagents for PCR

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

Sodium chloride	80.00 g
Potassium chloride	2.00 g
Disodium hydrogen phosphate	11.33 g
Potassium dihydrogen 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 lbs pressure and stored at 4°C. The stock solution was diluted to 1x before use.

3.4.1.2 Primers

3.4.1.2a Primers for Pasteurella multocida Specific PCR (PM– PCR)

Species-specific primers, to detect *Pasteurella multocida* designed by Townsend *et al.* (1998) 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 Sigma Aldrich.

3.4.1.2b Primers for Multiplex PCR

Multiplex capsular PCR typing system for capsular serogrouping was carried out by the primer sets designed by Townsend *et al.* (2001). The sequences of the primers were as follows:

Serogroup A: CAPA-FWD: 5'-TGCCAAAATCGCAGTCAG-3'

CAPA-REV: 5'-TTGCCATCATTGTCAGTG-3'

Serogroup B: CAPB-FWD: 5'-CATTTATCCAAGCTCCACC-3'

CAPB-REV: 5'-GCCCGAGAGTTTCAATCC-3'

Serogroup D: CAPD-FWD: 5'-TTACAAAAGAAAGACTAGGAGCCC-3'

CAPD-REV: 5'-CATCTACCCACTCAACCATATCAG-3'

Serogroup F: CAPF-FWD: 5'-AATCGGAGAACGCAGAAATCAG-3'

CAPF-REV: 5'-TTCCGCCGTCAATTACTCTG-3'

The primers were custom synthesized by M/s Sigma Aldrich.

3.4.1.2c 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:

FWD: 5'- TGT GGC AAA GAA AAG CAC AG - 3'

REV: 5'- AAC CGC TCT GTC GTT AAT GG - 3'

The primers were custom synthesized by M/s Integrated DNA Technologies

3.4.1.2d 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 sequences of the two primers were:

REP 1 5'- NNN NCG NCG NCA TCN GGC- 3' 18 mer

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

The primers were custom synthesized by Integrated DNA Technologies

3.4.1.3 PCR reaction buffer (10x)

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

3.4.1.4 Taq DNA polymerase

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

3.4.1.5 Deoxy ribonucleotide triphosphate

Deoxy ribonucleotide triphosphate (dNTP) mix

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

3.4.1.6 Magnesium chloride

Magnesium chloride with strength of 25 mM

3.4.1.7 DNA molecular size markers

3.4.1.7a 100base pair ladder (M/s Bangalore Genei (INDIA)).

3.4.1.7b O'Range ruler 500base pair ladder (M/s Fermentas Life Sciences)

3.4.1.7c λDNA/*EcoR I/Hind III* digest (M/s Fermentas Life Sciences)

With fragments of 21,226, 5148, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 base pairs

3.4.2 Method

3.4.2.1 Preparation of samples for PCR analysis

***3.4.2.1a* Preparation of template DNA from *P. multocida* cultures**

Polymerase Chain Reaction was conducted using bacterial culture lysates as template DNA. A pure colony of *P. multocida* was inoculated into 5 ml of BHI broth and incubated at 37°C for 18 h and centrifuged at 3000 x g for 10 min at 4°C. The pellet was washed twice in PBS and the 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 sample was then thawed and centrifuged at 3000 x g for 5 min at 4°C. The supernatant was stored at -20°C for further use as template DNA.

3.4.2.1b Blood sample 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 and centrifuged at 3000 x g for 15 min. The supernatant was discarded and cell pellet was washed twice with sterile PBS 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 -20ºC for further use as template for PCR reactions.

Blood smears were scraped with a blade into an eppendorf tube containing one point five milliliters of sterile distilled water and the mixture was kept at 37ºC for 30 min. It was then centrifuged at 3000 x g for 15 min, the cell pellet was washed twice with sterile PBS 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 ºC.

3.4.2.1c Nasal and pharyngeal swabs and lung samples

Nasal and pharyngeal swabs and representative portion of lung samples were inoculated into three milliliters of brain heart infusion broth and incubated at 37ºC for 3 h. The tubes containing inoculated broth were mixed thoroughly in a vortex mixer for three minutes and centrifuged at 3000 x g for 15 min. The pellet was washed twice with sterile PBS 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 –20ºC.

3.4.2.2 Setting up of PCR (test proper)

3.4.2.2a 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.* (1998).

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 p mol 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 5 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 at 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).

3.4.2.2b Multiplex PCR

Polymerase Chain Reaction (PCR) was carried out with five sets of primers *i.e*., PM specific , serogroup A-specific, serogroup B-specific serogroup D-specific and serogroup F- specific primer pairs simultaneously, as per method described by Townsend *et al*. (2001).

Polymerase chain reaction 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 (serogroup A-specific)	10µl
Reverse primer (serogroup A-specific)	10µl
Forward primer (serogroup B- specific)	10 µl
Reverse primer (serogroup B- specific)	10 µl
Forward primer (serogroup D-specific)	10µl
Reverse primer (serogroup D-specific)	10µl
Forward primer (serogroup F- specific)	10 µl
Reverse primer (serogroup F- 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 Serogroup A specific forward and reverse primers; Serogroup B specific forward and reverse primers ; Serogroup D specific forward and reverse primers; Serogroup F specific forward and reverse primers	
	First cycle	Next 29 cycles
Denaturation	95°C for 5 min	95°C for 30 sec
Annealing	55°C for 30 sec	55°C for 30 sec
Extension	72°C for 30 sec	72°C for 30 sec
		Final extension at 72°C for 5 min
Total number of cycles	30	

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

3.4.2.2c 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.

To each PCR tube 20 µl of master mix and 5 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:

	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 at 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).

3.4.2.2d 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 5 µl of template DNA were added. PCR amplification was carried out in an automated thermal cycler (Eppendorf, Master Cycler, Germany).

The programme of amplification was 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 at 72°C for 5 min
Total number of cycles	30	

3.5 DETECTION OF PCR PRODUCTS

3.5.1 Materials for Submarine Agarose Gel Electrophoresis

3.5.1.1 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.

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

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

Added triple distilled water to make one litre. Autoclaved at 121°C and 15 lbs pressure for 15min and stored at room temperature. The stock solution was adjusted to 1x before use.

3.5.1.3 Agarose Gel (1.5 per cent)

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

3.5.1.4 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.

3.5.1.5 Gel loading buffer (6x) (Genei), ready to use.

3.5.2 Method

The amplified PCR products of PM- 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 50ºC. 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 and samples were loaded along with DNA molecular size marker into respective slots carefully. Electrophoresis was carried out at 100volts 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.

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

3.6 RESTRICTION ENDONUCLEASE ANALYSIS OF GENOMIC DNA

3.6.1 Isolation of Genomic DNA

3.6.1.1 Materials

3.6.1.1a Tris EDTA (TE) *buffer*

Tris base	10 mM	1.2114 g
EDTA	1 mM	(2ml of 0.5M EDTA)

Dissolved in 900 ml distilled water and pH adjusted to 8.0. The volume was made up to 1000 ml. Filtered and autoclaved at 121°C and 15 lbs pressure for 15min in batches of 100 ml and stored at 4°C.

3.6.1.1b 3M Sodium acetate solution (pH 5.5)

Sodium acetate	40.824 g
Triple distilled water	70 ml

Adjusted the pH to 5.5, made up the volume to 100ml and autoclaved at 121°C and 15 lbs pressure for 15min and stored at 4^0 C.

3.6.1.1c Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Phenol 25ml
Chloroform 24ml
Isoamyl alcohol 1ml
Mixed and stored in amber coloured bottles

3.6.1.1d Chloroform: Isoamyl alcohol (24: 1)

Chloroform 24 ml
Isoamyl alcohol 1 ml

Mixed and stored in amber coloured bottles

3.6.1.1e Ethanol 70 per cent

Ethanol 70 ml

Distilled water 30 ml

Mixed and stored in amber coloured bottles

3.6.1.1f Ribonuclease A

Ribonuclease A 10 mg

Distilled water 1 ml

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

3.6.1.2 Method

Genomic DNA of all the isolates used in this study was extracted by the method of Wilson (1987), with slight modifications. About 2ml of 18 h broth culture was inoculated in 50ml of BHI broth and incubated at 37 ^{o}C, under constant shaking for 24 h.

The bacterial pellet was obtained by centrifugation at 5500 x g for 10min at 4˚C. Resulting pellet was washed twice in PBS and suspended in lysis buffer [2ml TE buffer, 300 µl of SDS (10 per cent W/V) and 5 µl of proteinase K]. It was mixed properly by rapid pipetting and kept at 37 ^{o}C for 1 h. The lysate was treated with 500 µl of 5M NaCl and 100 µl of cetyl trimethylammonium bromide (CTAB) (10 per cent W/V in 0.7M NaCl W/V) and the tubes were kept at 68 ^{o}C, in a water bath for 10min. Equal volume of chloroform: isoamyl alcohol were

added in each tube, mixed gently and then centrifuged at 8500 x g for10 min. The supernatant was collected in a separate tube and the DNA was precipitated by adding 1/10 volume of sodium acetate (3M) and double volume of chilled absolute ethanol. The tubes were kept at -20 ºC for 1 h and the DNA was spooled out with a sterile micropipette tip to another sterile tube. DNA was dissolved in 500 µl of sterile triple distilled water containing RNase A (20 µg/ml) and incubated at 37 ºC for 1 h. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added in each tube, mixed gently and centrifuged at 850 x g for 10 min. The upper aqueous phase was transferred to a fresh micro centrifuge tube and the DNA was precipitated as mentioned earlier. The DNA pellet was obtained by centrifugation at 18,800 x g for 20 min at 4˚C. Pellet was washed with 70 per cent ethanol, dried and resuspended in 100 µl of TE buffer and finally stored at -20 ºC in small aliquots. Purity of the DNA was checked by taking OD value at 260nm and 280nm.

3.6.2 Restriction Enzyme Analysis

3.6.2.1 Materials - Restriction Enzymes

Two restriction enzymes viz., *Hpa II* and *Hha I* were used in this study and were obtained from M/s Bangalore Genei Laboratories Private Limited, Bangalore and M/s Fermentas Life Sciences, respectively.

Hpa II (*Haemophilus parainfluenzae*) 5'C/CGG-3' 10 U/µl

10x Assay buffer L

Hha I (*Haemophilus haemolyticus*) 5'/GCG/C-3' 10 U/µl

10 x Buffer Tango

3.6.2.2 Method

Method of Sambrook *et al.* (1989) was followed for restriction enzyme digestion of all DNA samples.

The restriction digestion mixture for *Hpa* II was prepared as follows:

DNA sample	: 5 µg
Restriction enzyme	: 1 µl (10 units)
10x RE assay buffer	: 2 µl
Sterile triple distilled water to	: 20 µl

The tube containing the reaction mixture was incubated at 37°C for 3 h and then the enzyme was inactivated by raising the temperature to 65°C for 30 min.

The restriction digestion mixture for *Hha* I was prepared in a similar manner except that the enzyme was inactivated by the addition of 0.5M EDTA, pH 8.0, to achieve a 20 mM final concentration.

3.6.3 Electrophoresis

The restricted DNA fragments were analysed by submarine agarose gel electrophoresis as detailed 3.5.2, except that 0.8 per cent agarose gel in 1 x TBE buffer stained with ethidium bromide was used. Four microlitres of 6x gel loading dye was mixed with the total volume of digested product and loaded in the wells. Electrophoresis was carried out at 2.5 v/cm till the bromophenol blue dye reached near the bottom of the gel. The gel was visualized under UV transilluminator and results were documented in a gel documentation system.

Results

4. RESULTS

4.1 ISOLATION OF *Pasteurella multocida*

Totally 284 number of samples were collected from different sources and processed for the isolation of *P. multocida*. The samples were collected from apparently healthy animals and birds, cases of field outbreaks, birds and animals (ailing/dead) brought to the Veterinary Microbiology and Pathology departments for disease diagnosis and post mortem examination. The details of the samples collected are summarized in table 1.

Biomaterials collected from the animals and birds were streaked on ovine/bovine blood agar. *Pasteurella multocida* could not be isolated from any of the nasal and pharyngeal swabs due to the overgrowth of contaminant organisms. Isolation was possible from liver, spleen, lungs, heart, heart blood and blood samples. The Petri plates were incubated at 37°C for 18-24 h in a candle jar. Following incubation, mucoid, convex, greyish-white and non-haemolytic colonies were obtained. A characteristic odour was observed when the Petri plates were opened following overnight incubation at 37°C.

The reference strain (P_{52}) of *P. multocida* obtained from IVRI, Izatnagar and *Pasteurella multocida* serotype A (DP1) maintained in the Department of Veterinary Microbiology were used for comparison in isolation and characterization studies.

From a total of 284 samples used for isolation trials, 20 samples yielded growth in pure form. The details of isolates are given in the table 2.

Table 1. Details of samples collected from different animals and birds

Type of sample	Number of samples collected	Species from which collected	Place of sample collection
Nasal swabs	51	Cattle, buffalo, pig and goat	a. University Livestock farm b. Osho Farm,Pattikkad c. University Veterinary hospital d. Livestock Research Station, Thumburmuzhi. e. Slaughter house, Kuriachira
Pharyngeal swabs	37	Cattle, buffalo, pig and goat	Slaughter house, Kuriachira
Post mortem samples	162	Cattle, pig, goat, rabbit, duck, chicken, quail, emu, turkey, guinea fowl and pigeon	a. Slaughter house, Kuriachira b. Department of Pathology c. Veterinary hospitals in Palakkad
Blood samples and Blood smears	34	Cattle, buffalo, goat, pig and rabbit	a. University Veterinary hospital b. Veterinary hospitals in Thrissur district c. Veterinary hospitals in Palakkad district
Total number of samples	284		

Table 2. Details of the isolates obtained from different animals and birds

Serial Number	Isolates	Species	Place
1	DP 36	Duck	Wayanad
2	DP 38	Duck	Thrissur
3	DP 39	Duck	Thrissur
4	DP 40	Duck	Thrissur
5	DP 41	Duck	Palakkad
6	DP 42	Duck	Thrissur
7	DP 43	Duck	Thrissur
8	DP 44	Duck	Thrissur
9	DP 45	Duck	Thrissur
10	DP 46	Duck	Thrissur
11	DP 47	Duck	Thrissur
12	DP 48	Duck	Ernakulam
13	DP 49	Duck	Thrissur
14	FP 3	Chicken	Thrissur
15	FP 4	Chicken	Thrissur
16	FP 5	Chicken	Thrissur
17	QP2	Quail	Palakkad
18	EP1	Emu	Palakkad
19	RP1	Rabbit	Thrissur
20	BP5	cattle	Thrissur

4.2 IDENTIFICATION OF THE ORGANISM

4.2.1 First Stage

All the twenty isolates and the reference strains P_{52} and DP1, were Gram-negative, capsulated, non-motile and coccobacillary. They grew aerobically and micro aerobically, 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).

4.2.2 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, ornithine decarboxylase positive and citrate utilization negative.

With regard to the fermentation of the sugars all isolates fermented glucose, galactose, mannose, mannitol and sucrose but could not utilize lactose, maltose and inositol. Salicin could be fermented only by isolates DP 38, 39, 40, 41, 47, 48, 49, FP4 and RP1 (Table 4). Results from the first and second stage biochemical tests revealed that the isolates were *P. multocida*.

4.3 BIOTYPING

Fermentation of sugars like arabinose, dulcitol, sorbitol, trehalose and xylose by the isolates were studied to biotype them. Variations in the fermentation patterns of these sugars allowed the recognition of eight distinct biochemical types, termed biovars A-H (Table 5). Based on the criteria followed by Mutters *et al.* (1985) three biotypes were observed among the twenty isolates of *P. multocida.* Isolate FP3 fermented both dulcitol and sorbitol hence was biotyped as *Pasteurella multocida* subsp *gallicida* and the isolates EP1 and RP1 did not ferment either sorbitol or dulcitol and hence were biotyped as *Pasteurella*

Table 3. First stage biochemical tests of isolates

Tests	DP36	DP38	DP39	DP40	DP41	DP42	DP43	DP44	DP45	DP46	DP1
Gram's reaction	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli
Presence of capsule	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-
Growth in air	+	+	+	+	+	+	+	+	+	+	+
Growth anaerobically	+	+	+	+	+	+	+	+	+	+	+
Growth on Mac Conkey's agar	-	-	-	-	-	-	-	-	-	-	-
Haemolysis on blood agar	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Oxidative/fermentative	F	F	F	F	F	F	F	F	F	F	F

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Tests	DP47	DP48	DP49	FP3	FP4	FP5	EP1	QP2	RP1	BP5	P52
Gram's reaction	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli	Cocco-bacilli
Presence of capsule	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-
Growth in air	+	+	+	+	+	+	+	+	+	+	+
Growth anaerobically	+	+	+	+	+	+	+	+	+	+	+
Growth on Mac Conkey's agar	-	-	-	-	-	-	-	-	-	-	-
Haemolysis on blood agar	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Oxidative/fermentative	F	F	F	F	F	F	F	F	F	F	F

Table 4. Second stage biochemical tests of isolates

Tests	DP36	DP38	DP39	DP40	DP41	DP42	DP43	DP44	DP45	DP46	DP1
Indole Production	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer Test	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-
Ornithine Decarboxylase	+	+	+	+	+	+	+	+	+	+	+
Sugar Fermentation											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	+	+	+	+	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	=
Dulcitol	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	-	-	-	-	-	-	+	-	+
Arabinose	-	+	+	+	+	+	+	+	+	+	+

Table 4. Continued

Tests	DP47	DP48	DP49	FP3	FP4	FP5	EP1	QP2	RP1	BP5	P52
Indole Production	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer Test	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-
Ornithine Decarboxylase	+	+	+	+	+	+	+	+	+	+	+
Sugar Fermentation											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	+
Lactose	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	-	+	-	-	-	+	-	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	+	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	-	+	-	+	+
Trehalose	-	+	-	+	-	-	-	-	-	-	-
Xylose	+	-	-	-	-	-	+	-	-	+	+
Arabinose	-	+	+	+	+	+	-	-	-	-	-

Table 5. Biochemical differentiation of isolates of *P.multocida*

Sugars	Biovar A	Biovar B	Biovar C	Biovar D	Biovar E	Biovar F	Biovar G	Biovar H
Arabinose	+	-	+	+	+	-	-	-
Dulcitol	-	-	-	-	+	-	-	-
Sorbitol	+	+	+	+	+	-	+	-
Trehalose	-	-	-	+	+	-	-	-
Xylose	+	+	-	-	-	+	-	-

Biovar A: isolates DP 38 and 45

Biovar B: isolates DP 36, 47 and BP5

Biovar C: isolates DP 39, 40, 41, 42, 43, 44, 46, 49, FP4 and FP5

Biovar D : isolate DP 48

Biovar E : isolate FP3

Biovar F : isolate EP1

Biovar G : isolate QP 2

Biovar H : isolate RP 1

multocida subsp *septica*. All other isolates fermented sorbitol, but not dulcitol and hence were biotyped as *Pasteurella multocida* subsp. *multocida*.

4.4 ANTIBIOGRAM

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

All isolates were sensitive to norfloxacin, gentamicin, cefotaxime, nitrofurantoin, erythromycin and chloramphenicol. Except FP4 all others were sensitive to tetracycline and except QP2 all others were sensitive to Co-trimoxazole. All were resistant to metronidazole and sulphadiazine. Antibiotic sensitivity pattern of all the isolates is presented in the Fig. 1.

4.5 PATHOGENICITY TESTING IN MICE

All the isolates of *P. multocida* 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. Re-isolation of *P. multocida* was done from the heart blood, lung, liver and spleen on ovine or bovine blood agar.

4.6 STORAGE OF ISOLATES

The *P. multocida* isolates, stored at -70^0C 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. They were found to be viable for the period of observation of six months.

Table 6. Antibiogram of Isolates

	ISOLATES																			
Antibiotics used	DP36	38	39	40	41	42	43	44	45	46	47	48	49	FP3	FP4	FP5	EP1	QP2	RP1	BP5
Ampicillin (10 mcg)	S	R	S	S	S	S	R	S	R	R	S	S	R	S	S	S	R	S	S	R
Amoxycillin (10 mcg)	R	S	S	S	S	S	S	S	R	R	S	R	S	S	R	R	S	S	S	S
Penicillin G (10 units)	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R
Enrofloxacin (10 mcg)	S	S	S	S	S	R	S	R	S	S	S	R	S	S	S	S	S	S	S	S
Cephotaxime (10 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin (30 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin (10 mcg)	R	S	S	R	S	R	S	R	R	S	R	R	S	S	S	R	R	R	R	R
Tetracycline (30 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
Metronidazole (5 mcg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Nitrofurantoin (300 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol(30mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Co-trimoxazole (25 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
Sulphadiazine (100 mcg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Erythromycin (10 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Norfloxacin (10 mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

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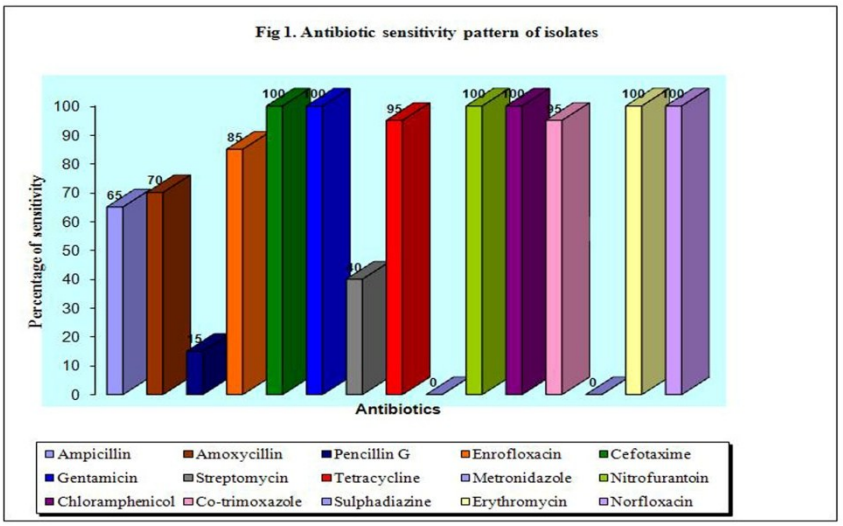
Fig 1. Antibiotic sensitivity pattern of isolates



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

4.7.1 *Pasteurella multocida* species specific PCR (PM-PCR) of culture lysates

All the twenty isolates of *P. multocida* obtained from cattle, rabbit, duck, chicken, emu and quail and the standard reference strain P_{52} and DP1, 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 (100 bp ladder) 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 twenty isolates as well as in the reference strain P_{52} (Fig.2). In the negative control, amplification product was not detected. Similar types of results were obtained with DNA extracts prepared from blood samples, blood smears and nasal swabs.

4.7.1.1Amplification of Pasteurella multocida DNA from Clinical Samples

4.7.1.1a 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 *P. 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) and results are presented in table 7.

4.7.1.1b Nasal and Pharyngeal Swabs and Lung samples

Nasal and pharyngeal swabs and lung samples were processed and amplified with primers KMT1SP6 and KMT1T7. Presence of *P. multocida* DNA in clinical samples was observed by the amplification of 460 bp fragment. In the

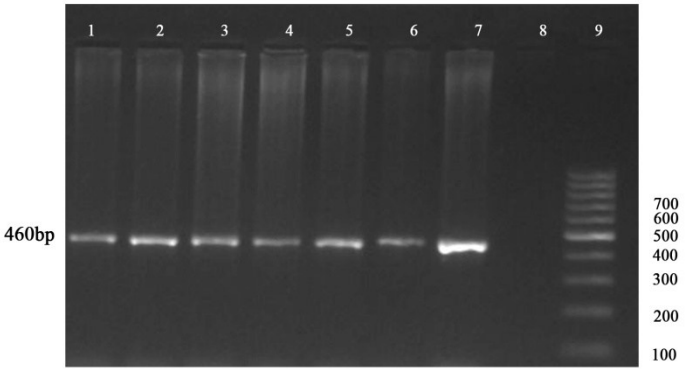


Fig 2. PM-PCR of culture lysates

Lane 1 P_{52}
Lane 2 DP 39
Lane 3 FP 4
Lane 4 EP 1
Lane 5 QP 2
Lane 6 RP 1
Lane 7 BP 5
Lane 8 Negative control
Lane 9 100bp ladder

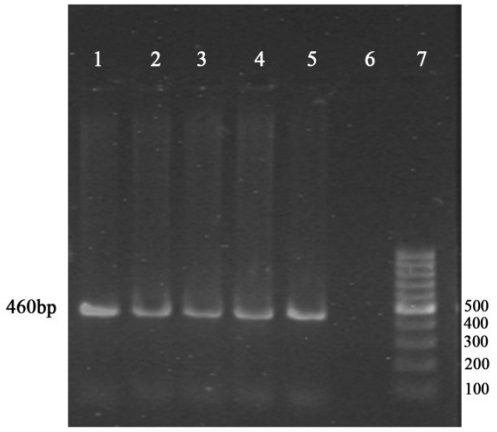


Fig 3. PM-PCR of clinical samples

Lane 1 P_{52}
Lane 2, 3 Blood samples
Lane 4 Blood smear
Lane 5 Nasal swab
Lane 6 Negative control
Lane 7 100bp ladder

Table 7. Results of clinical samples tested by PM-PCR

Sample	Polymerase chain reaction		
	Number tested	Number positive	Per cent positive
a. Blood samples	14	4	28.57
b. Blood smears	6	1	16.67
c. Nasal swabs	15	1	6.67
d. Pharyngeal swabs	9	Nil	--
e. Lung samples	31	Nil	--

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.

4.7.2 Multiplex PCR

Clinical samples that were found to be positive by PM-PCR were subjected to multiplex PCR, using PM– specific, Cap A, Cap B, Cap D and Cap F primer pairs with amplimer size 460 bp, 1044 bp, 760 bp, 657 bp and 851 bp respectively. *Pasteurella multocida* specific-PCR positive blood samples showed two bands each, corresponding to 460 bp and 1044 bp respectively (Fig.4). Multiplex PCR assay of all the positive isolates and DP1 also showed two bands corresponding to 460 bp and 1044 bp. Serogroup B reference strain P_{52} showed two bands each, corresponding to 460 bp and 760 bp respectively. Hence all the twenty isolates were grouped to serogroup A. (Fig.5).

4.7.3 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 samples found positive by PM-PCR when subjected to nested PCR, gave an amplified product of size 214 bp (Fig.6).

Since most of the clinical samples like nasal 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 *P. 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.7). The details of samples tested and the results are presented in table 8.

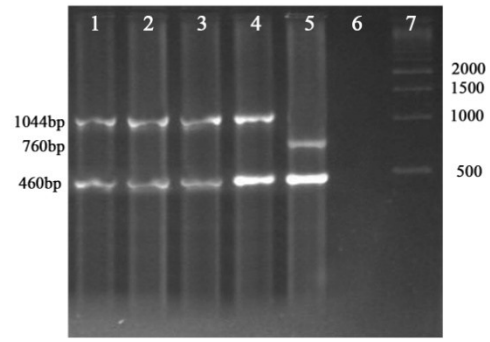


Fig 4. Multiplex PCR of clinical samples using cap primers

Lane 1 to 3 Blood samples
Lane 4 DP1
Lane 5 P_{52}
Lane 6 Negtive control
Lane 7 500bp ladder

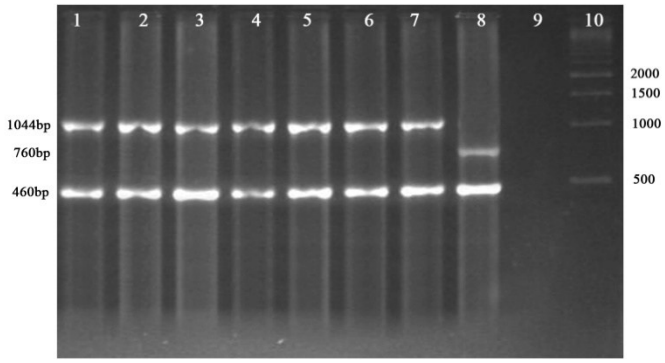


Fig 5. Multiplex PCR of culture lysates using cap primers

Lane 1 DP 39
Lane 2 FP 4
Lane 3 EP 1
Lane 4 QP 2
Lane 5 RP 1
Lane 6 BP 5
Lane 7 DP 1
Lane 8 P_{52}
Lane 9 Negative control
Lane 10 500bp ladder

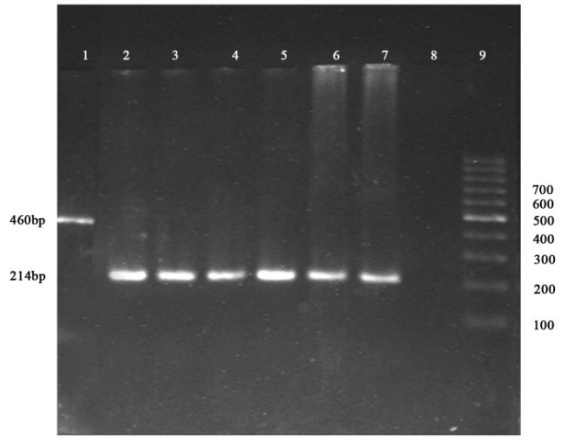


Fig 6. Nested PCR of culture lysates

Lane 1 P_{52} PM-PCR amplified product
Lane 2 DP 39
Lane 3 FP 4
Lane 4 EP 1
Lane 5 QP 2
Lane 6 RP 1
Lane 7 BP 5
Lane 8 Negative control
Lane 9 100bp ladder

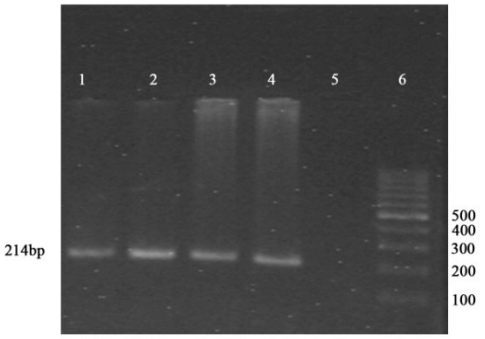


Fig 7. Nested PCR of clinical samples

Lane 1, 2 Blood samples
Lane 3 Nasal swab
Lane 4 Lung sample
Lane 5 Negative control
Lane 6 100bp ladder

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	26	26	Nil
	a. Positive isolates	20	20	Nil
	b. Blood samples and blood smears	5	5	Nil
	c. Nasal swab	1	1	Nil
Negative samples	1.Total nasal swabs	14	6	8
	2. Lung samples	31	24	7
	3. Total blood samples and blood smears	15	8	7

4.7.4 Repetitive Extragenic Palindromic Sequence PCR (REP-PCR)

All the 20 isolates of *P. multocida* as well as DP1, which were found to be positive by PM- PCR, were subjected to REP- PCR, using the primer pairs REP-1 and REP-2. Repetitive Extragenic Palindromic profiles showed amplicons ranging in size from 350 bp to 2.5 kbp. Only bands showing intense fluorescence were considered for analysis. *Pasteurella multocida* isolates demonstrated three distinct REP profiles. The isolates DP47, RP1 and BP5 with an extra band of the size about 500 bp were grouped into profile I. The isolate DP36 showed a unique profile with an extra band of approximate size 600 bp but it was lacking the 1000 bp size band which was present in all the other isolates and hence was grouped as profile II. All the other isolates formed profile III (figs 8 and 9), which lacked 500 and 600 bp bands but it contained all the other bands ranging from 350 bp to 2.5 kbp.

4.8 RESTRICTION ENDONUCLEASE ANALYSIS OF GENOMIC DNA.

The genomic DNA isolated from all the twenty isolates of *P. multocida* from different animals and birds were subjected to REA with restriction enzymes *Hpa* II and *Hha* I.

Four different banding patterns were observed among the 20 isolates of *P. multocida* when they were subjected to restriction enzyme analysis with *Hpa* II enzyme. Isolates DP 36, 38, 39, 40, 42, 44, 45, 46, 48, 49, FP3 and FP4 could be grouped into profile I. The isolates DP 41, 43, 47, FP5 and QP2 showed a common banding pattern hence were grouped to profile II. The isolates RP1 and BP5 formed profile III. Profile IV consisted of a single isolate *viz*., EP 1. (Fig. 10).

Restriction analysis of genomic DNA of all isolates with *Hha* I, yielded five profiles. Isolates DP 36, 38, 39, 40, 42, 44, 45, 46, 48, 49, FP 3 and FP4 were placed in profile I, QP 2 and EP1 both had unique profiles and were grouped into profile II and profile III respectively and profile IV included DP 41, 43, 47 and FP5. The rest of the isolates, *viz.,* RPI and BP5 were categorized as profile V (Fig. 11).

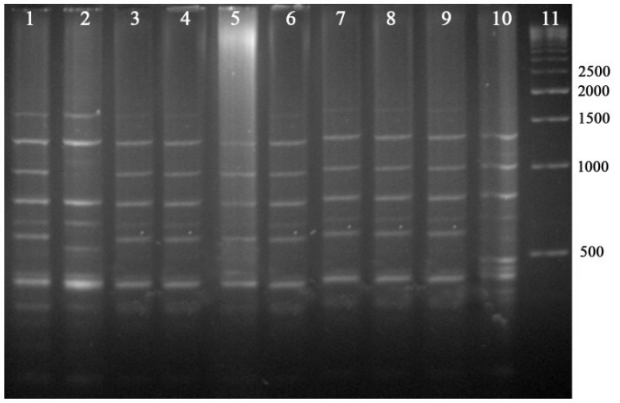


Fig 8. REP-PCR profiles of *Pasteurella multocida*

Lane 1 DP 38	Lane 4 DP 40	Lane 7 DP 43	Lane 10 DP 47
Lane 2 DP 36	Lane 5 DP 41	Lane 8 DP 44	Lane 11 500bp ladder
Lane 3 DP 39	Lane 6 DP 42	Lane 9 DP 45	

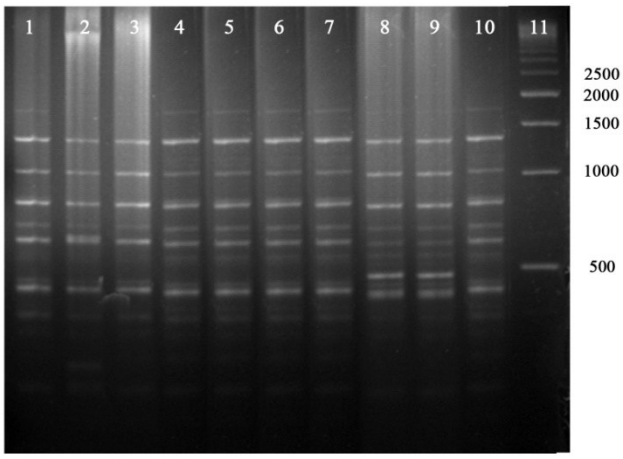


Fig 9. REP-PCR profiles of *Pasteurella multocida*

Lane 1 DP 46	Lane 4 FP 3	Lane 7 QP 2	Lane 10 EP 1
Lane 2 DP 48	Lane 5 FP 4	Lane 8 RP 1	Lane 11 500bp ladder
Lane 3 DP 49	Lane 6 FP 5	Lane 9 BP 5	

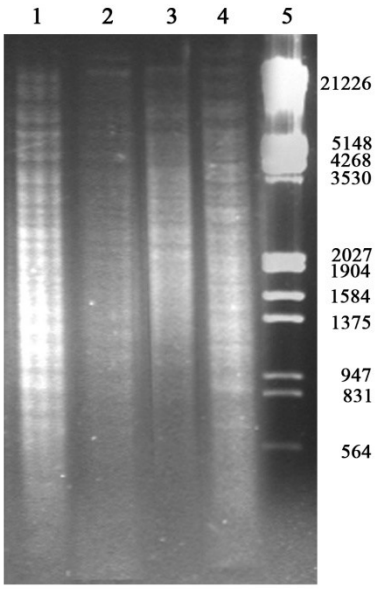


Fig 10 . REA profiles of genomic DNA of *Pasteurella multocida* digested with ***Hpa*** II

Lane 1 Profile I
Lane 2 Profile II
Lane 3 Profile III
Lane 4 Profile IV
Lane 5 Lambda DNA/ ***EcoR*** I/ ***Hind*** III Digest

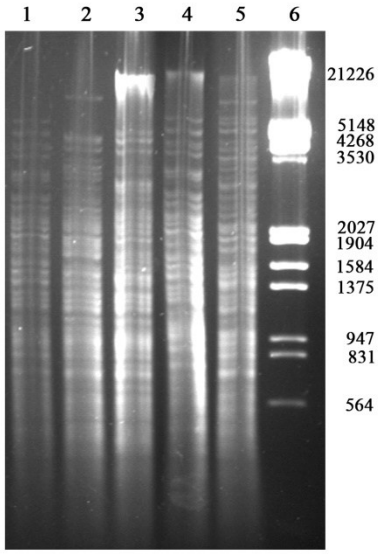


Fig 11 . REA profiles of genomic DNA of *Pasteurella multocida* digested with ***Hha*** I

Lane 1 Profile I
Lane 2 Profile II
Lane 3 Profile III
Lane 4 Profile IV
Lane 5 Profile V
Lane 6 Lambda DNA/ ***EcoR*** I/ ***Hind*** III Digest

Discussion

5. DISCUSSION

Pasteurella multocida is an important veterinary pathogen responsible for a variety of diseases in animals and birds, *viz*., haemorrhagic septicaemia and shipping fever in cattle, fowl cholera in chickens and turkeys, atrophic rhinitis in piglets and snuffles in rabbits. Thus it is responsible for heavy economic losses in a wide spectrum of hosts. Molecular techniques have now been developed both to identify bacterial types, and for further strain differentiation within serotypes. It has been possible to characterize isolates on a genotypic basis, providing a firmer and more stable basis than groupings based on phenotypic characteristics, for epidemiological studies. Phenotypical characterization of isolates of *P. multocida* provides insufficient information for epidemiological studies. Phenotypically similar isolates could be differentiated by using molecular methods and this is critically important in epidemiology to identify the vaccine strains. The present study was undertaken to elucidate the most prevalent genotypes associated with pasteurellosis, with a view to develop effective vaccines against the disease in animals and birds.

5.1 ISOLATION OF *Pasteurella multocida*

Isolation trials were carried out from specimens like tracheal, nasal and pharyngeal swabs, heart blood and tissues like liver, spleen and lungs from different species of animals and birds.

Primary isolation of the organism was done in five per cent bovine/ ovine blood agar. Rimler and Rhoades (1989) have suggested the use of bovine, equine or ovine blood in the media for isolation of *P. multocida*. Zhao *et al.* (1992) found five per cent ovine blood agar useful for primary isolation of *P. multocida* from swine, while Mehrotra and Bhargava (1999), found it useful for the

isolation of *P. multocida* from cattle, sheep and goats. Christensen *et al.* (1998) used bovine blood agar for the isolation of *P. multocida* from avian species.

The blood agar plates, streaked with the suspected material were incubated at 37°C 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), Rajalakshmi (2001), Antony (2004) and Karunakaran (2004).

5.2 IDENTIFICATION

All the isolates produced colonies that were mucoid, convex, greyish-white and non-haemolytic. There was a characteristic odour on opening the plates. The highly mucoid nature of the colonies might probably be due to the high amount of capsular material. Similar observations have been made by Mutters *et al.* (1989).

All the twenty isolates and the reference strains P_{52} and DP1, 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. These results are in agreement with those of Shigidi and Mustafa (1979), Kawamoto *et al.* (1990) and Mohan *et al*. (1994).

The second stage biochemical reactions used for characterization of *P. multocida* (Barrow and Feltham, 1993) were almost identical for the 20 isolates of *P. multocida*. Variations were observed only in the fermentation of the 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 fermented glucose, galactose, sucrose, mannose and mannitol. Rimler and Rhoades (1989) had identified the sugars such as glucose, mannose, galactose and fructose as those most commonly fermented by *P.*

multocida. Shigidi and Mustafa (1979) reported the fermentation of sugars such as xylose, glucose, fructose, galactose, mannose, sucrose and sorbitol. Other workers (Wijewardana *et al*.,1986; Blackall *et al.,* 1995 and Murthy and Kaushik, 1965) observed fementation of mannitol by *P. multocida.*

The twenty isolates in this study could not utilize lactose, maltose and inositol. Several workers have observed that none of the *P. multocida* isolates in their study fermented lactose (Murthy and Kaushik ,1965; Chandrasekaran *et al*.,1981; Wijewardana *et al*., 1986; Blackall *et al*., 1995; Kumar *et al*.,1996 and Shigidi and Mustafa ,1979) inositol (Heddleston *et al*.,1976; Kumar *et al*.,1996; Mohan *et al*.,1994; Chandrasekaran *et al*.,1981) and maltose (Chandrasekaran *et al*.,1981 and Kumar *et al*.,1996). Salicin could be fermented by only 45 per cent of isolates. Such variations in sugar fermentation had been reported by earlier workers also (Mohan *et al.*, 1994; and Antony, 2004).

Positive reactions to indole and ornithine decarboxylase have been described as the most useful biochemical indicators in the identification of *P. multocida* (OIE, 2008).

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

5.3 BIOTYPING.

Separation of isolates of *P. multocida* into subgroups or biotypes is based upon variation in biochemical characteristics. This subgrouping 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).

In the present study all the 20 isolates of *P. multocida* from different animals and birds could be grouped into eight biovars A-H (Table 5), based on the fermentation patterns of arabinose, dulcitol, sorbitol, xylose and trehalose.

Mohan *et al.* (1994) have grouped 60 Zimbabwean isolates of *P. multocida* form different host species into eight biovars based on fermentation patterns of dulcitol, mannitol, sorbitol and trehalose.

Mutters *et al*. (1985) divided *P. multocida* into three subspecies based on fermentation patterns of sorbitol and dulcitol. The sorbitol and dulcitol positive variety became *Pasteurella multocida* subsp. *gallicida*; those strains negative for both became *Pasteurella multocida* subsp. *septica* and those positive for sorbitol but negative for dulcitol were *Pasteurella multocida* subsp. *multocida*

Based on this the isolate FP3 which fermented both dulcitol and sorbitol was biotyped as *Pasteurella multocida* subsp *gallicida* and the isolates EP1 and RP1 which did not ferment either sorbitol or dulcitol and were biotyped as *Pasteurella multocida* subsp *septica.* All other isolates fermented sorbitol, but not dulcitol and hence were biotyped as *Pasteurella multocida* subsp. *multocida*.

In the present study 85 per cent of isolates tested could be classified as *P. multocida* subsp. *multocida*, based on the fact that they fermented sorbitol but not dulcitol. In several studies, investigators who have used sorbitol and dulcitol in biotyping *P. multocida*, have on an average found 82 per cent of the strains to be sorbitol-positive and dulcitol negative (Heddleston, 1976; Blackall *et al.*, 1995 and Muhairwa *et al.*, 2001).

5.4 ANTIBIOGRAM

All isolates were sensitive to norfloxacin, gentamicin, cefotaxime, nitrofurantoin, erythromycin and chloramphenicol. Ninty five per cent of isolates were sensitive to tetracycline and co-trimoxazole and 85 per cent was sensitive to enrofloxacin. Percentage of sensitivity to amoxicillin, ampicillin, streptomycin

and pencillinG were 70, 65, 40 and 15 per cent respectively. All of them were resistant to metronidazole and sulphadiazine. A 100 per cent resistance to metronidazole had been reported by earlier workers also (Balakrishnan 1998; Antony, 2004; Karunakaran, 2004). Similarly (Donahue and Olson, 1972) reported 100 per cent resistance to sulphadiazine.

Diallo *et al.* (1995) reported that all the strains of their study were resistant to streptomycin, lincomycin and trimethoprim and only one strain was resistant to tetracycline. The strains were susceptible to ampicillin, penicillin, gentamicin, erythromycin and nitrofurantoin. With regard to chloramphenicol, ampicillin and streptomycin similar results were obtained by Diker *et al*. (1994).

Zahoor *et al*. (2006) observed variable responses to different antimicrobial agents. All the six isolates of *P. multocida* were susceptible to chloramphenicol and ciprofolxacin and resistant to cephradine and streptomycin. Trimethoprim Sulpha, neomycin and nitrofurantoin gave variable results for all the isolates.

In the present study most of the isolates were sensitive to gentamicin, chloramphenicol, tetracycline, sulphonamide-trimethoprim combination, erythromycin, nitrofurantoin, penicillin, ampicillin and streptomycin. Several workers had reported similar results (De Alwis ,1984; Rammanath and Gopal, 1993 and Karunakaran, 2004).

Prescott and Yielding (1990) reported that *P. multocida* isolate from pig was highly susceptible to norfloxacin, enrofloxacin and ciprofloxacin. Most of the isolates of the present study also showed high degree of sensitivity to norfloxacin and enrofloxacin.

5.5 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.

Several workers have reported that *P. multocida* of bovine origin killed mice within 24 h post-inoculation (Kumar *et al*., 1996; Shivshankara *et al*., 2000; Sastry and Rajeswari, 2000; Goto *et al.,* 2001 and Anupama *et al*., 2003). 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.

5.6 STORAGE OF ISOLATES

The storage of *P. multocida* in defibrinated ovine/bovine blood at –70ºC 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 laborious, time consuming and requires sub culturing at more frequent intervals (Antony, 2004 and Karunakaran, 2004).

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

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

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

In the present study template DNA prepared from different sources were used for PCR amplification. These included purified genomic DNA, 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 compared to preparation of purified genomic DNA, 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 rapid detection of toxigenic *P. multocida* directly from clinical specimens like nasal swab using PCR. However 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 less. This might be due to very low number of organisms present in these samples. Several workers have reported similar findings (Pijoan *et al*., 1984; Amigot *et al*., 1998; Choi and Chae, 2001 and Karunakaran, 2004).

Use of template DNA prepared from blood smears and blood samples for performing PCR greatly reduces the time required for a specific diagnosis, although the band intensity was less. 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. Similar results were reported by Karunakaran (2004) and Antony (2004).

Blood samples 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 blood samples and smears. Hunt *et al.* (2000) have opined that modifications to sample preparations have allowed PCR analysis to be performed

on clinical specimens, dramatically reducing the time required for bacterial identification.

The observations made in the present study indicate that PM-PCR offers a rapid and specific method for diagnosis of pasteurellosis from animals and birds. Similar observations were made by Townsend *et al*. (1998); Townsend *et al*. (2000); Shivshankara *et al*. (2001) and Dutta *et al.* (2004).

5.7.2 Multiplex PCR

Multiplex PCR assay performed using PM– specific, Cap A, Cap B, Cap D and Cap F primer pairs generated products with two bands of approximately 460 bp and 1044 bp size. Only serotype A *P. multocida* isolates were observed to have these two bands. These results are in accordance with those of Townsend *et al*. (2001); Davies *et al.*, (2003) and Jaglic *et al*. (2005).

Townsend *et al*. (2001) reported multiplex capsular PCR typing system as a rapid and highly specific alternative to conventional capsular serotyping.

All the twenty isolates obtained from different animals and birds in this study were grouped to serotype A.

Isolates belonging to the serogroup A had the highest incidence (n = 74) and they were the most common in each of the host (pigs, calves, rabbits, chickens, cat, manul and human) as reported by Jaglic *et al*.(2005).

Kumar *et al*. (1996) reported the isolation of A:1, A:3, F:3, F:3, 4 from cattle and A:3 from buffalo for the first time in India.

Serotyping of avian *P. multocida* isolates from Australia and from Vietnam revealed that majority of them belonged to capsular type A (Gunawardana *et al*., 2000).

5.7.3 Nested PCR

Majority of the clinical samples like nasal swabs, lung samples, blood samples and blood smears when tested using *Pasteurella multocida* species specific primers KMT1SP6 and KTSP61 designed by Townsend *et al.* (1998), to detect *P. multocida* DNA, gave a negative result. So 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 well as from positive isolates, as indicated by the amplification of 214 bp fragment from within 460 bp region. The results of present study indicated that more than 50 per cent of the clinical samples previously found negative by PM-PCR gave positive results in nested PCR. But with a small proportion of samples screened, it is difficult to arrive at a definite conclusion. Karunakaran (2004) reported similar observations.

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). Calsamiglia *et al.* (1999) also reported 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. Hunt *et al.* (2000) opioned that additional primer set within the *tox* A gene for use in the nested PCR might enhance the sensitivity and specificity of the assay.

Karunakaran (2004) 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 the high sensitivity obtained needed further investigation before recommending the use of this PCR assay as a promising

diagnostic tool where conventional PM-PCR failed to detect *P. multocida.* The results obtained in the present study also agree with this.

5.7.4 REP–PCR

Repetitive Extragenic Palindromic elements are present in a wide range of enteric bacteria and are distributed widely across the genome. So the amplification of *P. multocida* genomic DNA fragments by outwardly directed primers based on the REP sequences generated complex profiles with multiplicity of bands (Blackall and Miflin, 2000). Analysis of the distribution of REP sequences in prokaryotic genome form the basis of a novel PCR based DNA finger printing technique known as REP-PCR.

In the present study all the 20 isolates of *P. multocida* from duck, chicken, emu, quail, rabbit and cattle generated 3 distinct profiles with minimal variation in the band pattern. The 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*., 1997).

All the isolates of the present study belonged to capsular serogroup A as evidenced by the multiplex PCR assay. Gunarwardana *et al.* (2000) reported that 22 isolates of *P. multocida* from chickens from Vietnam (majority of them were capsular type A) yielded 3 different profiles.

Saxena *et al*. (2006) observed that somatic and antigenic typing of the isolates did not reveal any correlation with REP-PCR profiles. Similar observations were made in the present study also. All the twenty isolates of the present study were of serogroup A and these could be grouped to 3 distinct REP-PCR profiles.

5.8 RESTRICTION ENDONUCLEASE ANALYSIS OF GENOMIC DNA

Restriction enzymes *Hpa* II (C/CGG) and *Hha* I (GCG/C) were used for the study of REA of genomic DNA. These enzymes were chosen since they cut

the genome of a bacterium such as *P. multocida*, which has a relatively low G + C content, less frequently than an enzyme whose recognition sequence that contains only adenine (A) and thymine (T) (Blackall and Miflin, 2000).

Among the several restriction enzymes used for DNA fingerprinting of *P. multocida* by REA, *Hpa* II and *Hha* I were found to be the most informative and yielded easily distinguishable profiles from a wide variety of serotypes (Wilson *et al.*, 1992; Diallo *et al.*, 1995 and Dutta *et al*., 2003).

Four different banding patterns were observed among the 20 isolates of *P. multocida* when they were subjected to restriction enzyme analysis with *Hpa* II enzyme. Isolates DP 36, 38, 39, 40, 42, 44, 45, 46, 48, 49, FP 3 and FP 4 could be grouped into profile I. The isolates DP 41, 43, 47, FP5 and QP2 showed a common banding pattern hence were grouped to profile II. Isolates RP1 and BP5 formed profile III and profile IV consisted of a single isolate *viz.*, EP 1. (Fig. 10).

Zhao *et al.* (1992) used restriction endonuclease *Hpa* II and observed four different REA patterns among the 156 serotype A strains of *P. multocida* isolated from swine. The present study also shows the high discriminatory power of *Hpa* II, yielding four distinct profiles among the 20 serogroup A isolates from different species.

Rubies *et al.* (2002) obtained 17 REA patterns from 218 isolates from swine in which one pattern was clearly predominant. The enzyme *Hpa*II was used to cleave the extracted DNA. They concluded that REA was a good epidemiological tool for identifying different strains of *P.multocida* with same phenotype.

Restriction analysis of genomic DNA of all isolates with *Hha* I, yielded five profiles. Isolates DP 36, 38, 39, 40, 42, 44, 45, 46, 48, 49, FP3 and FP4 were placed in profile I, QP2 and EP1 both had a unique profile were grouped in to profile II and Profile III respectively. Profile IV included DP 41, 43, 47 and FP5. While rest of the isolates, RPI and BP5 were categorized as profile V (Fig. 11).

Seventy-six avian isolates of *P. multocida*, belonging to 12 somatic serotypes, could be grouped into 28 DNA fingerprint profiles using restriction endonuclease *Hha* I (Wilson *et al.*, 1993).

All the 314 isolates of *P. multocida*, serotype A:1, isolated from wild birds in North America showed a single REA profile with *Hha* I. However, *Hpa* II generated three profiles in the same isolates. (Wilson *et al.*, 1995).

In another study, 27 avian isolates of *P. multocida* serogroup A, were analysed by REA using *Hha* I and *Hpa* II. Each of these enzymes yielded three different profiles and it was concluded that both the enzymes were equally discriminatory (Antony, 2004).

In the present study, the enzyme, *Hpa* II yielded four distinct profiles and *Hha* I yielded five distinct profiles from 20 isolates. The isolates of the profiles I, III and IV generated by *Hpa* II and the profiles I, V and III generated by *Hha* I were found to be the same. The enzyme *Hpa* II yielded a single profile (profile II) for the isolates DP 41, 43, 47, FP5 and QP2 whereas, *Hha* I yielded 2 separate profiles *viz.,* profile II (QP2) and profile IV (DP41, 43, 47 and FP5) and hence the results obtained from the present study indicated that *Hha* I had more discriminatory power than *Hpa* II.

Summary

6. SUMMARY

Pasteurellosis is an endemic disease in India. It has a very broad range of host spectrum affecting many domestic and wild animals and birds. Pasteurellosis produces high economic loss to the animal and poultry sector in the country. The key factors in prevention and control of pasteurellosis would be timely and correct reporting, accurate and rapid diagnosis and strategic use of the most effective vaccine. The present study was undertaken to isolate and identify *P. multocida* from different animals and birds, using conventional and molecular techniques and to determine the homogeneity/ heterogeneity between the isolates.

A total of 284 samples comprising of tracheal, nasal and pharyngeal swabs, heart blood and tissues like liver, spleen, heart and lungs were processed for isolation of *P. multocida.* This included 51 nasal swabs, 37 pharyngeal swabs, 162 post mortem samples and 34 blood samples and blood smears.

A total of 20 isolates were obtained from different species of animals and birds. This included 13 isolates from ducks (DP 36, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49), 3 from chicken (FP 3, 4, 5) and one each from quail (QP 2), emu (EP1), rabbit (RP1) and cattle (BP5). They have been characterized as *P. multocida* by morphological, cultural and biochemical tests. Reference strains of *P. multocida* P_{52} and DP1were used for comparison.

Based on the variation in fermentation patterns of arabinose, dulcitol, sorbitol, xylose and trehalose the 20 isolates could be grouped into eight biovars.

Three biotypes were observed among the twenty isolates of *P. multocida.* Isolate FP3 fermented both dulcitol and sorbitol hence was biotyped as *P. multocida* subsp *gallicida* and the isolates EP1 and RP1 did not ferment either sorbitol or dulcitol and hence were biotyped as *P. multocida* subsp *septica*. All

other isolates fermented sorbitol, but not dulcitol and hence were biotyped as *P. multocida* subsp. *multocida*.

All isolates were uniformly sensitive to norfloxacin, gentamicin, cefotaxime, nitrofurantoin, erythromycin and chloramphenicol. Except FP4 all others were sensitive to tetracycline and except QP2 all others were sensitive to Co-trimoxazole. All were resistant to metronidazole and sulphadiazine.

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

Storage of *P. multocida* in defibrinated ovine/bovine blood at –70^{o} 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 twenty isolates of *P. multocida* obtained from cattle, rabbit, duck, chicken, emu and quail and the standard reference strain P_{52} and DP1, when subjected to specific amplification by PM-PCR using species-specific primer pair KMT1SP6 and KMT1T7, generated an amplified product of size 460 bp.

All the isolates obtained were confirmed as serogroup A *P. multocida* when subjected to multiplex PCR, using PM– specific, Cap A, Cap B, Cap D and Cap F primer pairs. All the positive isolates showed two bands corresponding to 460 bp (PM specific) and 1044 bp (Serogroup A specific).

Pasteurella multocida could be detected in only 6 out of 75 clinical samples tested by species specific PCR (PM-PCR). This included one nasal swab and four blood samples and one blood smear. The entire samples tested positive by PM-PCR were confirmed as type-A *P. multocida* by multiplex PCR.

A nested PCR assay using the primer pairs designed by Primer 3 software, was used to detect *P. multocida* in clinical samples tested positive and negative by PM-PCR. When the PCR product (460 bp) of 20 isolates and 5 clinical

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 60 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.

All the 20 isolates of *P. multocida*, which were found to be positive by PM-PCR, were subjected to REP-PCR, using the primer pairs REP-1 and REP-2. *Pasteurella multocida* isolates demonstrated three distinct REP profiles. The isolates DP47, RP1 and BP5 with an extra band of the size about 500 bp were grouped into profile I. The isolate DP36 showed a unique profile with an extra band of approximate size 600 bp but it was lacking the 1000 bp size band which was present in all the other isolates and hence was grouped as profile II. All the other isolates formed profile III, which lacked 500 and 600 bp bands but it contained all the other bands ranging from 350 bp to 2.5 kbp. This indicated heterogeneity among the isolates.

The genomic DNA isolated from all the twenty isolates of *P. multocida* from different animals and birds were subjected to REA with restriction enzymes *Hpa* II and *Hha* I.

Four different banding patterns were observed among the 20 isolates of *P. multocida* when they were subjected to restriction enzyme analysis with *Hpa* II enzyme. Isolates DP 36, 38, 39, 40, 42, 44, 45, 46, 48, 49, FP3 and FP4 could be grouped into profile I. Isolates DP 41, 43, 47, FP5 and QP2 showed a common banding pattern hence were grouped to profile II. The isolates RP1 and BP5 formed profile III. Profile IV consisted of a single isolate *viz*., EP 1.

Restriction analysis of genomic DNA of all isolates with *Hha* I, yielded five profiles. Isolates DP 36, 38, 39, 40, 42, 44, 45, 46, 48, 49, FP 3 and FP4 were placed in profile I, QP 2 and EP1 each had a unique profile and were grouped in

to profile II and profile III respectively. Profile IV included DP 41, 43, 47 and FP5. The rest of the isolates, RPI and BP5 were categorized as profile V.

In conclusion, the results of the present study provide evidence for the ability of the PCR assays to provide rapid identification and serogrouping of *P. multocida* from both cultures and clinical samples. This technique could be implemented in regional laboratories to rapidly confirm a field diagnosis of pasteurellosis without the need to obtain pure cultures, perform extensive biochemical tests and serological determination. The different molecular techniques used in the present study showed genetic heterogeneity among the isolates of *P.multocida* serogroup A. The results suggested that there was no correlation between the results obtained in REP-PCR and REA. Also among the enzymes used in DNA fingerprinting of *P. multocida* isolates by REA, *Hha* I was found to have more discriminatory power. Since only lesser number of samples were used in the current study, a definite conclusion could not be drawn on this basis.

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ISOLATION AND CHARACTERIZATION OF *Pasteurella multocida* FROM ANIMALS AND BIRDS

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ABSTRACT

A total of 284 samples comprising of tracheal, nasal and pharyngeal swabs, heart blood and tissues like liver, spleen, heart and lungs were processed for isolation of *P. multocida.*

Twenty isolates were obtained from different species of animals and birds, which were characterized as *P. multocida* by morphological, cultural and biochemical tests. Reference strains of *P. multocida* P_{52} and DP1were used for comparison. All the isolates were found to be pathogenic for mice.

Based on the variation in fermentation patterns of arabinose, dulcitol, sorbitol, xylose and trehalose the 20 isolates could be grouped into eight biovars. Three biotypes *P. multocida* subsp *gallicida*, *P. multocida* subsp *septica* and *P. multocida* subsp. *multocida* were observed among the twenty isolates.

All isolates were uniformly sensitive to norfloxacin, gentamicin, cefotaxime, nitrofurantoin, erythromycin and chloramphenicol. All were resistant to metronidazole and sulphadiazine.

A species-specific PCR assay using primer pair KMT1SP6 and KMT1T7 was used to confirm the identity of the isolates. All the isolates obtained were confirmed as serogroup A *P. multocida* when subjected to multiplex PCR, using PM– specific, Cap A, Cap B, Cap D and Cap F primer pairs. *Pasteurella multocida* could be detected in only 6 out of 75 clinical samples tested by species specific PCR (PM-PCR). The entire samples tested positive by PM-PCR were confirmed as type-A *P. multocida* by multiplex PCR.

The PCR product (460 bp) of 20 isolates and 5 clinical samples amplified by using primer pairs KMT1SP6 and KMT1T7 when used for reamplification with nested PCR primers, a product of 214 bp size was observed. A high

proportion of the clinical samples previously found negative by PM-PCR gave positive results in nested PCR.

All the 20 isolates of *P. multocida*, which were found to be positive by PM-PCR, were subjected to REP-PCR, using the primer pairs REP-1 and REP-2. *Pasteurella multocida* isolates demonstrated 3 distinct REP profiles, indicating heterogeneity among the isolates.

The genomic DNA isolated from all the twenty isolates of *P. multocida* from different animals and birds were subjected to REA with restriction enzymes *Hpa* II and *Hha* I. Four different banding patterns were observed among the 20 isolates of *P. multocida* when they were subjected to REA with *Hpa* II, while five REA profiles were obtained with *Hha* I.

In conclusion, PCR assays could be used for the rapid identification and serogrouping of *P. multocida* from both cultures and clinical samples. The different molecular techniques used in the present study showed genetic heterogeneity among the isolates of *P.multocida* serogroup A. The results suggested that there was no correlation between the results obtained in REP-PCR and REA. Also among the enzymes used in DNA fingerprinting of *P. multocida* isolates by REA, *Hha* I was found to have more discriminatory power. Since only lesser number of samples were used in the current study, a definite conclusion could not be drawn in this basis.