MOLECULAR CHARACTERIZATION OF *Pasteurella multocida* ISOLATED FROM DUCKS IN KERALA

P.X.ANTONY

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

Doctor of Philosophy

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

2004

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR – 680 651

KERALA, INDIA

DECLARATION

I hereby declare that the thesis entitled **"MOLECULAR CHARACTERIZATION OF *Pasteurella multocida* ISOLATES FROM DUCKS IN KERALA"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy

6/08/04 **P.X. ANTONY**

CERTIFICATE

Certified that this thesis, entitled **"MOLECULAR CHARACTERIZATION OF *Pasteurella multocida* ISOLATES FROM DUCKS IN KERALA"** is a record of research work done independently by **P.X. Antony,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to him.

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

Mannuthy
6-8-04

CERTIFICATE

We, the undersigned, members of the Advisory Committee of **P.X. Antony**, a candidate for the degree of **Doctorate of Philosophy in Microbiology,** agree that the thesis entitled **"MOLECULAR CHARACTERIZATION OF *Pasteurella multocida* ISOLATES FROM DUCKS IN KERALA"** may be submitted by **P.X. Antony,** in partial fulfilment of the requirement for the degree.

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

Dr. V. Jayaprakasan,
Associate Professor and Head,
Department of Microbiology.
(Member)

Dr. M.Mini,
Assistant Professor,
Department of Microbiology
(Member)

Dr. Sisilamma George,
Associate Professor and Head
Department of Biochemistry
(Member)

Dr. T.V. Aravindakshan,
Assistant Professor,
Department of Animal Breeding and Genetics,
(Member)

EXTERNAL EXAMINER

ACKNOWLEDGEMENTS

Words are hard to find when the heart is too full to speak. I am at a loss of words to express my deep sense of gratitude to ***Dr. G. Krishnan Nair****, Associate Professor, Department of Microbiology and Chairman of the Advisory Committee for the able guidance and constant encouragement that helped bring out the best in me. I can never forget the wholehearted support rendered to me throughout the period of study. I hope and pray that this association would last a life time.*

It is my good fortune that ***Dr. V. Jayaprakasan****, Associate Professor and Head, Department of Microbiology is a member of the Advisory Committee. His thought provoking ideas, helped shape my research programme. I admire him for his leadership qualities and for his never say die attitude.*

Dr. M. Mini*, Assistant Professor, Department of Microbiology has always been a source of great help and encouragement. I am thankful to her for her keen interest in my work.*

I am indebted to ***Dr. Sisilamma George****, Associate Professor and Head, Department of Biochemistry, for her valuable guidance and critical suggestions and incessant encouragement. Her ability to trouble shoot problems is amazing. Her eagerness to help at every stage of my work is gratefully acknowledged.*

I am extremely privileged to be associated with ***Dr. T. V. Aravindakshan****, Assistant Professor, Department of Animal Genetics and Breeding. The myriad ways in which he helped me can never be forgotten. A scientist in every sense of the word, he shall always remain my inspiration.*

I place on record my sincere thanks to ***Dr. G. Butchaiah****, Dean, Rajiv Gandhi College of Veterinary and Animal Sciences, Pondicherry, for his generosity in granting me deputation and study leave for the doctoral programme*

and also for his keen interest in the progress of my work. I shall always remain grateful to you Sir for this great favour.

*I remember with gratitude the help and encouragement extended by **Dr. R. M. Pillai**, Professor and head, **Dr. Mukhopadhyay**, Associate Professor, Depart of Microbiology, Rajiv Gandhi College of Veterinary and Animal Sciences, Pondicherry.*

***Dr. Koshy John**, Assistant Professor, Department of Microbiology has always provided a helping hand. His gentle nature is inimitable.*

*I place on record my sincere thanks to **Dr. N. D. Nair** and **Dr. Vijayan** and all others at the Department of Pathology for their help and support.*

*The help rendered by **Smt..Sujatha**, Assistant Professor, Department of Statistics, is gratefully acknowledged.*

*I am grateful to the **Dean**, Faculty of Veterinary and Animal Sciences, for providing the facilities for the study.*

The faculty members of the COVAS, Mannuthy had always been a great source of help and encouragement. I fondly remember their help and concern at every stage of my work.

*I am indebted to **Dr. Chandrasekaran Nair**, Instrumentation Engineer, for his eagerness to help at all times. The dedication shown by him in the upkeep of the instruments is indeed commendable.*

*I thank **Dr. M.R. Saseendranath,** Associate Professor and Head, Department of Epidemiology and Preventive Medicine, for his concern and keen interest in the study.*

*This research work would not have been possible but for the constant help, advice and encouragement accorded to me by **Dr. Chintu Ravishankar**. He is a true friend in every sense of the word. I could count on him for anything. I guess the word help and Chintu are synonymous.*

I am indebted to **Dr .Dhinakraj**, Associate Professor, Department of Animal Biotechnology for his advice and encouragement.

I fondly remember the timely help rendered by **Drs. Binu, Elankumaran** and **Gurushanker**, my friends in USA. It's amazing how the Internet has not only made the world smaller but has brought people closer.

I am thankful to **Drs. Elaiyaraja, Sanjeetha, Manju Soman, Suja Rani, Sajitha** and **Deepa Surendran**, for the sheer joy of their company and for accepting me as one among them in spite of vast difference in age.

Without the unstinted co-operation of the **Drs. K.G. Biju, Saji Thomas, Rajeev**, **Sunilkumar**, **Mrs. Jayalakshmi** and **Miss. Sindhu Soman** SRFs of the AINWP on HS, this work would not have been possible. I thank them for their help and companionship.

I thank **Dr. Ranjan Prasad**, School of Biotechnology, Madurai Kamaraj University, Madurai, for his help and advice in the sequencing of the OmpH gene.

I am grateful to **Mrs. Smitha** and **Jolly** and **Miss Nisha** and **Sindhu**, SRFs at the Department of Animal Genetics and Breeding for their help and co-operation.

I have gained immensely from my interaction with **Drs. Arun, Muthu, Dipu, Josemi, Sunitha, Dhanya and Aparna** and I wish to thank them for the same.

The constant morale boosting words of **Dr. S. Bhaskaran**, Professor and Head, Central Training Institute, Mannuthy is gratefully acknowledged.

What a joy it is to interact with youngsters! I have benefited a lot from my interaction with my colleagues at the P.G. Hostel. Their friendly attitude has made my stay a truly memorable one. I thank you from the bottom of my heart for your kindness. The atmosphere at this hostel is indeed congenial for studies. Thanks to the pains taken by the hostel committee for maintaining high

standards. I thank my friends ***Drs. Sachin, Sethu, Jith, Cijo, Rahul, Arun, Rajeesh, Sujith, Pradeep, Philip, Laiju, Renjith, Ragavan, Jayanth*** *and* ***Ratish Babu*** *for the warmth of their friendship.*

I had the unique opportunity of being a colleague to my own students, ***Drs. Kantharaj, Bala, Prasana, Afsal and Prejith****. Theirs as well as the companionship and the help rendered by* ***Drs Sekar, Kowsigaraj, Sasi, Deepak Mathew, Suresh, Satha, Raja, Cyel, Anoop, Kalaiselvan, Yuvaraj, Giri, Hari, Jeri, Sivanesan, Rishi, Jega and Senthil*** *is gratefully acknowledged. They have made life enjoyable.*

I am thankful to the staff of the P.G. Hostel, ***Mr. Sathi, Mr. Paraman, Mr. Mohan***, ***Mr. Andrews*** *and* ***Reji*** *for making the stay and memorable..*

The help and co-operation extended by ***Ms Kumari, Mrs Lathika, Mrs Chandramathi, Mrs Leela*** *and* ***Mr Balu*** *is gratefully acknowledged.*

The love and affection showered on me by my parents and in laws have indeed been the elixir of life for me. I am at loss for words to thank them for their prayers and support.

I am deeply moved by the patience, love and unstinted support extended by my wife ***Nalini*** *and daughter* ***Anou****. Their morale boosting emails and phone calls have helped tide over many a dull moment.*

Without the help of you O ***Lord*** *nothing would have been possible. I bow before you and thank you for the innumerable blessing, which has helped me in this endeavour, and also for bringing me closer to you.*

P.X. Antony

CONTENTS

Sl. No.	Title	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	5
3	MATERIALS AND METHODS	49
4	RESULTS	87
5	DISCUSSION	104
6	SUMMARY	127
	REFERENCES	131
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No
1	First stage biochemical tests of isolates	89
2	Second stage biochemical tests of isolates	90
3	Antibiogram of isolates	92
4	Biochemical differentiation of isolates	94
5	Serotyping of isolates	96

LIST OF FGURES

Figure No	Title	Between pages
1	Epicardial haemorrhage	87-88
2	Enlarged liver with necrotic foci	87-88
3	Blood smear showing bipolar stained organisms (Leishman's stain 1000 x)	87-88
4	Liver impression smear showing bipolar stained organism (Leishman's stain 1000 x)	87-88
5	Pure culture of *P. multocida* (Gram's stain 1000 x)	93-94
6	Blood smear from experimentally infected mouse (Leishman's stain 1000 x)	93-94
7	Detection of *P. multocida* by PM-PCR	96-97
8	PCR-RFLP of 460 bp amplified PCR product digested with *Hae* III	97-98
9	REA profiles of genomic DNA of *P. multocida* digested with *Hpa* II	97-98
10	REA profiles of genomic DNA of *P. multocida* digested with *Hha* I	97-98
11	Plasmid profiles of *P. multocida*	98-99
12	REP-PCR profiles of *P. multocida*	98-99
13	Whole cell proteins profiles of *P. multocida*	99-100
14	Resolution of OMPs of *P. multocida* at different temperatures	99-100
15	SDS-PAGE analysis of OMPs of *P. multocida*	100-101
16	Immunoblots of *P. multocida*	100-101
17	Amplification of *OmpH* gene of *P. multocida*	101-102
18	REA of OmpH-PCR product of avian isolates with *Hinf* I	102-103
19	REA of OmpH-PCR product of avian isolates with *Dra* I	102-103
20	Restriction enzyme profiles of OmpH-PCR product (Serotypes A:1,A:3 & B:2) with *Hinf* I and *Dra* I	102-103
21	REA of OmpH-PCR products from inactivated FC vaccine	102-103
22	Sequence of *OmpH* gene of DP 1	103-104
23	Results of sequence alignment	103-104

Introduction

1. INTRODUCTION

The poultry sector plays an important role in the national economy and in the socio-economic development of the country. It also has a significant role in supplementing family incomes and generating gainful employment in rural sector, particularly among the landless, small and marginal farmers.

Ducks occupy the second position among the domesticated avian species. India has some inherent natural advantages for duck farming. A 5700 kilometer-long coastline and extensive water shed areas in several parts of the country offer excellent natural habitat for ducks. Moreover they are easy to rear, forage for most of their food and do not require expensive housing. Even then, duck farming in India has been a continual saga of neglect, though duck rearing is more economical than chicken. Duck population in India is around 24 million, which constitutes 10 per cent of the poultry population.

Kerala has a sizeable population of ducks, around 11.87 lakhs (Anon., 1996). Ducks are bred and reared on a large scale in Alappuzha, Thrissur, Kottyam and Pathanamthitta districts. The agro-climatic conditions prevailing in these areas provide a natural habitat, which is conducive for duck rearing, an important occupation of the rural and socially backward and deprived sections of our society.

Ducks, especially the indigenous varieties, are generally considered to be more hardy than chicken. However, disease outbreaks do appear in them from time to time. Severe mortality in ducks in the state has been reported since 1990. A study conducted by the department of Microbiology, as part of the ICAR scheme on “Development of Cell Culture Duck Plague vaccine for simultaneous vaccination with duck pasteurellosis”, has revealed that outbreaks were either due to simultaneous infection with duck plague virus (DPV) and Pasteurella or Pasteurella alone. During the year 2001-2002 there were disease outbreaks in

foraging ducks in different parts of the state. Most of these outbreaks have been attributed to pasteurellosis (Anon., 2001). Ducks in Kerala are regularly vaccinated against duck plague.

There are also reports of fowl cholera (FC) in the state, although the incidence is far less compared to duck pasteurellosis. Effective commercial vaccines are available for FC, but there is no information regarding the use of these vaccines in ducks. Hence, there is no systematic vaccination for pasteurellosis in ducks in the state and the disease continues to be a major threat to duck farming.

To treat a particular disease and to adopt effective preventive measures against the same, the causative agent has to be found out as quickly as possible. Avian pasteurellosis is caused by the bacterium *Pasteurella multocida (P. multocida)*. Conventional methods for detection of *P. multocida* rely on the detection of the organism by microscopy and its isolation and identification. These methods although confirmatory, are time consuming, often taking at least a week and are labour intensive.

Molecular approaches have allowed bacterial detection directly from clinical specimens, dramatically reducing the time required for identification. Since the initial development of the Polymerase Chain Reaction (PCR) in 1985, the basic principle of *in vitro* nucleic acid amplification through repetitive cycling has had extensive application in all aspects of fundamental and applied clinical sciences (Rapley *et al*., 1992). This nucleic-acid based technique enables rapid, sensitive and specific detection of micro-organisms. It could be used to obtain a rapid and confirmatory diagnosis of *P. multocida*, without the need to obtain pure cultures and conduct biochemical tests (Townsend *et al*., 1998a).

Pasteurella multocida is characterized serologically by capsular serotyping with passive haemagglutination test and somatic antigen type with gel diffusion precipitation test. Five capsular serotypes (designated A, B, D, E and F)

and 16 somatic serotypes (designated 1 to 16) have been identified. However in the past decade, it has been shown that Restriction Endonuclease Analysis (REA) of chromosomal DNA is more discriminatory than conventional serotyping in establishing clonality of strains of *P. multocida* (Wilson *et al*., 1995).

Plasmid profiling has also been used as an epidemiological tool for identifying different strains of *P. multocida* belonging to the same serotype (Rubies *et al*., 2002).

Porins are pore-forming outer membrane proteins (OMPs) of Gram-negative bacteria which are strong immunogens and have been demonstrated to be able to induce immunity in animal models against Gram-negative bacterial infections, thus making them attractive vaccine candidates. Zhang *et al*. (1994) have showed that polyclonal antibodies to a major outer membrane protein conferred immunity to chicks against avian pasteurellosis. Luo *et al*. (1997) showed that OmpH is a protective outer membrane protein of *P. multocida* X-73 (A:1 serotype isolated from domestic fowl) and protection studies indicated that it was able to induce homologous protection in chicken. They also isolated and sequenced the gene (*OmpH*) encoding OmpH of *P. multocida* of chicken origin. But no such work has been undertaken on the outer membrane proteins of *P. multocida* isolated from ducks.

In Kerala, so far very little work has been done on *P. multocida* isolated from ducks, especially with regard to its molecular characterization. Work has been carried out on the plasmid (Balakrishnan, 1998), nucleic acid and protein profile (Rajalakshmi, 2001) of avian isolates of *P. multocida*. However, no effort has been made to study the molecular characteristics of the duck and chicken isolates of *P. multocida* to establish the homogeneity/ heterogeneity amongst them.

Such a study would help in evolving better strategies to combat the disease, which continues to plague the duck farmers in the state, by the development of more effective vaccines.

Hence the present study was undertaken with the following objectives:

1. Rapid and confirmatory diagnosis of *P. multocida* isolates from ducks by PCR.

2. To study the molecular characteristics of *P. multocida* isolated from ducks in Kerala, by restriction endonuclease and plasmid profile analysis and comparison with fowl isolates.

3. To extract and identify the major antigenic fractions of OMPs of duck isolates of *P. multocida* and compare them with those of chicken by SDS-PAGE and Western blotting.

4. To amplify the outer membrane protein gene (*OmpH*) of duck isolates of *P. multocida* by PCR, using primers designed based on the published *OmpH* gene sequence of chicken isolates.

5. The amplified PCR products obtained from amplification of *OmpH* gene will be subjected to REA to detect heterogeneity/homogeneity between *OmpH* genes of chicken and duck isolates of *P. multocida.*

Review of Literature

2. REVIEW OF LITERATURE

Fowl cholera (avian pasteurellosis) is a septicaemic disease affecting both domestic and wild birds. The high mortality associated with this disease has resulted in significant economic losses to the poultry industry (Hansen and Hirsh, 1989). The severity of the disease and its incidence is influenced by environmental factors such as overcrowding, climate, nutrition and concurrent disease (Alberts and Graham, 1948).

Pasteurella multocida, the causative agent of fowl cholera (FC) is a Gram-negative facultative anaerobe (Holmes, 1998). *Pasteurella multocida* subsp. *multocida* is the most common cause of fowl cholera, although *P. multocida* subsp. *septica* and *gallicida* may cause fowl cholera to some extent. Carrier birds seem to play a major role in the transmission of the disease (Christensen and Bisgaard, 2000).

2.1 NOMENCLATURE

This organism had many names, beginning with *Micrococcus gallicidus* (Buchanan, *et al.*, 1966). For a while, isolates of *P. multocida* were named according to the animal from which they were isolated, i.e., *P. avicida* or *P. aviseptica, P. bovicida* or *P. boviseptica.* In 1929, it was suggested that all isolates be referred to as *P. septica* (Topley and Wilson, 1929). The name *P. multocida* proposed by Rosenbusch and Merchant (1939), has now been widely accepted and it is listed as the type species of the genus (Mannheim and Carter, 1984).

The genus found its place in the family *Pasteurellaceae* under section five (facultatively anaerobic Gram-negative bacilli) in the ninth edition of Bergey's Manual of Systematic Bacteriology (Mannheim, 1984).

Rimler and Rhoades (1989) considered *P. multocida* as the only name to represent this heterogenous species.

The binomial nomenclature *Pasteurella multocida* has been universally accepted as the name of the etiological agent of avian pasteurellosis (Holmes, 1998).

2.2 HISTORY

The disease was first studied by Chabert (1782) and Mailet (1836), who first used the term "fowl cholera". Lignieres (1900) used the term "avian pasteurellosis". Toussaint (1879) isolated the bacterium and proved it as the sole causative agent of the disease.

The organism was first isolated and grown in pure cultures in chicken broth by Pasteur (1880).

2.3 PREVALENCE

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed world-wide (OIE, 2000). A report concerning the international distribution of fowl cholera in 1986 indicated outbreaks in Africa, America, Asia, Europe and Oceania (FAO, 1987).

2.3.1 Prevalence of the Disease Outside India

Alberts and Graham (1948) reported losses of over 68 per cent within six days, in an outbreak of fowl cholera, in a flock of turkeys in USA.

Dougherty (1953) reported that FC in ducks was a serious problem in Long Island (USA), where it was diagnosed in 32 of the 68 commercial duck farms.

Fowl cholera was first recognized in Indonesia in 1972 and there had been many subsequent outbreaks in ducks (Mariana and Hirst, 2000).

Faddoul *et al.* (1967) recorded pasteurellosis in wild birds such as robins, starlings, common grackle and screech owls in and around Massachusetts, USA.

Glisson *et al.* (1989) reported an unusually high mortality due to FC in Japanese quails in Georgia.

Pasteurellosis in turkeys has been reported from several countries including Britain (Curtis, 1979), Australia (Blackall *et al.,* 1995) and Japan (Goto *et al.*, 2001).

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

The first outbreak of fowl cholera in Sudan was reported by El-Ghali *et al.* (1997).

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

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

Concurrent infections with *P. multocida* and *Ascaridia galli* on free ranging chicken in Denmark had been reported by Dahl *et al.* (2002).

Parveen *et al.* (2003) isolated *P. multocida* from a breeder flock in Lahore District in Pakistan.

2.3.2 Occurrence in India

In India, the incidence of fowl cholera in ducks has been reported as early as 1947 (Mulbagal *et al.,* 1972).

The disease has been reported from several parts of the country.

It occurred in Andhra Pradesh from time to time (Rao, 1964; Rajini *et al.*, 1995 and Devi *et al.*, 2000).

Panda *et al.* (1981) isolated *P. multocida* from the oedema fluid of wattles and heart blood from affected birds in an outbreak of FC in Central poultry breeding farm, Bhubaneswar.

An outbreak of FC in six-day-old chicks in an organized poultry farm in Parbhani, Maharashtra, was reported by Kulkarni *et al.* (1988).

Sambyal *et al.* (1988) isolated and characterized *P. multocida* from an outbreak of FC in ducks in Srinagar, Jammu and Kashmir.

Rammanath and Gopal (1993) reported the isolation of *P. multocida* from Khaki Campbell ducks in Karnataka State.

An outbreak of FC in Khaki Campbell ducks has been reported from Agartala (Bhaumik and Dutta, 1995). Murugkar and Ghosh (1995) investigated an outbreak of FC in ducks in Tripura, which caused the mortality of 360 birds.

Mortality rate of 40-50 per cent in Japanese quails was reported due to FC from Madras by Chandran *et al.* (1995).

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).

2.4 ISOLATION OF *P. multocida*

Pasteur (1880) was the first to isolate the organism in pure culture.

Curtis and Ollerhead (1981) used cotton wool swabs that were rotated in the cloacal slit of chicken and turkeys for isolation of *Pasteurella*.

Generally, the organism is easily isolated from tissues such as liver, spleen, lung and from the heart blood of birds which succumbed to the acute form

of the disease and from localized lesions in chronically affected birds (Rhoades and Rimler, 1989).

Waltman and Horne (1993) showed that no single organ was entirely satisfactory, but isolation from different organs was most likely to yield *P. multocida*.

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

2.4.1 Media used for Isolation

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

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

The nutritional requirement of *P. multocida* was more exacting at 37°C than at lower temperatures (Burrows and Gillett, 1966).

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) 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.*

Recovery of the organism from specimens that were grossly contaminated was achieved by the use of selective media.

Das (1958) developed a selective medium for isolation that had crystal violet and cobalt chloride as inhibitors of contaminants.

Carter (1967) found that even though media containing five per cent ovine or bovine blood were useful for routine isolation of *Pasteurella*, such media were not useful in identification of colony variants. Brucella agar containing two per cent haemolysed rabbit serum, tryptic soy blood agar and tryptose blood agar containing five per cent ovine or bovine blood were found useful for the purpose.

A transport semi-solid medium containing thioglycolic acid and methylene blue was employed by De Alwis (1973) for isolation of *Pasteurella* from animals several hours after their death.

Pasteurella multocida is a facultative anaerobe which grows best at temperatures of 35-37°C in air or air plus five per cent carbon dioxide (Carter, 1981). However, Smith and Phillips (1990) concluded that *P. multocida* grew best at an optimum temperature of 37°C.

Nafcillin was found to be useful for isolation of *P. multocida* from clinical specimens. (Neter and Dryja, 1981)

Knight *et al.* (1983) developed a selective medium termed as CGT medium for isolation of *P. multocida* from human specimens. The medium contained clindamycin, gentamicin, potassium tellurite and amphotericin-B in five per cent horse blood agar.

De Jong and Borst (1985) employed a selective medium for the isolation of *P. multocida* and *Bordetella bronchiseptica*. This medium contained tryptose soy agar, five per cent defibrinated ovine blood, gentamicin sulphate, potassium tellurite, amphotericin-B and bacitracin. It proved useful in the isolation of toxigenic strains of *P. multocida*.

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

Moore *et al.* (1994) proposed a new selective medium for isolation of avian *P. multocida,* containing Gentamicin, potassium tellurite and Amphotericin B. The medium was termed as *Pasteurella multocida* sensitive agar (PMSA).

Warner (1996) developed a transport enrichment medium (TEM), which consisted of brain heart infusion agar, incorporating amikacin, gentamicin, potassium tellurite and amphotericin B. This medium greatly enhanced the possibility of isolating *P. multocida* from field specimens.

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

A higher rate of isolation of *P. multocida* from avian alimentary tract was possible by using a new selective medium that contained, polymyxin B, crystal violet, thallous acetate, bacitracin and cycloheximide in ten per cent ovine blood dextrose starch agar (Lee *et al.*, 2000b).

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

2.5 IDENTIFICATION OF THE ORGANISM

2.5.1 Cultural Characteristics

Identification of *P. multocida* is done by observing the phenotypic characteristics of the isolate. These include colony morphology, staining reactions and biochemical tests. These characteristics form the basis for the taxonomy and identification of bacteria.

Mucoid type A *P. multocida* was identified employing hyaluronidase producing *Staphylococcus aureus* (Carter and Rundell, 1975).

Mutters *et al.* (1989) observed that the colonies of *P. multocida* on blood agar were indistinguishable from many other non mucoid *Pasteurellae*. They 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.

Pasteurella multocida grew fairly well on nutrient agar, typically forming circular colonies about 0.5-1 mm diameter after 24 h at 37°C. They were convex, amorphous, greyish yellow and translucent, with a smooth, glistening surface and an entire edge. The consistency was butyrous and easily emulsifiable (Holmes, 1998).

Recently isolated *P. multocida,* formed colonies that ranged from 1-3 mm in diameter after 18-24 h of incubation. They were usually discrete, circular, convex and butyraceous (OIE, 2000).

2.5.2 Biochemical Characteristics

The primary biochemical tests for identifying *P. multocida* are tests for catalase and oxidase and oxidative or fermentative utilization of glucose (Cowan, 1974). Growth on Mac Conkey's agar, haemolysis on blood agar and urease activity were also included as primary biochemical tests for identification of *P. multocida* by Buxton and Fraser (1977).

Isolates of *P. multocida* from fowl cholera outbreaks in turkeys from Georgia were characterized based on biochemical reactions, *in vitro* drug sensitivity and serology (Walser and Davis, 1975).

Curtis (1979) examined 102 avian isolates of *P. multocida,* based on biochemical characteristics and serology.

Fegan *et al.* (1995) characterized 110 isolates of *P. multocida* from poultry, based on biochemical parameters.

Phenotypic characterization of 150 isolates of *P. multocida* from Australian pigs, up to the subspecies level, was carried out by Blackall *et al.* (1997).

Isolation and characterization of *P. multocida* from ovine lungs were carried out by Das and Bhagwan (1997).

Differentiation of *P. multocida* from *Riemerella anatipestifer* is based on indole production, glucose and sucrose utilization and orinithine decarboxylase reaction; while the former is positive for all the three reactions, the latter gives a negative reaction for all three tests (OIE, 2000).

One hundred and seven *Pasteurella* isolates from porcine respiratory tract were characterized based on phenotypic criteria such as haemolysis on blood agar, urease, catalase and indole formation, as well as other fermentative activities (Kielstein *et al.*, 2001).

Studies conducted by Muhairwa *et al.* (2001b) revealed phenotypic similarities between chicken and duck isolates of *P. multocida*.

Papova and Tzvetkov (2002) characterized a *P. multocida* isolate from pheasants, based on phenotypic and biochemical properties.

2.5.3 Biotyping

Separation of isolates of *P. multocida* into subgroups or biotypes based upon variations in biochemical characteristics had been reported (Schneider, 1948).

P. multocida could be divided into three groups based on the differences in the ability of the strains to ferment xylose, arabinose and dulcitol (Dorsey, 1963).

Donahue and Olson (1972), while using the Dorsey's system of classification, found that 14 per cent of the turkey isolates could not be grouped, as they failed to ferment any of the three sugars.

Walser and Davis (1975) observed that 28 of the 30 isolates of *P. multocida* from turkey exhibited similar biochemical patterns.

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

Mohan *et al.* (1994) were of the opinion that reactions of the organisms with dulcitol and sorbitol should be treated as variable characters of *P. multocida,* rather than to be used to split the taxon into different sub species.

Twenty-two field isolates of *P. multocida* from turkeys were grouped into four distinct biochemical types called biovars. Three of these biovars matched the property of *P. multocida* subsp. *multocida* (Blackall *et al.*, 1995).

Seven different biochemical biovars were recognized among 150 isolates of *P. multocida* of porcine origin. Sixty seven per cent of these isolates were identified as belonging to *P. multocida* subsp. *multocida* (Blackall *et al.*, 1997).

Christensen *et al.* (1998) could biotype 45 isolates of *P. multocida* from two outbreaks of fowl cholera as *P. multocida* subsp. *multocida*.

2.6 ANTIBIOGRAM

Numerous reports on antimicrobial agents describe sensitivity and resistance of *P. multocida, in vitro,* as well as their effects on the organism and animals *in vivo*.

In vitro drug sensitivity of 181 isolates of *P. multocida* of turkey origin revealed that 96 per cent of the isolates were sensitive to chlortetracycline, chloromycetin, erythromycin, novobiocin, penicillin, polymyxin B, terramycin, tetracycline, furadantin, furacin and furoxone. Most isolates were resistant to sulphadiazine and bacitracin (Donahue and Olson, 1972).

Panda *et al.* (1981) while testing for the antibiotic sensitivity pattern of avian isolates of *P. multocida* found that all the isolates were highly sensitive to tetracycline.

Borkataki *et al.* (1987) opined that gentamicin and kanamycin were the most sensitive drugs for majority of the strains of *P. multocida*. The isolates were resistant to sulphadimidine, triple sulpha, neomycin and streptomycin.

Sambyal *et al.* (1988) reported that *P. multocida* isolates from ducks were highly sensitive to chloramphenicol, nitrofurantoin and tetracycline.

Dhillon *et al.* (1989) tested the antibiotic sensitivity pattern of thirty isolates of *P. multocida* from poultry and one from duck and found that all the isolates were sensitive to chlortetracycline, doxycycline, oxytetracycline, ampicillin, co-trimoxazole, nitrofurazone, chloramphenicol and polymyxin B. All of them were resistant to penicillin G, cloxacillin, bacitracin and triple sulpha.

All isolates of *P. multocida* from Japanese quails were found sensitive to sulfonamides and tetracycline (Glisson *et al.*, 1989).

Lee *et al.* (1991) reported that all strains of *P. multocida* of avian origin were resistant to sulfisoxazole, rifampin and bacitracin. They also concluded that

antibiotic resistance of these isolates had no correlation to the presence of plasmids.

P. 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).

Murugkar and Ghosh (1995) observed that *P. multocida* isolates from ducks in Tripura were highly sensitive to sulphadiazine, trimethoprim, erythromycin, ampicillin and gentamicin.

Balakrishnan (1998) found that antibiotic sensitivity patterns could help in differentiating strains of *P. multocida*. He showed most isolates were sensitive to oxytetracycline, pefloxacin and streptomycin.

Pefloxacin and gentamicin were found to be highly sensitive to *P. multocida* isolates from ducks and they were used for treatment successfully (Devi *et al.,* 2000).

Isolates of *P. multocida* from pheasants (*Phasianus colchicus*) were highly sensitive to thiamphenicol (Popova and Tzvetkov, 2002).

2.7 SEROTYPING OF *Pasteurella multocida*

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).

2.7.1 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.

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

2.7.2 Capsular Serogrouping

The system most commonly used for specific capsular serogrouping is based on passive haemagglutination of erythrocytes by capsule antigen (Carter, 1955). Five serogroups A, B, D, E and F have been reported in the Carter system (Carter, 1967). All but serogroup E have been isolated from avian hosts (OIE, 2000).

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

2.7.3 Somatic Serotyping

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

The determination of somatic serotypes was accomplished by agar gel immuno diffusion (AGID) test (Heddleston, 1962). Serotypes 1-16 have been reported. All but serotypes 8 and 13 have been isolated from avian hosts (OIE, 2000).

Of the 258 *P. multocida* isolates tested 33 were found to be type 1, 157 were type 3, 28 type 4, 4 type 5 and 4 type 6, using a gel precipitation test for somatic serotyping (Heddleston *et al.,* 1972).

Walser and Davis (1975) reported that 17 of the 30 isolates of *P. multocida* from turkey reacted with antiserum to type 3 only, one with type 4 only and twelve were precipitated by antisera of both types 3 and 4.

A representative strain of *P. multocida* isolated from an outbreak of fowl cholera in ducks was characterized as belonging to serotype 1 on the basis of somatic antigens (Sambyal *et al.*, 1988).

All the isolates of *P. multocida* from three flocks of Japanese quails were found to belong to serotype 3 and 4 (Glisson *et al.*, 1989).

Majority of the 520 isolates of *P. multocida* collected from turkeys and wild birds in California were classified as serotype 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).

An isolate of *P. multocida* from an outbreak of FC in ducks in Tripura was serotyped as A:1 (Murugkar and Ghosh, 1995).

During their studies on *P. multocida* isolates of avian origin, Kumar *et al.* (1996) found that three poultry isolates were serotyped as A:1, one as A:3 and two duck isolates as F:3.

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).

2.7.4 Other Typing Techniques

Rundell (1975) developed a simple test in which serogroup A strains were recognized by depolymerization of the capsule after growth in proximity to hyaluronidase- producing strain of *Staphylococcus aureus.*

Rimler (1978) developed a co-agglutination procedure for recognition of serogroups B and E.

Counter-immunoelectrophoresis was developed as a rapid serological method for recognition of serogroups B, D and E (Carter and Chengappa, 1981).

2.8 DIAGNOSIS

Diagnosis of fowl cholera is based on isolation and identification of *P. multocida* from birds with typical signs and lesions of the disease. Presumptive diagnosis can be made by observing characteristic signs and lesions, by microscopic demonstration of bipolar stained bacteria in blood smears or impressions stained with Leishman's/Wright's or by isolating *P. multocida* from tissues (Rhoades and Rimler, 1989).

However, culture conditions can influence the expression of phenotypic attributes, diminishing the stability and reliability of phenotypic methods for strain differentiation (Matsumoto and Strain, 1993).

Immunofluorescent microscopy can be used to demonstrate the organism in infected tissues (Sulong and Maheswaran, 1976), but this is generally not used for routine diagnosis (Rhoades and Rimler, 1989).

Wang *et al.* (1984) developed an indirect fluorescent antibody technique for diagnosis of FC.

2.9 MOLECULAR METHODS ADOPTED FOR DIAGNOSIS

In recent years, identification and characterization have favoured analysis that reflects one of the most fundamental properties of an organism, its genetic information. Molecular approaches such as DNA hybridization and nucleic acid amplification have allowed bacterial detection directly from clinical samples, dramatically reducing the time required for identification.

2.9.1 Nucleic Acid Hybridization

Pande *et al.* (1997) developed a DNA probe for diagnosing fowl cholera, using a 4.4 kbp *Hind* III fragment of *P. multocida*. The probe hybridized with two *P. multocida* fragments corresponding to 4-9 and 9-22 kbp.

A *P. multocida* species-specific oligonucleotide probe *pmbhy* 449, targeting 16s rRNA was designed by Mbuthia *et al.* (2001). The probe proved suitable for rapid detection of *P. multocida* in histological formalin fixed tissues. It was a useful tool for studies on pathogenesis of fowl cholera.

2.9.2 Amplification of Nucleic Acid

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

Modifications to sample preparation have allowed PCR analysis to be performed on clinical specimens, considerably reducing the time required for bacterial identification (Hunt *et al.,* 2000).

A PCR protocol, which resulted in the amplification of an 846 nucleotide segment of the *tox A,* gene was developed by Lichtensteiger *et al.* (1996). This PCR protocol was specific for toxigenic *P. multocida* and could detect fewer than 100 bacteria.

A PCR based assay using primers constructed to amplify the *Psl* gene encoding the p6-like protein of *P. multocida* was developed. After Southern blotting and hybridization with *Psl,* the assay (PCR-H) was found to be specific and sensitive. The PCR-H assay detected 11 infected turkeys out of 178 samples collected (Kasten *et al.,* 1997).

Townsend *et al.* (1998a) developed a *Pasteurella multocida* specific PCR (PM-PCR) that identified all subspecies of *P. multocida* viz. subsp *multocida*, subsp. *gallicida* and subsp. *septica,* through specific amplification of an approximately 460 bp DNA fragment within the KMTI gene. Even though DNA from *Pasteurella canis* biovar 2 was also amplified by this PCR assay, this species could be readily distinguished by indole fermentation patterns. They also showed that PCR amplification could be performed directly on bacterial colonies, representing an extremely rapid and sensitive method for *P. multocida* identification.

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

A multiplex PCR was introduced as a rapid alternative to conventional capsular serotyping system by Townsend *et al.* (2001). The serogroup specific primers used in this assay were designed following identification, sequence determination, and analysis of capsular biosynthetic loci of each capsular serogroup. This PCR assay was successfully used for serotyping chicken, duck and turkey isolates of *P. multocida*.

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.

Using the primers designed by Townsend *et al.* (2001), Davis *et al.* (2003) characterized one hundred avian *P. multocida* isolates recovered from cases of FC and related infections in England and Wales, over a thirteen-year period. Using this technique, 68 per cent of the isolates were typed as A, 14 per cent as type F, five per cent as type D and four per cent as type B. Nine per cent of the isolates could not be typed by this technique.

2.10 MOLECULAR CHARACTERIZATION OF *P. multocida*

During the last decade genomic characterization techniques have supplemented or replaced traditional typing methods for the discrimination of isolates from a wide range of bacterial pathogens (Tenover *et al*., 1995).

The development of DNA-based techniques has provided alternative methods of characterization that overcame the limitations of phenotyping, while identifying precisely individual strains of closely related bacteria (Owen, 1989).

The ability to differentiate phenotypically similar isolates is critically important in epidemiology (Stull *et al.,* 1988).

Molecular characterization, or DNA fingerprinting as we know it today, encompasses a large range of methods with variable specificity and discriminatory powers, most of which have been used to differentiate phenotypically similar *P. multocida* isolates. The application of these techniques to the epidemiology of *P. multocida* is on the rise.

2.10.1 Isolation of Genomic DNA from Bacterial Cultures

Several techniques have been introduced to separate bacterial genomic DNA from pure culture of bacteria, which was suitable for digestion with restriction enzymes.

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) separated genomic DNA from *P. multocida* isolated from turkeys, using phenol: chloroform extraction procedures. They used Triton X-100 instead of SDS. Ribonuclease (RNase) was added to eliminate ribonucleic acid.

In another method, bacterial cells were lysed 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. The final concentration of DNA was estimated relative to known concentration of DNA electrophoresed in agarose gel (Carpenter *et al.,* 1991).

Frozen bacterial pellets were thawed and lysed using lysozyme and SDS. This was followed by RNase treatment and proteins were removed by addition of proteinase K. The DNA was extracted by phenol: chloroform treatment and precipitated with ethanol (Wilson *et al.*, 1992).

Zhao *et al.* (1992) recovered the cell extract of *P. multocida* and suspended it 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, and its concentration measured spectrophotometrically at 260 nm.

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.*, 2001a).

Morishita *et al.* (1996a) 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.

2.10.2 Restriction Endonuclease Analysis of Genomic DNA

Restriction endonuclease analysis (REA) of chromosomal DNA provides an alternative method for characterization and differentiation of isolates belonging to the same serotype (Marshall *et al.*, 1981; Thiermann *et al.*, 1985 and Langenberg *et al.,* 1986). This 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).

Restriction endonucleases cleave the DNA at specific sites in the nucleotide sequences and produce a set of DNA fragments which, when separated by electrophoresis, provides a characteristic band pattern or fingerprint of the respective genome (Kim and Nagaraja, 1990).

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

Snipes *et al.* (1990) using restriction enzyme *Sma*1 revealed eight different restriction patterns 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*1 restriction endonuclease. They also found that all 13 serogroup E isolates had identical DNA fingerprint profiles when *Hha* 1 endonuclease was used.

Sixty-three avian isolates of *P. multocida* and 13 attenuated vaccine strains could be grouped into 28 distinct DNA profiles using restriction enzyme *Hha* I (Wilson *et al.,* 1993).

Fingerprinting of DNA with *Sma* I of *P. multocida* isolates associated with progressive atrophic rhinitis supported the hypothesis that a common infectious source existed in Australian swine herds (Gardner *et al*., 1994) and that the disease was associated with the importation of breeder pigs.

Wilson *et al.* (1995) examined *P. multocida* isolates from 21 raptors using restriction endonucleases *Hha* I and *Hpa* II. Nineteen isolates that expressed somatic type 1 antigen had similar *Hha* I profile that was identical to those of reference somatic type 1, strain X-73, while *Hpa* II digestion yielded four profiles, one of which was identical to the *Hpa* II profile of strain X-73.

Diallo *et al.* (1995) evaluated different restriction endonucleases such as *EcoR*I, *Hind* III, *Bgl* II, *Bam H*1, *Pst* 1 and *Hpa* II and concluded that *Hpa* II gave clear patterns that allowed the 39 avian isolates to be placed in 10 groups, three of which contained a single isolate each and 11 of them falling into a single group

Christensen *et al.* (1998) proved that two outbreaks of FC in the avifauna of Denmark was caused by the same clone of *P. multocida* sub sp. *multocida*, using enzymes *Hpa* II and *Hha* 1.

Twenty-two field isolates of *P. multocida* from outbreaks of FC in turkeys when subjected to restriction endonuclease analysis with *Hpa* II yielded 7 profiles (Blackall *et al*., 1995).

Restriction endonuclease analysis with *Hpa* II of strains from Muscovy ducks showed the two strains of *P. multocida* subsp *multocida* from outbreaks in 1996 had identical REA profiles (Muhairwa *et al.,* 2000).

Nine *P. multocida* isolates from avian cholera outbreaks in Indonesia could be grouped into eight and seven distinct REA profiles using enzymes *EcoRI* and *Hind III* respectively (Jonas *et al*., 2001).

Muhairwa *et al.* (2001a) while studying the relationships among Pasteurellae isolated from free ranging chicken and their animal contacts found all the *P. multocida* isolates from cat in a village had the same REA-type, indicating a common origin.

Restriction endonuclease analysis with *Hpa* II of 218 isolates of *P. multocida* from swine revealed 17 patterns. The authors opined that REA was a useful epidemiological tool for identifying different strains of *P. multocida* (Rubies *et al.,* 2002).

Each of the eleven *P. multocida* strains of different serotypes, had unique restriction enzyme profile generated by digestion with *Hpa* II and *Hha* I (Dutta *et al.,* 2003).

2.10.3 Ribotyping

The banding patterns produced by REA are often complex, making visual interpretation of the results difficult. Ribotyping, like REA, utilizes restriction enzyme digestion of genomic DNA and agarose gel electrophoretic separation of DNA fragments. The additional use of Southern blotting and hybridization with a labeled DNA probe reduces the complexity of the restriction patterns and highlights restriction fragment length polymorphisms (RFLPs) within the

bacterial genome. Ribosomal RNA (rRNA) molecules are highly conserved ubiquitous molecules that constitute the major proportion of RNA in the bacterial cell (Grimont and Grimont, 1986). As rRNA operons vary in copy number and genomic location between strains and species, DNA probes specific for rRNA gene sequences can be used to identify RFLPs within and/or around the ribosomal operon, thus providing the basis for bacterial strain differentiation.

Eight different ribotypes were detected from among the 55 isolates of *P. multocida* from turkeys. Majority of the isolates from flocks vaccinated with attenuated M-9 strain had a profile that matched that of the vaccine strain (Snipes *et al.,* 1990).

Carpenter *et al.* (1991) observed that ribotyping proved a useful tool in understanding the epidemiology of *P. multocida*. *EcoR*I digestion of DNA, followed by ribotyping with *E. coli* rRNA probe, revealed 11 distinct ribotypes among 49 *P. multocida* isolates from turkeys.

Zhao *et al*. (1992) examined 156 serotype A strains of *P. multocida* isolated from swine herds, by REA with *Hpa* II and ribotyping. Four patterns were observed by REA while ribotyping revealed two ribotype profiles.

Twenty-two field isolates of *P. multocida* from outbreaks of FC in turkeys could be grouped into seven ribotypes (Blackall *et al.,* 1995). Ribotyping was performed using *Hpa* II digests probed with 16s rRNA operon of *Haemophilus paragallinarum*.

Morishita *et al.* (1996b) detected various restriction site heterogeneities of *P. multocida* chromosomal DNA among isolates from raptors, by ribotyping.

Christensen *et al.* (1998) reported great diversity among the 30 back yard poultry strains of *P. multocida* in Denmark. Restriction enzyme *Hpa* II generated 12 ribotypes while *Hha* I generated 10 ribotypes.

By ribotyping, 107 isolates of *P. multocida* from Australian pigs could be grouped into 13 and 12 unique ribotypes respectively, using restriction enzymes *Hind* III and *Hpa* II (Bowles *et al.,* 2000).

2.10.4 Pulsed Field Gel Electrophoresis (PFGE)

Field alternation electrophoretic method, more commonly known as pulsed field gel electrophoresis (PFGE), remains the 'gold standard' finger printing method for molecular epidemiology as polymorphisms throughout the chromosome are examined without the complexity of REA patterns and the restricted view of genetic variation produced by ribotyping (Goering, 1993).

Conventional agarose and polyacrylamide gels are only capable of resolving DNA fragments up to 40 kb in length (Dawkins, 1989). The technique has overcome the size limitations of conventional electrophoretic methods by forcing the DNA molecules to reorient periodically between two electrical fields, allowing the separation of DNA fragments up to 10 mega bases (Townsend *et al.,* 1993).

Analysis using PFGE has consistently shown greater discrimination in identification and differentiation of bacterial species than ribotyping (Prevost *et al.*, 1992).

Townsend *et al.* (1997a) compared ribotyping and field alternation gel electrophoresis (FAGE) to examine 19 *P. multocida* isolates from outbreaks of haemorrhagic septicaemia and concluded that FAGE was more discriminatory than ribotyping.

While examining *P. haemolytica* isolates from different geographical locations, Kodjo *et al.* (1999) observed that PFGE was more discriminatory than ribotyping and Random Amplified Polymorphic DNA–PCR (RAPD-PCR). Isolates belonging to the same ribotype-RAPD group could be further separated into unique entities using PFGE.

Twenty-one distinct PFGE profiles were evident among 73 avian isolates of *P. multocida* of Australian origin, while the 22 avian isolates from Vietnam could be grouped into only three PFGE profiles (Gunawardana *et al.*, 2000).

2.10.5 PCR Fingerprinting

Although PFGE is recognized as the 'gold standard' in molecular epidemiology the technique is time consuming, requiring specialized equipment. The PCR fingerprinting methods offer a relatively uncomplicated alternative to PFGE (OIE, 2000). This technique is feasible for any laboratory with PCR capability.

2.10.5.1 Random Amplified Polymorphic DNA Polymerase Chain Reaction Assay (RAPD-PCR)

Random amplified polymorphic DNA offers a unique technique that detects DNA polymorphisms without the need for prior knowledge about the genome. Polymorphism within the genome is determined based on the different patterns generated (Welsh and McClelland, 1990 and Williams *et al.,* 1990).

Chaslus-Dancla *et al.* (1996) performed RAPD-PCR on forty strains of *P. haemolytica* isolated from bovine and rabbits. They concluded that the result of RAPD was in accordance with those of ribotyping, thus validating the use of RAPD assay for epidemiological studies of *Pasteurella* strains.

Three main RAPD clusters and three subclusters were observed among the majority of 81 isolates of *P. multocida,* from a variety of diseases in animals in Zimbabwe. Serogroups A and D were found to be more closely related to the reference strain of *P. multocida* subsp. *multocida.* The technique seemed to separate subsp. *multocida* from subsp. *septica.* (Dziva *et al.,* 2001).

Huber *et al.* (2002) compared RAPD and Amplified fragment length polymorphism (AFLP) fingerprinting for identifying genetic relatedness amongst *P. multocida* isolates from fatal FC infections. They concluded that organisms

isolated from vaccinated flocks were significantly closer to the M-9 vaccine strain.

Dutta *et al.* (2003) reported that each of the eleven isolates of *P. multocida* strains of different serotypes of Indian origin had a unique banding pattern by RAPD-PCR assay.

2.10.5.2 Repetitive Extragenic Palindromic (REP) - PCR

Repetitive extragenic palindromic sequences were initially described as genetic elements of approximately 35 bp in length, common to intercistronic regions from several multicistronic operons of *Salmonella typhimurium* and *E. coli* (Higgins *et al.* 1982). These elements have been reported to be present throughout the eubacterial kingdom, although REP elements are more commonly found in Gram-negative enteric bacteria and related species (Versalovic *et al.,* 1991).

Analysis of the distribution of repetitive extragenic sequences in prokaryotic genomes forms the basis of a novel PCR based DNA fingerprinting technique known as REP-PCR.

Townsend *et al.* (1997b) performed REP-PCR on 38 *P. multocida* strains. They observed a high degree of homogeneity of REP-PCR fingerprints in haemorrhagic septicaemia - causing strains of *P. multocida,* providing support for the existence of a disease associated REP profile that was distinct from isolates implicated in other pasteurellosis. They also reported the presence of a one kilo base pair *P. multocida* species specific band.

Gunawardana *et al.* (2000) compared PFGE and REP-PCR for strain identification and epidemiological studies of avian *P. multocida* and concluded that REP-PCR could provide a competent alternative to the more labour intensive PFGE system. Seventy-three avian isolates could be grouped into 21 distinct profiles.

Seventeen isolates of *P. multocida* from swine, subjected to REP-PCR, revealed nine distinct profiles. The amplified products ranged from 350 bp to 4.4 kb. Heterogeneity was observed among fragments between 300 and 900 bp (Townsend *et al.,* 2000).

Amonsin *et al.* (2002) used REP-PCR and AFLP to characterize 43 field isolates and four attenuated vaccine strains of *P. multocida* and concluded that REP-PCR and AFLP techniques could be used for rapid fingerprinting of *P. multocida* isolates to establish their genetic relatedness.

2.10.5.3 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

Enterobacterial repetitive intergenic consensus PCR (ERIC–PCR) is a technique that involves the amplification of genomic DNA enclosed between conserved repetitive elements (ERIC sequences) and was described first for the genomes of enterobacteria and later for those of many other bacterial species. The number and location of ERIC sequences vary not only between species but also between strains of the same species (Hulton *et al*., 1991).

Loubinoux *et al.* (1999) evaluated this technique for strain differentiation of 56 *P. multocida* strains isolated from dogs in France and compared the results with RFLP. They concluded that RFLP was more discriminatory than ERIC-PCR for differentiating *P. multocida* strains.

2.10.5.4 Single Enzyme Amplified Fragment Length Polymorphism (SE-AFLP)

A rapid PCR-based technique called single enzyme amplified fragment length polymorphism (SE-AFLP) was used for typing of *P. multocida* subsp. *multocida* isolated from pigs in Brazil. Ninety-seven strains could be grouped into 18 SE-AFLP profiles. There were 7 to 12 DNA fragments ranging in size from 400 to 1400 bp (Moreno *et al.*, 2003).

2.11 PLASMID STUDIES OF *P. multocida*

2.11.1 Plasmid DNA Isolation

Many methods have been developed for the isolation and purification of plasmid DNA. These methods use one of the many properties of plasmids such as differences in base composition of chromosomal DNA, relatively smaller size compared with chromosomal DNA, transferability and circularity (Freifelder, 1970).

The different methods for isolation and purification of small scale preparations of plasmid DNA called "minipreps" usually involves three basic steps viz., the growth of bacterial cultures, followed by harvesting, extraction and finally purification of plasmid DNA (Sambrook *et al*., 1989).

A rapid alkaline extraction procedure for the isolation of plasmid DNA was developed by Birnboim and Doly (1979). In this technique the plasmid-containing cells were treated with lysozyme to weaken the cell wall, followed by complete lysis with SDS and sodium hydroxide. The lysates were then neutralized with acidic sodium acetate, which causes the precipitation of chromosomal DNA, protein-SDS complex and the high molecular weight RNA. The plasmid DNA and residual low molecular weight RNA were recovered from the supernatant by ethanol.

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

Sambrook *et al*. (1989) modified the procedure of Birnboim and Doly (1979) by using potassium acetate instead of sodium acetate. They found that the

plasmid DNA extracted with phenol:chloroform only was amenable to treatment with restriction enzymes.

A method popularly termed as "lysis by boiling" was introduced by Holmes and Quigley (1981). In this method bacterial cells were suspended in a buffer containing ehthylene diamine tetra acetate (EDTA) and Triton X. Addition of lysozyme caused the weakening of the cell wall. The samples were then boiled briefly. The chromosomal DNA and proteins precipitated were separated by centrifugation. Plasmid DNA in the supernatant was recovered following the addition of sodium acetate.

2.11.2 Plasmid Profiles of *P. multocida*

Plasmids are covalently closed circular DNA stably inherited in an extrachromosomal state. They harbour the genes responsible for transmissible drug resistance, colicin, haemolysin production, symbiosis and nitrogen fixation by bacteria (Stanisich, 1988).

The most useful classification of naturally occurring plasmids is based on the main characteristics coded by the plasmid genes. The five main types of plasmids according to this classification are (1) fertility or '*F*' plasmids, (2) resistance or '*R*' plasmids, (3) '*col*' plasmids, (4) degradative plasmids and (5) virulence plasmids (Brown, 1990).

Several workers have undertaken studies to determine the presence of plasmids in numerous strains of *P. multocida* isolated from several species of animals and birds and to investigate the correlation between antibiotic resistance profiles and the presence of plasmids.

Berman and Hirsh (1978) isolated two non-transmissible R-plasmids, from a turkey strain of *P. multocida,* having molecular weights of 4.4 and 3.44 megadaltons (MDa), which coded for resistance to tetracycline, streptomycin and sulphonamides.

Hirsh *et al.* (1981) described a turkey fowl cholera strain of *P. multocida* capable of conjugal transfer of streptomycin and sulphadiazine resistance to other *P. multocida* and *E. coli*. Genes necessary for transfer of resistance were associated with a 28.5 MDa plasmid and resistance genes were associated with a second plasmid of 7.2 MDa.

Haghour *et al.* (1987) found plasmids in 35 of 163 strains of *P. multocida* from different animal species. The plasmids ranged from 1.3 to 28.8 MDa, but antimicrobial resistance did not seem to be associated with the plasmids.

Three isolates of *P. multocida* from turkeys were found to contain a cryptic plasmid of approximately 2 MDa (Snipes *et al.,* 1989).

A classical study conducted by Christiansen *et al.* (1992) on three turkey farms in California, revealed that wildlife were a possible reservoir of infection. They showed that on two premises *P. multocida* isolates from turkeys and captured wildlife carried a 2 MDa plasmid. Their findings were also based on biochemical, serological and restriction endonuclease analyses.

Price *et al.* (1993) examined 12 small plasmids isolated from avian strains of *P. multocida* by restriction enzyme mapping. All plasmids contained sites for several commonly used restriction enzymes and ranged in size from 3.4 - 3.8 kb. They concluded that the restriction enzyme maps of the 12 plasmids were similar.

Of the 45 strains of *P. multocida* of avian origin from Australia, Diallo *et al.* (1995) showed that 20 of these did not carry a plasmid. Seven of them contained a single plasmid of 1.3 kbp and 18 contained two plasmids of 2.4 and 7.5 Kbp. There was no correlation between plasmid content and resistance to antimicrobial agents.

Christensen *et al.* (1998) did not find any plasmids in *P. multocida* isolates from outbreaks of FC in Denmark, but in 6 out of 30 strains originating

from backyard poultry a cryptic plasmid of approximately 3.4 Kb could be detected.

Lee *et al.* (2000a) concluded that FC outbreaks in vaccinated birds were not vaccine related. The RFLPs of *P. multocida* carrying plasmids from outbreaks in vaccinated flocks were dissimilar to those of the vaccine strain.

On examination of twelve *P. multocida* isolates of Indian origin Shivshankara *et al.* (2000) found that seven isolates showed a single plasmid each, while three possessed two plasmids and the remaining two lacked a plasmid. They could not establish any correlation between plasmid carriage and virulence, as all the isolates were pathogenic for mice.

Rubies *et al.* (2002) demonstrated plasmids in 81 out of the 218 isolates of *P. multocida* from swine. The plasmid containing isolates were assigned to six profile types. They showed that REA and plasmid profiles could be used as epidemiological tools for identifying different strains of *P. multocida* belonging to the same serotype.

2.12. PROTEIN PROFILES OF *P. multocida*

2.12.1 Whole Cell Protein Profiles of *P. multocida*

Most of the bacterial genomes are expressed and hence a study of the protein profiles of organisms may help in the differentiation of strains of *P. multocida.*

Ireland *et al.* (1991) analysed soluble protein extracts from field isolates of *P. multocida* serotype A:1 on 12.5 per cent acrylamide gels and found the protein profiles to be similar. The major difference between isolates was in the position of one of the major proteins in the 34-38 kDa regions.

The whole cell protein profiles of 18 field isolates of *P. multocida* of avian origin and the reference strain were separated on 10 per cent sodium-

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separation showed 18-31 clearly visible bands with various molecular weights. Proteins bands in the 52, 50 and 37 kDa regions were stained intensely (Kedrak and Opacka, 2002).

Sonicated cells of *P. multocida* strain P:52 on ammonium sulphate precipitation yielded a protein rich material, which when analysed on SDS-PAGE revealed 14 polypeptide in the molecular weight range of 30 to 91 kDa (Srivastava, 1998).

Anon (1998) analysed the SDS-PAGE profile of whole cell proteins of *P. multocida* and *Rimerella anatipestifer*. The *P. multocida* isolate had unique protein bands of 43, 23 and 10.5 kDa which were absent in *Rimerella anatipestifer*. Protein bands of 34, 33, 26 , 9 and 4 kDa were unique to *Rimerella anatipestifer*. Ten other bands were similar to both strains.

2.12.2 Outer Membrane Proteins of *P. multocida*

2.12.2.1 Extraction of Outer Membrane Proteins

Several workers have developed various techniques for the extraction of outer membrane proteins (OMPs) of *P. multocida*.

Syuto and Matsumoto (1982) purified crude soluble antigens of *P. multocida* by chromatographic methods. They used Sephadex G-200 for initial purification, followed by adsorption on to DEAE-cellulose and elution of fractions using linear gradient solutions of sodium chloride.

Membrane residues from lysed suspensions of turkey isolates of *P. multocida* were treated with various solubilizing agents such as dimethyl sulfoxide, Triton X-100, M-butanol and sodium lauroyl sarcosinate, to release proteins (Brogden and Rimler, 1983).

Cell envelopes were isolated by differential centrifugation, after disruption of stationary phase cells by sonication. Then they were extracted with Triton X-100, in the presence of 10 mM $MgCl_2$ (Lugtenberg *et al.,* 1986).

Extraction of outer membrane proteins from *P. multocida* isolated from rabbits was achieved by the use of hyaluronidase, DNase and RNase and disruption of cells by French pressure cell, followed by purification by ultracentrifugation on sucrose gradients (Lu *et al.,* 1988).

Choi *et al.* (1991) extracted the outer membrane proteins of *P. multocida* using sonication of bacterial suspension and sodium lauroyl sulphate to dissolve the inner membranes, followed by precipitation of outer membranes by ultracentrifugation.

Davis *et al.* (1991) used 0.5 per cent sodium N-lauroyl sarcosine to solubilize the inner membranes, while outer membranes were precipitated by ultracentrifugation

Ireland *et al.* (1991) prepared soluble boiled antigens using buffer containing glycerol, phenyl methyl sulphonyl fluoride and SDS. It offered a simple technique for studying the protein profiles of the organism.

Kennet *et al.* (1993) isolated outer membrane proteins of *P. multocida* 6:B strain by disrupting the cells suspended in HEPES buffer, using French pressure cell. Sodium N-lauroyl sarcosinate was used to dissolve the inner membranes and the outer membranes were precipitated by ultra centrifugation.

Ramdani and Adler (1993) prepared sub-cellular fractions of sonicated *P. multocida* cultures using sucrose density gradient centrifugation.

Wang and Glisson (1994) used Zwittergent to completely solubilize the *P. multocida* bacterial lysates. The detergent soluble and insoluble fractions were separated by centrifugation and the protein content of each sample was determined using Lowry's method.

Dabo *et al.* (1997) fractionated the OMP-enriched fraction of bovine *P. multocida* serogroup A isolates by treating the cell extract serially with sucrose phenyl methylsulphonyl fluoride, lysozyme, DNase and Rnase, followed by density gradient centrifugation.

Luo *et al.* (1997) prepared outer membrane proteins using sonication, followed by two per cent sodium N-lauroyl sarcosinate treatment, ultracentrifugation and purification on Superdex 200 columns.

Srivastava (1999) purified ammonium sulphate precipitated proteins of *P. multocida* serotype 6:B by ion-exchange chromatography. They used DEAE Sepharose CL-6B and sodium chloride gradients to elute the fractions.

Outer membrane proteins from four avian isolates of *P. multocida* were prepared by Rajalakshmi (2001) using the protocol of Barenkamp *et al.* (1981)

Singh and Goel (2002) prepared outer membrane vesicles using lithium chloride acetate, followed by filtration chromatography using Sepharose 6B-100.

Pal *et al.* (2002) prepared OMPs from *P. multocida* serotype B using the method of Choi *et al.* (1991).

Gatto *et al.* (2002) extracted OMPs from N-lauryl sarcosine insoluble protein preparations by a combination of detergent fractionation with Zwittergent 3-14 and chromatography.

2.12.2.2 Outer Membrane Protein Profiles of P. multocida

The outer membranes of Gram-negative bacteria form an interface between the bacteria and the host environment. It harbours various molecules like proteins, polysaccharides and lipids, which are surface-exposed or embedded in the membrane. These components are presently being investigated for their role in pathogenicity and ability to elicit a protective immune response.

Bacterial porins are channel forming transmembrane proteins found in the outer membranes of Gram-negative bacteria. They function as molecular sieves to allow the diffusion of small hydrophillic solutes through the outer membrane and also serve as receptors for bacteriophages and bacteriocins (Jap and Walian, 1990).

Porins are highly immunogenic, exposing epitopes on the bacterial surface (Roy *et al.,* 1994).

Several studies have been carried out to identify the potentially important outer membrane proteins (OMPs) of *P. multocida*.

The outer membrane proteins of *Haemophilus influenzae* (*H.influenzae)* type B was extracted by sonication of concentrated bacterial culture, followed by treatment with sodium lauroyl sarcosinate (Barenkamp, 1981).

Lugtenberg *et al.* (1984), while examining 34 isolates of *P. multocida* of porcine origin, classified membrane protein and lipopolysaccharide (LPS), analysed by SDS-PAGE, into three and six patterns respectively.

Lugtenberg *et al.* (1986) further studied the biochemical and immunological properties of the cell surface proteins and LPS of the 34 porcine isolates and identified a major protein with a molecular weight of 34-38 kDa, which they termed as protein H and it was found to be immunogenic.

Using Barrenkamp's procedure for extraction of OMPs, Choi *et al.* (1991) isolated OMPs from avian strains of *P. multocida*. They showed that culture medium did not have any significant effect on the OMP profiles of the strains, but *in vivo* propagation had an appreciable effect on OMP profile composition, expressing several additional OMP bands at 27 kDa, 48 kDa, 56 kDa, 60 kDa, 80 kDa and 94 kDa regions. All reference strains of the 16 serotypes of *P. multocida* except strains representing serotypes, A:1 and B:2 expressed major OMP in the 34.5 kDa and 38 kDa regions.

Protein profiles of sonicated antigens of *P. multocida* serotype 1 isolates from fowl cholera revealed quantitative differences in the molecular weights of proteins present in the range of 34-38 kDa. Immunoblotting revealed a major immunogenic polypeptide in the 34 kDa region. Sera from experimentally infected chicks reacted strongly with purified LPS and showed a rising titre over the course of the infection (Ireland *et al.*, 1991).

Lu *et al.* (1991b) demonstrated that the OMP of *P. multocida* contained immunogens, which elicited protective immunity in rabbits against homologous challenge. They immunized rabbits with sucrose density gradient purified OMPs. Significant levels of nasal and pulmonary IgA were recorded in the rabbits vaccinated with OMPs.

Davis *et al.* (1992) examined the OMP profiles of two *P. haemolytica* isolates and found that they comprised of four to five major proteins and at least twenty minor proteins. Proteins of apparent molecular masses 18, 29, 39.5, 40.5 and 42 kDa could be classified as major proteins.

Chevalier *et al.* (1993) identified protein H as the major polypeptide of the outer membrane of *P. multocida*, which had a molecular mass that ranged from 37 to 41.8 kDa, depending upon the electrophoretic system used for analysis.

The protein profiles of six field strains of *P. multocida* isolated from cattle and buffalo in Philippines were analysed using PAGE and were found to be similar to those of other Asian strains. On the basis of staining intensity the main protein bands had apparent molecular masses of 27, 32, 45 and 47 kDa (Johnson *et al.* 1993).

Kennet *et al.* (1993) found that outer membrane and associated proteins (OMAP) prepared from *P. multocida* grown in iron restricted conditions were superior immunogens compared to those prepared under iron-replete conditions. Coomassie blue staining of SDS-PAGE gels showed one major OMAP band with

a molecular mass of 35 kDa and three faintly staining OMAP bands at 30 kDa, 23 kDa and 17 kDa.

Ramdani and Adler (1993) produced three monoclonal antibodies (Mabs) against protein antigens of *P. multocida* strain M 1404. Two of these reacted with three protein bands at 29 kDa, 33 kDa and 42 kDa, while one Mab reacted with a protein band at 36 kDa based on immunoblotting. But they found that none of the Mabs protected against a lethal dose of 100 organisms of homologous *P. multocida*. They concluded that antibodies to these protein antigens were not protective.

Kasten *et al.* (1995) developed an antibody specific for a 16 kDa outer membrane protein of a rabbit strain of *P. multocida* and found that it reacted with all 16 somatic serotypes of *P. multocida* as well as vaccine strains CU and M9, indicating that the protein was expressed in all *P. multocida* strains.

The SDS-PAGE analysis of whole cell antigens of capsular serotypes A, B:1, E and F indicated that the molecular masses of the OMPs were 37, 32, 38 and 36 kDa respectively. Capsular type D exhibited two OMP types, type I (32 kDa) and type II (37 kDa) (Marandi and Mittal, 1995).

Confer *et al.* (1996) reported that several OMPs of *P. multocida* type A:3 might be important for stimulating immunity to the organism in cattle. Their findings were based on Western blot analysis that indicated antibodies to 11 prominent antigenic bands (100, 97, 90, 85, 74, 53,46, 35, 32, 21 and 16 kDa).

Field isolates of *P. haemolytica* exhibited major outer membrane proteins that ranged in size from 41 to 30.5 kDa, depending upon the isolate (Davis and Donachie, 1996).

Marandi and Mittal (1996) demonstrated the heat modifiable nature of a major OMP. They found that when the OMP-preparation was solubilized at 60°C, it had a molecular mass of 28 kDa, which increased, to 37 kDa when it was

solubilized at 100° C. Since it showed a significant N-terminal amino acid homology with OMPA family it was termed as OmpA.

Analysis of OMP from *P. multocida* serotype B:2, by SDS-PAGE indicated 10 major polypeptide bands ranging in size from 88 to 25 kDa. Immunoblotting indicated that three polypeptides, viz., 44, 37 and 30 kDa, were major immunogens. Vaccine prepared from outer membrane proteins was found to be protective in cattle (Pati *et al.*, 1996).

Ruffolo and Adler (1996) identified an 87-kDa common OMP in all serotypes of *P. multocida*. This antigen was able to elicit antibodies in mice that protected them against homologous challenge.

Mahasreshti *et al.* (1997) purified and characterized two major outer membrane proteins of *P. haemolytica,* designated as PomA and PomB, with molecular weights of 35 and 32 kDa. They found out that PomA had significant N-terminal sequence homology with OmpA protein of *Escherichia coli.* No homology between the N-terminal amino-acid sequence of PomB and those of other known bacterial proteins was found.

Marandi and Mittal (1997) showed that monoclonal antibodies specific for 32 kDa ompH were involved in highly significant protection of mice against lethal challenge infection, by both intra-peritoneal and intra-nasal routes, with homologous *P. multocida* strain. The results indicated that ompH-specific Mab inhibited proliferation of *P. multocida* in the lungs.

An enzyme immunoassay for the detection of antibodies to *P. multocida* in rabbits was developed by Peterson *et al.* (1997). They raised a murine immunoglobulin G Mab to a major outer membrane protein with a molecular weight of 37 kDa. The test had a sensitivity of 98 per cent and a specificity of 92 per cent.

The production of OMP by *P. multocida* serotype B (strain P:52) was studied based on its ability to bind Congo red dye (Srivastava, 1997). He suggested the usage of this technique to select a *P. multocida* strain that had a greater capacity to produce OMPs, which could be used for improving the efficacy of the vaccine.

Lu *et al.* (1998) demonstrated that rabbits mounted major antibody responses against five *P. multocida* OMPs (27, 37.5, 49.5, 58.7 and 64.4 kDa), using Western blot analysis.

The role of OMP in conferring protection in animals against *P. multocida* was investigated by Srivastava (1998). He compared a commercial whole cell vaccine with OMP vaccine in mice and found a satisfactory level (67 per cent) of survival in the mice vaccinated with the latter. He also found OMP to be antiphagocytic, interfering with phagocytosis of opsonized *Candida albicans* by murine peritoneal cells *in vivo*.

Gatto *et al.* (2002) studied the major antigenic heat modifiable 28 kDa outer membrane protein (Omp 28). Their studies indicated that although Omp 28 was a surface protein and antigenic in nature, mice vaccinated with purified Omp 28 succumbed to homologous intra-peritoneal challenge. By contrast, treatment group vaccinated with *P. multocida* or a commercial vaccine were significantly protected from a similar challenge.

Investigation of outer membrane proteins of *P. multocida* B:2 by Pal *et al.* (2002) revealed a major band of 32 kDa size that was heat modifiable, apart from two minor bands at 39 and 28 kDa. They were of the opinion that solubilization in sample buffer in presence of β mercaptoethanol and boiling at 100º C for five minutes were sufficient to break the disulphide bonds.

Singh and Goel (2002) fractionated outer membrane vesicles of *P. multocida* P:52 on Sepharose 6-B 100 columns, which yielded three peaks. Analysis of peak one by SDS-PAGE, yielded six protein bands. Of these, the 32

kDa OMP was identified as the marker for Asian isolates of *P. multocida* causing haemorrhagic septicaemia.

Tomer *et al.* (2002) identified three major OMPs of molecular weights 31, 33 and 38 kDa in *P. multocida* P:52 strain. The profiles of the field isolates showed minor differences when compared with that of the vaccine strain P:52. By immunoblotting using rabbit hyperimmune serum against P:52 strain, the OMP of 37 kDa was found to be the main antigenic protein in all the field isolates, as well as in the vaccine strain.

Borrathybay *et al.* (2003) observed a correlation between capsule thicknesses and amount of 39 kDa protein, since heavily capsulated strains exhibited the greatest amount, whereas non-capsulated strains including a non-capsulated and low virulent variant-P-1059B possessed little 39 kDa protein. Thus the capsule thickness and quantity of 39 kDa capsular protein on avian *P. multocida* correlated with their pathogenicity for chicken.

Dabo *et al.* (2003) cloned and characterized *P. multocida OmpA* – like gene (*Pm OmpA*). The mature protein, which had molecular mass of 35,015 Da, showed significant similarity with *E. coli* OmpA proteins. Using immunoblotting techniques they showed that Pm OmpA was immunogenic. Their studies also indicated that the protein might play a vital role in adherence to host cells.

Analysis of outer membrane profiles of 100 *P. multocida* isolates from FC and related infection in England and Wales revealed 19 distinct OMP profiles. Fifty six per cent of the isolates were represented by 15 OMP profiles, while 44 per cent of the isolates were associated with four OMP types. The OmpA and OmpH proteins of avian isolates of *P. multocida* were shown to be heterogeneous since numerous molecular mass variants were identified. However, the OmpH protein (33.1-38.3 kDa) was clearly more heterogenous than the Omp A protein (36.9-37.9 kDa) (Davis *et al*., 2003).

2.13 OUTER MEMBRANE PROTEIN GENES

Kasten *et al.* (1995) isolated and sequenced a gene from *P. multocida* that encoded a 16-kDa protein, which had extensive sequence homology with the gene encoding the P6 protein of *H. influenzae*. All somatic serotypes carried and expressed this gene, indicating that this gene is conserved.

Ruffolo and Adler (1996) identified an 87 kDa outer membrane antigen, Oma 87, which was present in all 16 serotypes of *P. multocida*. The gene encoding this protein was cloned and sequenced. It had significant similarity to the D15 protective surface antigen of *H. influenzae* (Loosmore *et al.,* 1997). Proteinase K treatment of this protein suggested that it was surface exposed. Antiserum raised against the protein protected mice against homologous lethal challenge.

Luo *et al.* (1997) cloned and characterized the major outer membrane protein gene of *P. multocida* X-73 (serotype A:1). They designated this gene as *OmpH* gene as it encoded major outer membrane protein OmpH. This gene was distributed in all the 15 serotypes including the Clemson University (CU) strain. Purified X-73 ompH protein induced 100 per cent protection in chicks, confirming its protective nature.

Mitchison *et al.* (2000) found the immunization of chicks with serogroup D GST-Oma 87 fusion protein failed to protect chicks against challenge with serotype A:1 strain, despite the fact that there was greater than 95 per cent homology in sequence of OMA 87 from the two strains.

Luo *et al.* (1999) designed a primer to amplify the *OmpH* genes of *P. multocida*. The primers amplified the gene from all the serotypes including the CU vaccine strain. A single PCR product with a similar molecular size of approximately one kilo base (Kb) was obtained from each strain. Multiple sequence alignment of OmpH amino acid sequences of different serotypes revealed high homology (72.3 per cent overall identity).

The complete genomic sequence of *P. multocida Pm*70, a common avian clone, has been established by May *et al.* (2001).

2.14 PCR RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)

The PCR-Restriction fragment length polymorphism (PCR-RFLP) is a technique wherein DNA sequence variation is identified by amplification of the region using PCR, followed by digestion of amplified product with a restriction endonuclease. The restriction fragments vary in size and can be revealed as different sized bands on agarose/acrylamide gels.

This technique has been used by several workers to detect polymorphism within a gene segment and such information has been useful for serotyping of isolates.

Restriction endonuclease analysis of a 285 bp product generated by G1/G2 primers yielded seven different patterns among 29 serovars of pathogenic *Leptospira*. A reasonable agreement between PCR-RFLP profiles and species could be observed (Brown and Levett, 1997).

Heinemann *et al.* (2000) investigated the use of PCR-RFLP for differentiation of serovars using 19 different restriction endonucleases and concluded that PCR-RFLP could be used as a rapid detection technique for differentiation of *Leptospira* sp. serovars.

Hirose *et al.* (2003) while studying the antibiotic resistance patterns of *Salmonella enterica* serovar *typhi* and serovar *paratyphi* to fluoroquinolones demonstrated the emergence of strains with mutations in the *gyrA* genes, using PCR-RFLP.

Hong *et al.* (2003) used a PCR-RFLP flagellar typing scheme to successfully serotype 112 *Salmonella* isolates obtained from poultry. Restriction enzymes *Sau*3A and *Hha* I were used for the purpose. They concluded that PCR-

RFLP was a rapid, accurate and economical approach for serotyping *Salmonella* species.

All the 50 isolates of *P. multocida*, serogroup A were shown to be identical by RFLP analysis of amplified PCR product after digestion with *Bgl* II. (Gautam *et al*., 2004)

2.15 DNA SEQUENCE DETERMINATION

Since the introduction of polymerase chain reaction, various methods of sequencing PCR-generated fragments directly have been described. These methods are mainly based on the enzymatic sequencing method of Sanger, employing chain terminating dideoxynucleotides (Sanger *et al.,* 1977) instead of chemical cleavage, or the Maxam-Gilbert sequencing method (Maxam and Gilbert, 1977).

The identity of the amplified PCR product may be confirmed more specifically as target DNA by direct sequencing of the amplified fragment (Murphy *et al*., 1999).

Luo *et al.* (1997) determined the DNA sequence of *OmpH* gene by dideoxy chain termination method. The coding region of this gene was 1059bp long. The sequence has been deposited in GenBank under accession No. U50907.

Luo *et al.* (1999) analysed the DNA sequence of *OmpH* genes of *P. multocida* serotypes 4-16. The PCR products were purified and directly sequenced. The study revealed a high degree of homology among different serotypes.

Partial sequencing of 16s ribosomal RNA gene of *P. multocida* isolated from a cat had been reported by Kuhnert *et al.* (2000). The size of the product was 1364 bp and the sequence has been assigned a GenBank Accession No. AF 294410.

Sequencing of the *P. multocida* genome has been undertaken by Advanced Genetics Analysis Centre at the University of Minnesota. The complete genomic sequence of *P. multocida* Pm70, a common avian clone, had been established by May *et al.* (2001).

The sequence of *P. multocida* A:3 outer membrane protein A (*OmpA*) gene has been determined by Gatto *et al.* (2002). The product was 2035 bp long. The sequence has been deposited in GenBank and has an accession No. of AY035341

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and Tarsons and Genei brand plasticware were used in this study. All chemicals used were of Molecular biology grade, obtained from Bangalore Genei and Sisco Research Laboratories Private Limited (SRL). Ready-made media were procured from Hi-Media Laboratories Private Limited, unless otherwise mentioned.

3.1 ISOLATION OF *PASTEURELLA*

3.1.1 Materials

3.1.1.1 Collection of Samples

Samples for the isolation of *Pasteurella* were collected mainly from apparently healthy birds, cases of field outbreaks, birds (ailing/dead) brought to the Microbiology and Pathology departments and from the University Poultry and Duck Farm, Mannuthy, for disease diagnosis and post mortem examination.

3.1.1.2 Media for Isolation of Pasteurella

Nutrient agar (NA) or brain heart infusion agar (BHIA) supplemented with sterile defibrinated ovine/bovine blood at five per cent level was used for isolation.

3.1.2 Method

3.1.2.1 Isolation of *Pasteurella multocida*

Samples of heart blood, lung, liver and spleen collected from the sacrificed/dead ducks 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. Colonies that were suggestive of *Pasteurella* sp. were stained by Gram's staining technique.

3.2 IDENTIFICATION

The bacterial isolates were identified based on morphology, cultural characteristics, tests for catalase and oxidase, growth on Mac Conkey's agar, haemolysis on blood agar, indole production, methyl red and Voges Proskauer reactions, urease activity, H_2S production, nitrate reduction, citrate utilization, lysine and ornithine decarboxylase activities, and production of acid from carbohydrates (glucose, galactose, inositol, lactose, maltose, mannitol, mannose, salicin, sucrose, dulcitol, sorbitol, trehalose, xylose and arabinose) as described by Barrow and Feltham (1993). The blood and tissue impression smears prepared from the ailing/dead birds 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 Materials

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

3.2.1.2 Method

Ten per cent stock solution of each sugar was prepared in sterile distilled water and sterilized by autoclaving at 110°C, 10 lbs pressure for 20 min. The stock solution was diluted to one per cent in Andrade's peptone water prior to use. Three milliliters of each diluted solution was inoculated with the culture under study. The tubes were incubated at 37°C for a minimum of seven days and examined for the production of acid 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 (µg) or international units (IU) per disc were used (Hi-Media Laboratories Private Limited, Mumbai, India).

1. Ampicillin(A) - 10 µg
2. Cloxacillin (Cx) - 5 µg
3. Penicillin G (P) - 10 units
4. Enrofloxacin (Ex) - 10 µg
5. Pefloxacin (Pf) - 5 µg
6. Gentamicin (G) - 30 µg
7. Streptomycin (S) - 10 µg
8. Tetracycline (T) - 10 µg
9. Metronidazole (Mt) - 5 µg
10. Nitrofurantoin (Nf) - 300 µg
11. Chloramphenicol (C) - 3 µg
12. Co-trimoxazole (Co) - 25 µg
13. Furazolidone (Fr) - 100 µg
14. Ciprofloxacin (Cf) – 10 µg
15. Bacitracin (B) - 10 units

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, 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 intra-peritoneally with 0.1 ml of inoculum containing 3 x 10^8 organisms per ml in sterile normal saline. A control mouse was injected with 0.1 ml of sterile saline. A total of six mice were used for each isolate. All the animals were observed for signs of infection. Blood smears were prepared from the dead mice and 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

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

3.3.2 Method

A single colony of *P. multocida* was added to three millilitres of Brain Heart Infusion Broth (BHIB) and incubated at 37°C for three to four hours.. A drop of this broth was added to the defibrinated blood and incubated for six hours at 37°C. The vials were then labeled 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 SEROTYPING

The isolates inoculated on ovine blood agar slant were sent to Indian Veterinary Research Institute, Izatnagar (UP), India for serotyping.

3.5 AMPLIFICATION OF NUCLEIC ACID

3.5.1 Preparation of template DNA

3.5.1.1 Materials

3.5.1.1a Phosphate Buffered Saline 10x

NaCl	80.00 g
KCl	2.00 g
Na_2HPO_4	11.33 g
KH_2PO_4	2.00 g
Distilled water to	1000 ml

3.5.1.1b PBS 1x

PBS 10x	100 ml
DW	900 ml

3.5.1.2 Method

3.5.1.2a 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 five millilitres of BHI broth and incubated at 37°C for 18 h. One point five millilitres of this broth culture was transferred to an Eppendorf tube and centrifuged at 3000 x g for 10 min. 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. The supernatant was stored at -20°C for further use as template DNA.

3.5.1.2b Preparation of template DNA from morbid materials

Morbid materials such as heart, liver, spleen collected from ducks during post mortem were used for preparation of template DNA.

Two grams of each tissue samples was homogenized in two millilitres of PBS in sterile mortar and pestle. The mixture was kept undisturbed in a refrigerator for 20 min to settle large tissue debris. The supernatant was boiled for 10 min and immediately chilled on ice for 30 min. It was then centrifuged at 3000 x g for 10 min. The supernatant fluid was collected and stored at -20°C.

3.5.1.2c Preparation of template DNA from blood smears

Blood smears and impression smears collected at post mortem were scrapped with a blade into an Eppendorf tube. To this tube 1.5 ml of sterile distilled water was added and the mixture kept at 37°C for 30 min. It was then centrifuged at 3000 x g for 15 min. The pellet was washed twice with PBS and the final pellet was resuspended in 50µl of triple distilled water, boiled for 10 min and immediately chilled on ice for 30 min, thawed and centrifuged at 3000 x g for 10 min. The supernatant was stored at -20°C.

3.5.1.2d Colony touch method

The PCR assay was also performed on suspected bacterial colonies, single bacterial colony was picked up with the help of a sterile pipette tip and added directly to the PCR master mix.

3.5.1.2e Preparation of culture lysates from bacteria other than *P. multocida*

For testing the specificity of PCR, culture lysates were prepared from bacterial cultures maintained in the Department of Microbiology. These included, *Streptococcus zooepidemicus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Leptospira* serogroup canicola serovar *canicola*. The procedure was similar to that described in section 3.5.1.2a

3.5.1.2f Genomic DNA

Genomic DNA from all the 27 isolates were prepared as detailed in 3.6.1.2

3.5.2 Polymerase chain reaction for detection of *P. multocida* (PM-PCR)

3.5.2.1 Materials

3.5.2.1a PCR reaction buffer (10x)

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

3.5.2.1b *Taq* DNA polymerase

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

3.5.2.1c Magnesium chloride

Magnesium chloride with strength of 25 mM

3.5.2.1d Deoxy ribonucleotide triphosphate

Deoxy ribonucleotide triphosphate (dNTP) mix

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

3.5.2.1e Primers for PM-PCR

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

KMTISP6 5'-GCT GTA AAC GAA CTC GCC AC-3'

KMTIT7 5'-ATC CGC TAT TTA CCC AGT GG-3'

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

3.5.2.2 Method

Polymerase Chain Reaction was conducted for the detection of *P. multocida* by the method as described by Townsend *et al*. (1998a) using the primer pair KMTIT7-KMTISP6, which amplified a 460 bp region within the KMT1 gene (Accession number AF 016259).

A 25 µl reaction mixture was prepared in 0.2 ml thin walled PCR tube (Genei, India). The reaction mixture consisted of the following.

Template DNA	5 µl
Primers	20 pmol of each primer
10x PCR buffer	2.5 µl
Taq DNA polymerase	1.0 unit
dNTP mix	1 µl
Triple distilled water to	25 µl

The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme.

Initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 45 sec and a final extension at 72°C for 6 min. The whole reaction was

conducted under the heated lid. The above-mentioned PCR technique was applied using the following materials as template DNA.

1. Direct bacterial colony
2. Bacterial culture lysates
3. Boiled extract of morbid materials obtained from infected ducks
4. Blood smears
5. Genomic DNA

The product was analysed by submarine agarose gel electrophoresis

3.5.3 Submarine Agarose Gel Electrophoresis

3.5.3.1 Materials

3.5.3.1a 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 1N NaOH. The volume was made upto 100ml, filtered, autoclaved and stored at room temperature.

3.5.3.1b TAE (Tris acetate EDTA) buffer (50x) pH 8.0

Tris base	48.40 g
Glacial acetic acid	11.42 ml
0.5 M EDTA pH 8.0	20.00 ml
Distilled water to	1000 ml

Autoclaved and stored at room temperature.

3.5.3.1c Agarose Gel (1.5 per cent)

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

3.5.3.1d Gel loading buffer (6x)

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

Stored at 4°C.

3.5.3.1e Ethidium bromide

Ethidium bromide (SRL)	100 mg
Distilled water	10 ml

Stored at 4°C in amber coloured bottles

3.5.3.1f DNA molecular size marker

pBR 322 DNA/*Alu 1* Digest

With fragments 908, 659, 521, 403, 281, 257, 226, 100, 90, 63, 57, 49

46, 19, 15 and 11 base pairs

3.5.3.2 Method

The PCR product was detected by electrophoresis in 1.5 per cent agarose gel in Tris acetate EDTA (TAE) buffer (1x). Agarose was dissolved in TAE buffer (1x) by heating. When the mixture cooled to around 50°C, ethidium bromide was added to a final concentration of 0.5 μg/ml. Agarose was then poured into clean, dry, gel platform, the edges of which were sealed with adhesive tape and the comb was kept in proper position. Once the gel was set, the comb and adhesive tape were removed gently and the tray containing the gel was placed in the buffer tank. Buffer (TAE 1x) was poured till the gel was completely covered.

Amplified PCR product (five microlitre) was mixed with one microlitre of 6x gel loading buffer and the samples were loaded in the wells. The pBR *322/Alu*

I digest was used as DNA molecular size marker. Electrophoresis was carried out at 5V/cm for one hour (or) until the bromophenol blue dye migrated more than two-third of the length of the gel.

The gel was visualized under UV transilluminator (Hoefer, USA) and the images were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3.5.4 Restriction Enzyme Analysis of PM-PCR Product

3.5.4.1 Restriction of PM-PCR Product

3.5.4.1.1 Materials

Restriction enzyme *Hae* III was used in this study. The enzyme was obtained from Bangalore Genei Pvt. Limited, Bangalore.

Hae III (10 units/µl) 5'GG↓CC -3'
(*Haemophilus aegyptius*)
10x Assay buffer C
The above enzyme was selected using Webcutter programme.

3.5.4.1.2 Method

The restriction digestion mixture was prepared as follows:

Amplified PCR product (PM-PCR) (from 3.5.2.2)	10 µl
10x RE buffer	2 µl
Restriction enzyme	1 µl (10 units)
Distilled water	7 µl

Restriction enzyme digestion was performed in Eppendorf Master Cycler, (Germany). The digestion was carried out at 37°C for two hours, followed by inactivation of the enzyme at 80°C for 20 min.

3.5.4.2 Electrophoresis of Restricted Product

The restricted PCR products were analysed by submarine agarose gel electrophoresis as detailed 3.5.3.2

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 50mM, 50mM EDTA)

Tris base	50 mM	0.61 g
EDTA	50 mM	(10 ml of 0.5M EDTA)
Distilled water to	100 ml	

Autoclaved and stored at 4°C.

3.6.1.1b TE (Tris 10mM, EDTA 1mM) 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 and in batches of 100 ml and stored at 4°C.

3.6.1.1c 10 per cent sodium dodecyl sulphate (SDS)

SDS	10 g
Distilled water to	100 ml

3.6.1.1d Tris Borate EDTA (TBE) 10x

Tris base	108.0g
Boric acid	55.0g
EDTA	9.3g
Distilled water to	1000ml.

pH was adjusted to 8.3, autoclaved and stored at room temperature.

3.6.1.1e Ammonium Acetate 7.5 M

Ammonium acetate	57.81 g
Distilled water to	100 ml

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

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

3.6.1.1g Chloroform: Isoamyl alcohol (24:1)

Chloroform	24 ml
Isoamyl alcohol	1 ml

Mixed and stored in amber coloured bottles at 4°C.

3.6.1.1h Ethanol 70 per cent

Ethanol	70 ml
Distilled water	30 ml

Mixed and stored in amber coloured bottles at 4°C.

3.6.1.2 Method

Genomic DNA of all the isolates used in this study was isolated by the method described by Christensen *et al*. (1993). About six millilitres of overnight grown broth culture (single colony inoculated in BHI broth) was centrifuged at 6000 x g for 10 min. The pellet was washed twice in PBS and resuspended in 500 µl of TE buffer (50 mM Tris, 50 mM EDTA). To this suspension 20 µl of 10

per cent SDS was added and incubated at 56°C for 30 min. The mixture was cooled to room temperature and 250 µl of 7.5M ammonium acetate was added and kept on ice for 15 min. To this mixture, added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mixed thoroughly by gentle inversion for about 10 min. The mixture was centrifuged at 8000 x g for 15 min. The aqueous phase was pipetted out into a fresh Eppendorf tube and equal volume of chloroform: isoamylalchohol (24:1) was added and mixed by gentle inversion for 10 min. The mixture was centrifuged at 8000 x g for 15 min. After the centrifugation, the aqueous phase was pipetted out and the previous step repeated once more. The aqueous phase was pipetted out into a fresh Eppendorf tube and 1/10 volume of 3 M sodium acetate and equal volume of ice-cold isopropyl alcohol was added. The mixture was kept at -70°C overnight. It was then thawed and centrifuged at 12,000 x g for 30 min. The pellet was washed twice with 70 per cent ethanol, dried and dissolved in 40 µl of TE buffer (Tris 10 mM, EDTA 1mM) and stored at -20°C in small aliquots. Purity of the DNA was checked by measuring the O.D. values at 260 nm and 280 nm. Those preparations showing a value (260/280) of 1.8 was taken for restriction enzyme analysis.

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 Bangalore Genei Laboratories Private Limited, Bangalore.

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

10x Assay buffer L

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

10 x Assay buffer E

100 x Nuclease free BSA

10 x Nuclease free BSA

100 x Nuclease free BSA	1 part
Triple distilled water	9 parts

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 three hours 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 10 x nuclease free BSA (2 µl) was also added to the reaction mixture.

3.6.3 Electrophoresis

The restricted DNA fragments were analysed by submarine agarose gel electrophoresis as detailed 3.5.3.2 except that 0.8 per cent agarose gel in 1 x TBE buffer was used. Four microlitre 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 gels were stained with ethidium bromide (0.5 µg/ml) for 30 min and destained in distilled water for 15 min. Gels were exposed to UV illuminator to visualize the DNA fragments and documented using a gel documentation system (Bio-Rad, USA). Standard molecular size marker λ DNA/*Hind III* digest with

fragments 23130, 9416, 6557, 4361, 2322, 2207, 564 and 125 base pairs was used to ascertain the size of the DNA fragments.

3.7 PLASMID PROFILE OF *P. multocida*

3.7.1 Isolation of Plasmid DNA

3.7.1.1 Materials

3.7.1.1a Luria Bertani Broth

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

3.7.1.1b 1M Tris HCl (pH 8.0)

Tris base	12.11 g
Conc. HCl	0.2 ml

The above ingredients were dissolved in 90 ml of triple distilled water. The volume was made upto 100 ml with distilled water and sterilized by autoclaving.

3.71.1c 3 M sodium acetate (pH 4.8)

Sodium acetate	40.81 g
Distilled water to	100 ml

Adjusted pH with glacial acetic acid and stored at 4˚C

3.7.1.1d 1N NaOH

NaOH	4 g
Distilled water to	100 ml

Stored at room temperature.

3.7.1.1e TEG buffer pH (8.0)

Tris (0.25M)	2.5 ml of 1M Tris
Glucose (50 mM)	9.008 g
EDTA (10 mM)	2.0 ml of 0.5 M EDTA

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

3.7.1.1f SDS-NaOH solution

10 per cent SDS	0.5 ml
1N NaOH	1.0 ml
Triple distilled water	3.5 ml

The solution was prepared fresh each time.

3.7.1.1g Ribonuclease A

Ribonuclease A	10 mg
Distilled water	2 ml

Distributed into aliquots and stored at -20°C.

3.7.1.2 Method

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

The broth was centrifuged at 8000 x g for 15 min. Resuspended the pellet in 100 µl of TEG buffer containing lysozyme at a concentration of 10 mg/ml and was kept on ice for 15 min. To this mixture 200 µl SDS-NaOH was added and gently mixed until the solution became translucent. The tube was further incubated on ice for 15 min. To this was added 150 µl of 3M sodium acetate incubated on ice for another 15 min. The mixture was centrifuged at 12000 x g for 30 min at 4°C.

The supernatant was carefully transferred into a fresh Eppendorf tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by gentle inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred to a fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred into a fresh Eppendorf tube, added double the volume of ice-cold ethanol and allowed the plasmid DNA to precipitate at -70°C overnight. The tube was then thawed and centrifuged at 10,000 x g for 15 min. The DNA pellet was washed in 70 per cent ethanol and the final pellet was resuspended in 20 µl of TE buffer. Ribonuclease A (10 mg/ml) 2 µl was added and incubated at 37°C for half an hour and then stored at -20°C. Plasmid DNA from *Escherichia coli* V517 maintained in the Dept of Microbiology was prepared in a similar manner.

Electrophoresis

The isolated plasmid DNA was analysed by submarine agarose gel electrophoresis as detailed under section 3.5.3.2 except that 0.8 per cent agarose gel in 1 x TBE buffer was used. Approximately 20 µl of plasmid DNA was mixed with 6x gel loading dye and loaded into the wells. *Escherichia coli* V517 plasmid DNA was used to ascertain the size of the plasmids. Electrophoresis was carried out at 40V till the dye reached near the bottom of the gel. The DNA fragments were viewed on a transilluminator and photographed using a gel documentation system (Bio-Rad, USA).

3.8. REPETITIVE EXTRAGENIC PALINDROMIC (REP) PCR

3.8.1 Materials

Except for the primers, the materials used for this technique were the same as those that were used for PM-PCR. (3.5.2.1)

3.8.1a Primers for REP - PCR

A primer set designed by Gunawardana *et al*. (2000) was used for the molecular typing of *P. multocida*. The primers amplified repetitive extragenic palindromic sequences throughout the entire genome.

The sequences of the two primers were:

REP 1	5'-NNNN CGN CGN CAT CNG GC -3'	18 mer
REP 2	5'-NCG NCT TAT CNG GCC TAC - 3'	18 mer

3.8.2 Materials

REP-PCR assay was carried out for typing *P. multocida* using the primer pairs REP 1 and REP 2.

A 25 µl reaction mixture was prepared in 0.2 ml PCR tubes with the following proportions.

Template DNA	5.0 µl (boiled culture lysates)
10x PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
dNTP mix	2.0 µl
Taq polymerase	1 unit
Primers	20 pmol each REP 1 and 2
Triple distilled water to	25 µl

The REP-PCR amplification was carried out in an automated thermal cycler (Eppendorf, Master Cycler, Germany) with the following programme.

Initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 41°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Products were analyzed on two per cent agarose gels in TAE 1 x by submarine gel electrophoresis as detailed in

3.5.3.2. Standard molecular weight markers, λ DNA/*Hind III* digest with fragments 23130, 9416, 6557, 4361, 2322, 2207, 564 and 125 base pairs and pUC19 DNA/*Msp*I digest with fragments 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34 and 26 base pairs were used as DNA molecular size markers to ascertain the size of the DNA fragments

3.9 WHOLE CELL PROTEIN PROFILES OF *P. multocida*

3.9.1 Extraction of Whole Cell Proteins

3.9.1.1 Materials

Antigen preparation buffer

Tris (0.1M)	1.200 g
Phenyl methylsulphonyl fluoride	0.034 g
Glycerol	15.00 ml
SDS	2.000 g

pH adjusted to 6.8 and the volume made up to 100 ml with distilled water.

3.9.1.2 Method

Whole cell proteins were prepared as per the method of Ireland *et al.* (1991). Pure cultures of *P. multocida* grown on ovine/bovine blood agar plates at 37˚C for 18 h were harvested in antigen preparation buffer. The cell suspensions were mixed, placed in a boiling water bath for five minutes, centrifuged at 10,000 x g for three minutes and the supernatant collected. The protein concentration of the preparations was determined by the method of Lowry *et al.* (1951) using a bovine serum albumin (BSA) standard.

3.9.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.9.2.1 Materials

3.9.2.1.a Acrylamide-bisacrylamide stock (30 : 0.8)

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Distilled water to make	100 ml

Filtered through Whatman No.1 filter paper and stored at 4°C.

3.9.2.1.b 1.5 M Tris pH 8.8

Tris base	181.7 g
Distilled water to	1000 ml

adjusted pH to 8.8 with 4N HCl. and stored at 4˚C.

3.9.2.1.c 0.5 M Tris

Tris base	60.6 g.
Distilled water to	1000 ml

Adjusted pH to 6.8 with 4 N HCl. and stored at 4˚C.

3.9.2.1.d Resolving gel (12.5 per cent)

Acrylamide : bisacrylamide (30: 0.8)	12.5 ml
Tris hydrochloride (1.5 M) pH 8.8	7.5 ml
Sodium dodecyl sulphate (ten per cent)	0.3 ml
Ammonium persulphate (ten per cent)	0.15 ml
N, N, N, N - tetra methyl ethylenediamine (TEMED)	0.01 ml
Distilled water	9.6 ml

3.9.2.1.e Stacking gel (four per cent)

Acrylamide stock (30.8 per cent)	0.67 ml
Tris hydrochloride (1.5 M) pH 6.8	1.25 ml
Sodium dodecyl sulphate (ten per cent)	0.05 ml
Ammonium persulphate (ten per cent)	25 µl
N, N, N, N - tetra methyl ethylenediamine (TEMED)	2.5 µl
Distilled water	3.00 ml

3.9.2.1.f Electrophoresis buffer

Tris base	3.0 g
Glycine	14.4 g
Sodium dodecyl sulphate	1.0 g
Distilled water to make	1000 ml

3.9.2.1.g Sample preparation buffer (2x)

0.5 M Tris hydrochloride, pH 6.8	2.5 ml
Glycerol	2.0 ml
Sodium dodecyl sulphate (ten per cent)	4.0 ml
2-mercaptoethanol	0.2 ml
Bromophenol blue	0.5 mg
Distilled water to make	10.0 ml

Distributed in small aliquots and stored at 4°C.

3.9.2.1h Destaining solution I

Glacial acetic acid	70 ml
Methanol	400 ml
Distilled water to	1000 ml

3.9.2.1i Destaining solution II

Glacial acetic acid	70 ml
Methanol	50 ml
Distilled water to	1000 ml

3.9.2.1j Coomassie brilliant blue (SRL) staining solution

Coomassie brilliant blue (R250)	0.5 g
Methanol	800 ml
Glacial acetic acid	140 ml
Distilled water to	2000 ml

3.9.2.2 Method

The whole cell protein extracts of *P. multocida* were analysed by discontinuous system of polyacrylamide gel electrophoresis (Laemmli, 1970)

Resolving gel solution, 12.5 per cent was prepared and degassed. Ten per cent ammonium persulphate and TEMED were added and poured between two glass plates so as to form a gel of 1 mm thickness and left for polymerization. Distilled water was layered on the top to ensure uniformity of the gel surface. The set up was left overnight at 4°C for complete polymerization. After polymerization the distilled water was pipetted out and four per cent stacking gel was prepared and poured between the glass plates. The comb was then inserted and the apparatus left as such for complete polymerization for one and half hours. After polymerization the comb was removed and the wells were washed thoroughly with running buffer. A small quantity of running buffer was added into each of these wells. Twenty micrograms of the sample was mixed with equal volume (10 µl) of sample preparation buffer and kept in boiling water bath for five minutes. The samples were loaded into individual wells under the column of buffer in each well. Standard high range molecular weight marker (GENEI) was

loaded in one of the wells. The glass plates were fixed onto the vertical slab gel electrophoresis apparatus and electrophoresis buffer was carefully poured into the top and bottom reservoirs and electrophorosed at 10 mA constant current till the bromophenol blue marker reached near the bottom of the resolving gel. The gel was then removed from the glass plate, the stacking gel was snipped off and transferred to a Petri plate containing Coomassie brilliant blue staining solution for two to three hours. The gels were then destained till the background became clear and were viewed in white light and photographed.

Estimation of molecular weight

The molecular weights of the different protein fractions were obtained by comparing the distance migrated by the different fractions with that of the standard marker proteins of known molecular weights. A standard graph was plotted with the log10 Da values of the marker protein on the Y-axis and the mobility of the known bands of the marker on the X-axis. The values of the distance migrated by the sample proteins were interpolated with the standard curve to determine the molecular weight of the polypeptide bands.

3.10 ANALYSIS OF OUTER MEMBRANE PROTEINS (OMP) OF *P. multocida*

3.10.1. Extraction of OMP

3.10.1.1 Materials

3.10.1.1a Phosphate buffered saline (PBS) pH 7.2

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate dihydrate	1.06g
Potassium dihydrogen phosphate	0.2 g
Distilled water to	1000 ml

pH adjusted to 7.2 and autoclaved at 121°C for 15 min at 15 lb pressure and stored at 4°C.

3.10.1.1b Tris-HCl 20 mM (pH 7.2)

Tris-HCl	3.15 g
Distilled water to	1000 ml

pH adjusted to 7.2 , autoclaved and stored at 4°C.

3.10.1.1c HEPES (N-2-hydroxyethyl Piperazine N-2 ethane sulphonic acid) buffer, 10mM , pH 7.4

HEPES buffer	0.238 g
Distilled water to	100 ml

pH was adjusted to 7.4, autoclaved and stored at 4°C.

3.10.1.1d Sodium N-lauroyl sarcosine (0.5 per cent) (Sarkosyl ,Sigma Aldrich, USA)

500 mg of sodium lauroyl sarcosinate was dissolved in 100 ml of sterile 10 mM HEPES buffer (pH 7.4) just prior to use.

3.10.1.2 Method

The outer membrane protein (OMP) enriched extract from *P. multocida* was prepared as per the method described by Davies and Donachie (1996).

The bacterial strains were sub cultured on Brain heart infusion agar containing 5 per cent defibrinated ovine/bovine blood. Pure culture was inoculated into 10 ml of sterile BHI broth and incubated over night at 37°C.

Two-litre flask containing 400 ml of BHI broth was pre-warmed overnight at 37°C. Overnight grown culture, approximately 0.4 ml was added to 400 ml of pre-warmed medium and incubated for 12 h at 37°C. The cultures were placed on ice to stop the bacterial growth.

The bacteria were harvested by centrifuging at 8000 x g for 30 min at 4°C. The bacterial pellet was washed twice in sterile PBS (pH 7.2) and the final pellet was dissolved in 7 ml of ice-cold HEPES buffer and kept for 20 min (pH 7.2). Bacterial cells were sonicated on ice for 5 min (Branson Sonifier 450) at 12 microns by placing the probe into the suspension to a depth of 1.0-1.5 cm to avoid frothing. The sonicated sample was poured into a 15 ml centrifuge tube, placed on ice for 15 min and centrifuged at 4000 x g for 20 min at 4°C to remove the intact cells and debris. The supernatant was carefully transferred to 10 ml polyallomer ultracentrifuge tubes and centrifuged at 1,00,000 x g for 1 h at 4°C in a Sorvall ultracentrifuge to pellet the cell envelopes.

The supernatant was discarded and the pellet was resuspended in seven millilitres of 0.5 per cent sodium N-lauroyl sarcosine and kept at room temperature for 20 min. The sarkosyl insoluble outer membrane enriched fraction was pelleted by centrifugation at 1,00,000 x g for 1 h at 4°C. The pellet was washed twice in distilled water and finally dissolved in 0.5 ml of Tris-HCl 20 mM pH 7.2. About 50 µl of the outer membrane protein was aliquoted into an Eppendorf tube for protein estimation. The remainder was stored at -70°C.

The protein content of the OMP-enriched extract of the isolates was determined by the method of Lowry *et al.* (1951) using a bovine serum albumin standard.

3.10.2 Determination of Optimal Conditions for Analysis of Outer Membrane Proteins

Six Eppendorf tubes, each containing OMPs of a the duck isolate DP 1, prepared as detailed in 3.10.1.2 were mixed with equal volumes of sample preparation buffer (3.9.2.1. g) and subjected to heat treatment at 50ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC for 10 min. These preparations were then analysed on 12.5 per cent SDS-PAGE. Electrophoresis and subsequent staining was carried

out as described in 3.9.2.2. Standard high range molecular weight marker (GENEI, Bangalore) was used to determine the size of the proteins.

3.10.3 Analysis of OMPs on SDS-PAGE

The outer membrane proteins of *P. multocida* were subjected to 100ºC for 10 min and analysed by discontinuous system of polyacrylamide gel electrophoresis as described in 3.9.2.2.

3.11 DETECTION OF ANTIGENIC OMPs OF *P. multocida*

3.11.1 Raising of Antiserum against *P. multocida* Whole Cell Proteins

3.11.1.1 Materials

3.11.1.1a Brain heart infusion broth

3.11.1.1b PBS pH 7.2 (3.10.1.1a)

3.11.1.1c 0.5 per cent formalin in PBS

Formaldehyde 0.5 ml
PBS pH 7.2 to 100 ml

3.11.1.1d Freund's complete and incomplete adjuvants (Sigma Aldrich, USA)

3.11.1.1e Rabbits

Adult male rabbits (New Zealand White) weighing about 1 kg were obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy.

3.11.1.2 Method

A pure culture of *P. multocida* (fowl isolate FP 1) was inoculated to 50 ml of BHI broth and incubated at 37°C for 18 h. The broth culture was centrifuged at 3000 x g for 30 min. The supernatant was discarded and the pellet washed thrice in PBS, pH 7.2. The final pellet was resuspended to a concentration of 10^8 cell per ml in PBS, pH 7.2 containing 0.5 per cent formalin. Formalin was allowed to act for 24 h at 37°C.

3.11.1.2a Sterility test

The sterility of the formalinized suspension was checked by streaking 0.2 ml of the suspension on BHI agar and incubating at 37°C for 7 days.

3.11.1.2b Preparation of antigen

The formalinized, sterility tested bacterial suspension was thoroughly emulsified with equal volume of Freund's complete adjuvant.

3.11.1.2c Inoculation of rabbits

One millilitre of the emulsified suspension was inoculated by subcutaneous route to each of two rabbits.

A booster dose was given on day 14, followed by another dose on day 21. The antigens used for booster doses were emulsified with Freund's incomplete adjuvant.

3.11.1.2d Test bleedings

On 28th day the rabbits were bled from the ear vein, the serum separated and tested for presence of specific antibody by agar gel precipitation test

3.11.2 Testing for Seroconversion by Agar Gel Precipitation Test (AGPT)

3.11.2.1 Materials

3.11.2.1a Gel for AGPT

Agarose	1.0 g
Sodium chloride	0.85 g
Sodium azide	0.01 g
Distilled water to	100 ml

To dissolve the agarose in saline, the solution was boiled for five minutes.

3.11.2.1b Agar coated slides

Clean glass slides were coated by smearing 0.5 per cent melted agarose in distilled water and drying in air by keeping the slides horizontally over glass rods.

3.11.2.2 Method

Agar gel precipitation test was done as per the method of Pati *et al*. (1996) with minor modifications. Three millilitres of melted agarose was poured onto precoated glass slides and allowed to set. One central well and five peripheral wells, each with three millimetre diameter were punched in such a way that the distance between the central well and any peripheral well was three millimetres. Distance between the adjacent peripheral wells was kept equal. The central well was filled with 20 µl each of sonicated *P. multocida*. The peripheral wells were filled with 20 µl of two-fold dilution of the serum samples. The slides were incubated at room temperature in a humid chamber for 24 h and were examined in diffuse light for the presence of precipitin lines.

3.11.3 Western Blotting

3.11.3.1 Materials

3.11.3.1a Nitrocellulose membrane (NCM)

3.11.3.1b Whatman filter paper No.1

3.11.3.1c PBS pH 7.2

3.11.3.1d PBS - Tween

This was prepared by adding 0.5 ml of Tween 20 to 1 litre of PBS, pH 7.2.

3.11.3.1e Two per cent BSA

Albumin bovine Fraction V (SRL) 2 g
PBS pH (7.2) to 100 ml

3.11.3.1f Blot buffer

Tris 0.1 M, pH 9	1200 ml
Absolute methanol	80 ml
Distilled water	120 ml

3.11.3.1g Conjugate

Anti-immunoglobulin G horse radish peroxidase (Anti-IgG HRP) conjugate against rabbit raised in goat, obtained from Bangalore GENEI was diluted 1:2500 in PBS, pH 7.2

3.11.3.1h Chromogen stock solution

50 mg of Diamino benzidine in 100 ml of PBS, pH 7.2
Stored at room temperature

3.11.3.1i Substrate solution

3 ml of chromogen stock solution in 9 ml of PBS, pH 7.2
150 µl of 1 per cent H_2O_2

3.11.3.2 Method

The *P. multocida* outer membrane proteins were analysed by SDS-PAGE Duplicate samples were loaded in the wells in the following fashion:

Lane 1 protein molecular weight marker

Lane 2 FP 1

Lane 3 DP 1

Lane 4 DP 25

Lane 5 FP 1

Lane 6 DP 1

Lane 7 DP 25

Electrophoresis was stopped when the bromophenol blue dye reached near the bottom of the gel. The gel was cut into two halves. The first half containing lanes 1 to 4 was stained in Coomassie brilliant blue staining solution for three hours, then transferred to destaining solution I for 45 min or till the background staining had cleared sufficiently. The gels were then transferred to destaining solution II and then photographed. The other portion of the gel was used for Western blotting.

Transfer of OMPs to NCM

Western blotting

Western blotting was performed following the method of Towbin *et al.* (1979). The second portion of the gel containing lanes 5, 6 and 7 was gently removed and placed on a moistened Whatmann filter paper.

A large staining glass tray was taken, in the centre of which was placed three micro-titration plates. Over these plates a glass plate was placed. The glass tray was filled with 400 ml of blot buffer On the glass plate a Whatmann (No 1) filter paper of a size larger than the gel, wetted with blot buffer was placed, whose ends were immersed in the blot buffer. The gel to be blotted was placed on the

filter paper. The NC membrane soaked in blot buffer for five minutes was placed over the gel. Care was taken to ensure that the correct side of the NCM was facing the gel. Over the NCM was placed a small sheet of wet Whatman (No.1) filter paper. A 10 ml glass pipette was then rolled over this to ensure that there was no air bubbles trapped between the gel and the NCM. Over the filter paper, stacks of filter paper were placed. On the top of this a glass plate was kept and on which a weight of two kilograms was placed. This set up was left as such overnight for complete transfer.

Identification of antigenic OMPs of *P. multocida*

Following complete transfer the NCM was washed in 100 ml of distilled water, followed by incubation in blot buffer for one hour at 37°C. This was followed by washing the NCM in PBS-T for one hour at 37°C. The NCM was then blocked by incubation in two per cent BSA in PBS, to prevent non-specific binding, for two hours at 37°C.

The NCM was then washed in PBS-T in a shallow Petri dish with four changes of 50 ml each of PBS-T over 30 min. The washed NCM was incubated with rabbit anti *P. multocida* serum (1:50 dilution in PBS) overnight at 4°C. This was followed by the washing step described earlier. Goat anti-rabbit HRPO conjugate (1:2500 dilution in PBS) was then added to the NCM and incubated at 37°C for one hour. The NCM was washed as described earlier and was then kept in substrate solution at room temperature with mild rocking until colour developed. After 10 min the NCM was washed in distilled water and allowed to dry. The dried NCM was photographed.

3.12 Polymerase chain reaction for detection of *OmpH* gene of *P. multocida*

3.12.1 Materials

Except for the primers, the materials used for this technique were same as that which was used for PM-PCR. (3.5.2.1)

3.12.1a Primers for OmpH-PCR

Two oligonucleotides based on the sequence of *P. multocida* X-73 *ompH* gene, Accession No. U50907 (Luo *et al*., 1997) were designed using Primer3 software. The primers were custom synthesized by M/s Bangalore Genei India.

The sequences of the two primers were as follows:

OmpH 1	5'-GCG TTT CAT TCA AAG CAT CTC-3'	- 21 mer
OmpH 2	5'-ATG ACC GCG TAA CGA CTT TC -3'	- 20 mer

3.12.2 Method

A 50 µl reaction mixture was prepared in 0.2 ml thin walled PCR tube (Genei, India). The reaction contained :

Template DNA	5 µl
Primers	40 pmol each
10x PCR buffer	5 µl
Taq DNA polymerase	2 units
dNTP mix	2 µl
Triple distilled water to	50 µl

The following programme was used - initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The template DNA used was bacterial culture lysate. The product was analysed by submarine agarose gel electrophoresis as detailed in 3.5.3.2. Standard

molecular size marker low range DNA ruler with fragments 3000, 2500, 2000, 1500, 1000, 600, 300 and 100 bp was used as DNA molecular size markers to ascertain the size of the amplified PCR product.

3.13 RESTRICTION ENZYME ANALYSIS OF OMPH-PCR PRODUCT

3.13.1 Restriction enzyme pattern of OmpH-PCR Product

3.13.1.1 Materials

Two restriction enzymes viz., *Dra I* and *Hinf I* were used in this study. The enzymes were obtained from Bangalore Genei Pvt. Limited, Bangalore.

Dra I (10 units/µl)	5’G TTT↓AAA - 3’
(*Dienococcus radiophilus*)	
10x Assay buffer E	
Hinf I (10 units/µl)	5’G↓ANTC-3’
10x Assay buffer C	

Both the above enzymes were selected using Webcutter programme.

3.13.1.2 Method

The restriction of PCR products was carried out as detailed in 3.5.4.1.2.

3.13.2 Electrophoresis of Restricted Product

The restriction fragments were analysed on eight per cent acrylamide gels (DNA-PAGE).

3.13.2.1 Materials

3.13.2.1a Preparation of 8 per cent Acrylamide gel

Acrylamide: bisacrylamide (30:0.8)	3.22 ml
TBE (5x)	3.00 ml
Distilled water	9.73 ml
10 per cent Ammonium per sulphate	50 µl
TEMED	3 µl

3.13.2.2 Method

Eight per cent acrylamide solution was prepared and degassed. Added ten per cent ammonium per sulphate and TEMED to this and poured between two glass plates. A comb was introduced in the top and the solution was allowed to polymerize. After complete polymerization the comb was removed and the wells were washed with 1x TBE and the wells were half-filled with 1x TBE. Five microlitres of the amplified PCR product digested with restriction enzyme was mixed with one microlitre of 6x gel loading buffer and carefully loaded in the wells under the buffer column. In one of the wells undigested amplified PCR product was added. The glass plates containing the polyacrylamide gel was transferred to the vertical slab gel electrophoresis system (Hoeffer, USA) and 1x TBE was filled in the upper and lower buffer tanks. Electrophoresis was carried out at 70 V till the bromophenol blue dye reached the bottom of the gel.

At the end of the electrophoresis, the glass plates were dismantled and the gel was stained with ethidium bromide as in 3.6.3. The gels were viewed on a transilluminator and photographed using a gel documentation system.

3.14. STUDIES ON INACTIVATED FOWL CHOLERA VACCINE (CHOLERIN TRIPLE)

3.14.1 Amplification of *OmpH* Gene from Inactivated Fowl Cholera Vaccine

3.14.1.1 Materials

Cholerin Triple – Fowl Cholera bacterin, inactivated oil adjuvant vaccine containing *P. multocida* serotypes 1,3 and 4. Manufactured by ABIC, Israel and marketed by M/s Sarabhai Chemicals, Baroda.

Isopropanol (SRL, Chemical, Bombay)

3.14.1.2 Method

Two millilitres of Cholerin Triple vaccine was mixed with eight milliliters of isopropanol in a test-tube and vortexed for five minutes. The mixture was then centrifuged at 3000 X g for 10 min. The oil phase on the top and the aqueous phase of the vaccine below, which was mixed with isopropanol were pipetted out. The pellet containing the bacterin was washed twice in PBS, pH 7.2. The PBS was decanted and the washed pellet was resuspended in 100 µl of triple distilled water and boiled at 100ºC for 10 min. The mixture was then frozen at -20ºC for 30 min, thawed and centrifuged at 3000 x g for 10 min. The supernatant was used as template DNA for PCR. Template DNA prepared from duck isolate DP1 was used as control. The OmpH-PCR was performed as described in 3.12.2

3.14.2 Restriction Endonuclease Analysis

Restriction endonuclease analysis of PCR product amplified from template DNA prepared from oil adjuvant fowl cholera vaccine (Cholera triple), as well as DP 1 was performed using two restriction endonucleases *Dra* I and *Hinf* I as described in 3.13.1.2.

Electrophoresis of the digested products was performed as detailed in 3.13.2.2. The DNA fragments were viewed using a transilluminator and photographed using a gel documentation system (BIO-RAD, USA)

3.15 DETERMINATION OF *OmpH* GENE SEQUENCE

3.15.1 Purification of PCR Product

3.15.1.1 Electrophoresis

The PCR products (OmpH) were electrophorosed in 1.5 per cent agarose gel as described for the detection of PCR products. The gel was transferred to the

UV transilluminator and the band was visualized under preparative UV source. Using a sharp scalpel blade (or) razor blade, the agarose containing the band of interest was cut with minimum amount of free agarose.

3.15.1.2 Electroelution

A piece of dialysis tubing about seven centimeters long was taken and one end was sealed. The slice of gel containing the band of interest was pushed into the dialysis tube. About 500 µl of TBE buffer was added to the dialysis tubing so that the gel slice was completely immersed without any bubbles and the other end of the dialysis tubing was sealed. The dialysis tubing containing the agarose slice was immersed in a shallow layer of 1x TBE buffer in an electrophoresis tank. Electroelution was carried out at 4-5V/cms for two to three hours. During this time the DNA was electroeluted out of the gel into the inner wall of the dialysis tube. The polarity of the current was reversed for one minute to release the DNA from the wall of the dialysis tube. At the end of electroelution the bag was opened and the entire buffer surrounding the gel slice was carefully transferred to a sterile Eppendorf tube.

3.15.1.3 DNA Cleaning and Precipitation

To Eppendorf tube containing the buffer was added an equal volume of chloroform:isoamylalchohol mixture (24:1) and spun at 12,500 x g for about three minutes at 4°C. The top aqueous phase containing the DNA was carefully transferred to a sterile Eppendorf tube without disturbing interphase. To this DNA in the Eppendorf tube 1/10 volume of 3M sodium acetate (pH 4.8) was added, followed by the addition of two volumes of ice cold ethanol and kept at -20°C for about 15 min. The mixture was then centrifuged at 12,500 x g for about 15 min at 4°C. The supernatant was decanted and 500 µl of 70 per cent ethanol (ice cold) was added to the pellet. The tube was spun at 12,500 x g for 2 min. The supernatant was removed and the DNA pellet was dissolved in sterile triple glass distilled water. The purified PCR product was then directly used for sequencing.

3.15.2 Sequencing of PCR Product

The purified PCR product was directly sequenced by Sanger' dideoxy-chain termination method using ABI PRISM Model 310 version 3.4.1. Primers OmpH 1 and OmpH 2 were used as sequencing primers. Sequencing was carried out at the School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu.

Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) network provided by the National Centre for Biotechnology Information (NCBI).

Results

4. RESULTS

4.1 ISOLATION OF *Pasteurella multocida*

Isolation of *P. multocida* was attempted from 157 samples originating from ducks and fowl. The samples were collected from apparently healthy birds, cases of field outbreaks, birds (ailing/dead) brought to the Microbiology and Pathology departments and from the University Poultry and Duck Farm, Mannuthy, for disease diagnosis and post mortem examination

The specimens collected for the isolation trials were tracheal swabs, heart blood and tissues like liver, spleen and lungs.

The gross lesions comprised of epicardial petechiae; enlargement, congestion and pinpoint, white, multiple necrotic foci of the liver; congested spleen; haemorrhagic tracheitis and diffuse haemorrhages in internal organs like proventriculus, lungs and intestine (Fig. 1 and 2).

Examination of the heart blood smears and impression smears from liver and spleen of necropsied birds, stained with Leishman's stain revealed the presence of bipolar organisms. Spleen and Liver impression smears contained more number of organisms as compared to heart blood smears (Fig. 3 and 4).

Biomaterials collected from the birds were streaked on ovine/bovine blood agar. The Petri plates were incubated at 37°C for 18-24 h in a candle jar. Following incubation, mucoid, convex, greyish-white and non-haemolytic colonies were obtained. A characteristic odour was observed when the Petri plates were opened following overnight incubation at 37°C.

A reference strain (LKO) of *P. multocida*, obtained from Indian Veterinary Research Institute, Izatnagar, was used for comparison. Reference

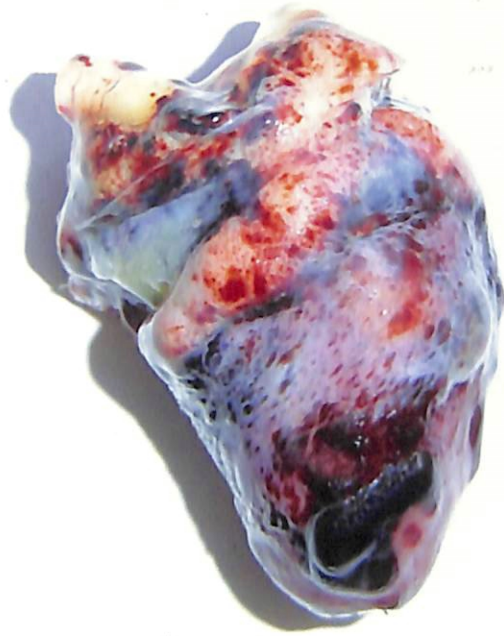


Fig. 1 Epicardial haemorrhage

Fig. 2 Enlarged liver with necrotic foci

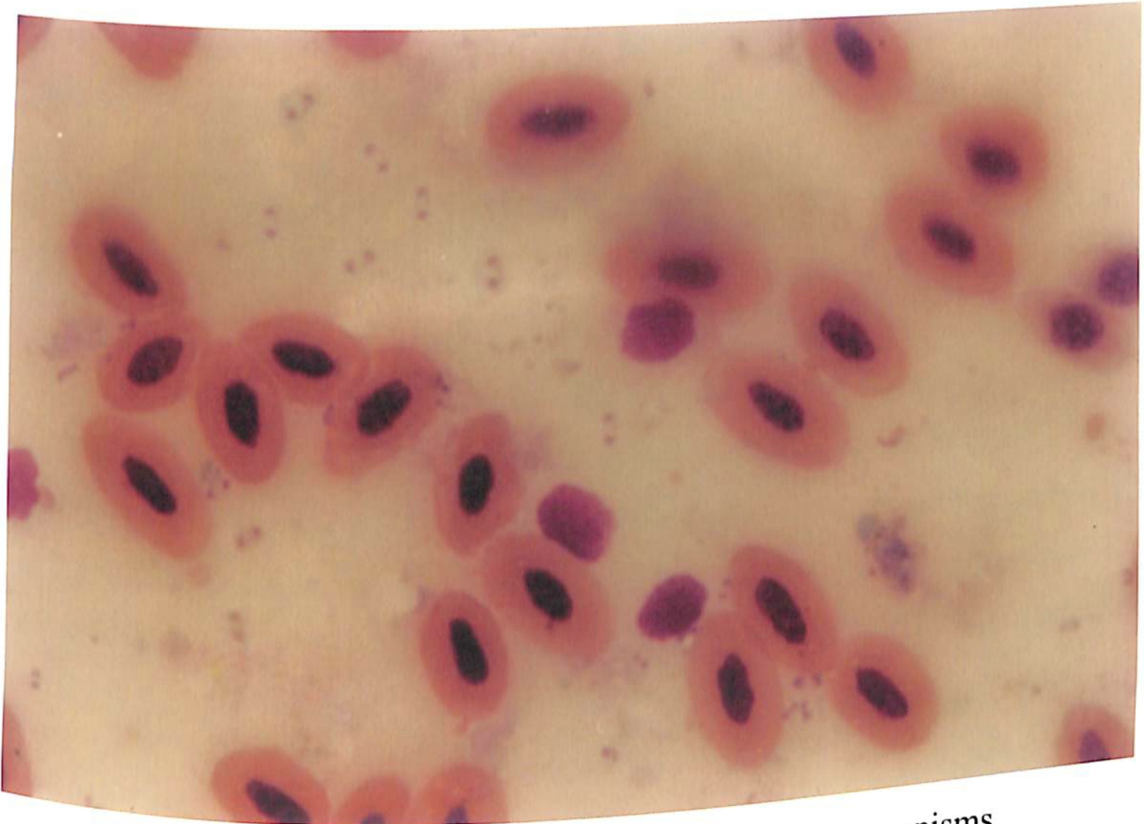


Fig. 3 Blood smear showing bipolar stained organisms.
(Leishman's stain 1000 x)

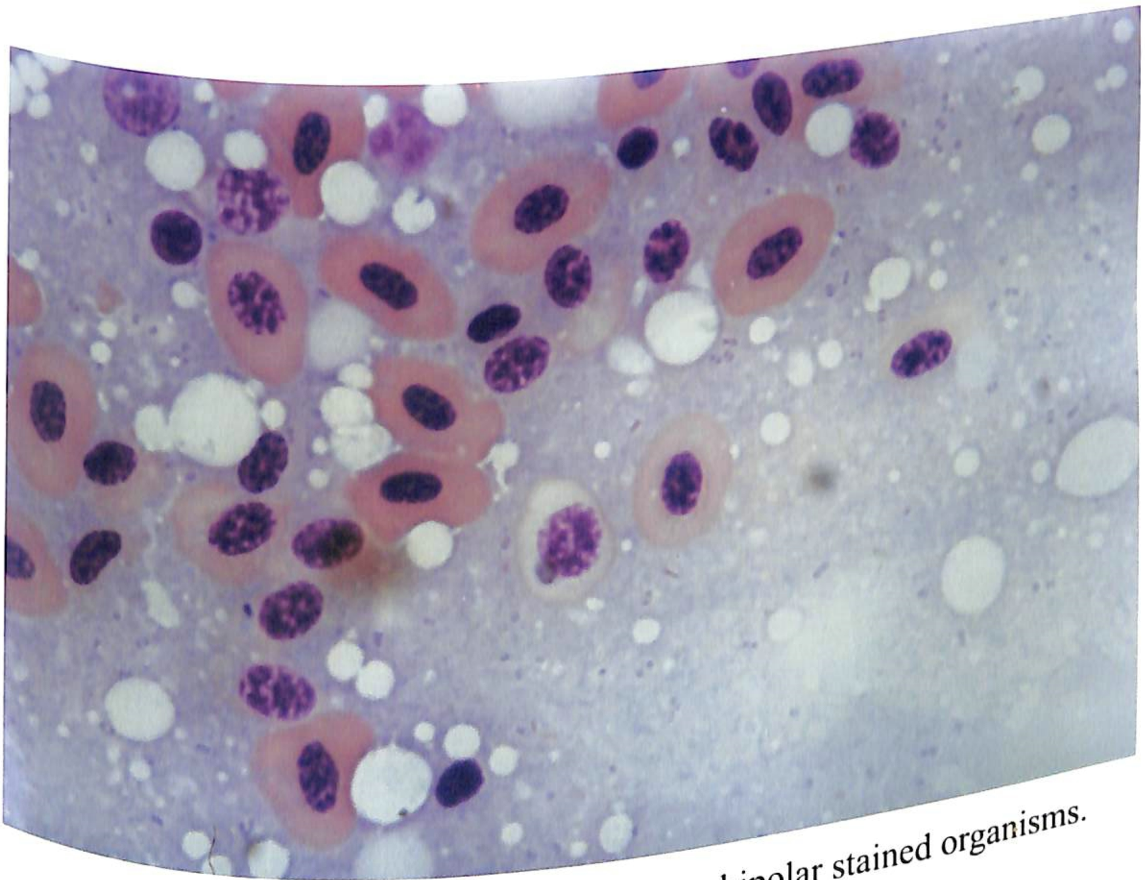


Fig. 4 Liver impression smear showing bipolar stained organisms.
(Leishman's stain 1000 x)

strains *P. multocida* serotype A:3 and bovine strain serotype B:2 also obtained from IVRI were also used in studies on outer membrane proteins and genes.

4.2 IDENTIFICATION OF THE ORGANISM

4.2.1 First Stage

Altogether 25 isolates were obtained from ducks (named DP1 – DP25) and one isolate from fowl (FP1). All isolates including the reference strain LKO were non-motile, Gram-negative and coccobacillary (Fig 5). They grew aerobically and anaerobically, did not grow on Mac Conkey's agar and were non-haemolytic on blood agar. All were catalase and oxidase positive and fermented glucose (Table 1).

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 and sucrose but could not utilize lactose. Inositol could be fermented only by isolates DP 18, 20 and 21. Isolates DP 4, 5, 6, 8, 18, 19, 20 and 25 and FP1 fermented maltose while salicin was fermented by DP 16, 18, 19 and 20. Three isolates DP 2, 3, 22 and LKO could not ferment mannitol (Table 2).

4.3 ANTIBIOGRAM

Antibiogram of *P. multocida* isolates indicating the susceptibility and resistance to various antibiotics/antibacterial agents is presented in table 3.

All isolates were sensitive to Enrofloxacin, Pefloxacin and Chloramphenicol.

Table 1. First stage biochemical tests of isolates

Tests	DP1	DP2	DP3	DP4	DP5	DP6	DP7	DP8	DP9	DP10	DP11	DP12	DP13	DP14
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	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	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	F	F	F

Tests	DP15	DP16	DP17	DP18	DP19	DP20	DP21	DP22	DP23	DP24	DP25	FP1	LKO
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	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	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	F	F

Table 2. Second stage biochemical tests of isolates

Tests	DP1	DP2	DP3	DP4	DP5	DP6	DP7	DP8	DP9	DP10	DP11	DP12	DP13	DP14
Indole Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-Galactosidase Activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine Decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sugar Fermentation														
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	+	+	+	-	+	-	-	-	-	-	-
Mannitol	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Xylose	+	+	+	-	+	+	-	-	-	-	-	-	+	+
Arabinose	+	+	+	-	-	-	-	-	-	-	-	-	-	-

Table 2. Continued

Tests	DP15	DP16	DP17	DP18	DP19	DP20	DP21	DP22	DP23	DP24	DP25	FP1	LKO
Indole Production	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer Test	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-Galactosidase Activity	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine Decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+
Sugar Fermentation													
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	+	-	+	+	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	+	+	+	-	-	-	-	+	+	-
Mannitol	+	+	+	+	+	+	+	-	+	+	+	+	-
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	+	-	+	+	+	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	+	-	+	-	-	-	+	+	-	-
Xylose	-	-	-	+	-	+	+	+	-	+	-	-	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Antibiogram of Isolates

	DP1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	FP1	LKO
Ampicillin	R	R	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R
Cloxacillin	R	R	S	S	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R
Penicillin	R	R	S	S	S	R	R	S	S	R	S	S	S	S	R	S	S	S	R	S	S	S	S	S	S	S	R
Enrofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Pefloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	R	R	S	S	S	S	S	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	R	R	R	R	R	R	R	S	R	S	S	S	R	R	S	R	R	R	R	R	R	R	R	R	S	R	R
Tetracycline	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
Metronidazole	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Nitrofurantoxin	S	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Co-trimoxazole	S	S	S	S	R	R	R	R	S	S	S	S	S	R	S	S	S	R	R	S	S	S	S	S	S	S	S
Furazolidone	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
Ciprofloxacin	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	R	S	S
Bacitracin	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S

Seven isolates representing 25.92 per cent of the isolates tested were found to be resistant to co-trimoxazole.

Furazolidine and streptomycin showed a sensitivity of only 22.2 per cent while all the isolates were resistant to metronidazole.

4.4 BIOTYPING

The avian isolates of *P. multocida* as well as the reference strain LKO differed in their ability to ferment arabinose, maltose, sorbitol, trehalose and xylose. Variations in the fermentation patterns of these sugars allowed the recognition of ten distinct biochemical types, termed biovars A-J (Table 4). Based on the criteria followed by Mutters *et al.* (1985) two biotypes were observed among the twenty-five duck isolates of *P. multocida* . Isolates DP4 and DP 5 did not ferment sorbitol and dulcitol and hence were biotyped as *Pasteurella multocida* subsp *septica* and all other duck isolates as well as the fowl isolates FP1 and LKO fermented sorbitol, but not dulcitol and hence were biotyped as *Pasteurella multocida* subsp. *multocida*.

4.5 PATHOGENICITY TESTING IN MICE

All the 25 duck isolates of *P. multocida* as well as the fowl isolates FP1 and LKO were able to kill weaned mice. A concentration of 0.3×10^8 organisms per 0.1 ml was able to kill mice within 24 h when injected by intra-peritoneal route.

The gross lesions observed in the internal organs of the dead mice were petechiae in the pericardium and congestion of lung, liver and spleen. Blood smear and impression smears from spleen and liver collected from dead mice, on staining with Leishman's stain, revealed the presence of bipolar shaped organisms (Fig 6). Re-isolation of *P. multocida* was done from the heart blood, lungs, liver and spleen on ovine/bovine blood agar.

Table 4. Biochemical differentiation of avian isolates of *P.multocida*

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

Biovar A isolates : DP 1,2 and 3

Biovar B isolates : DP 13,14 and 24

Biovar C isolates : DP 18 and 20

Biovar D isolate : DP 4

Biovar E isolate : DP 5

Biovar F isolates : DP 7,9,10,11,12,15,16,17 and 23

Biovar G isolates : DP 8, 19 and FP1

Biovar H isolate : DP 25

Biovar I isolates : DP 21, 22 and LKO

Biovar J isolate : DP 6

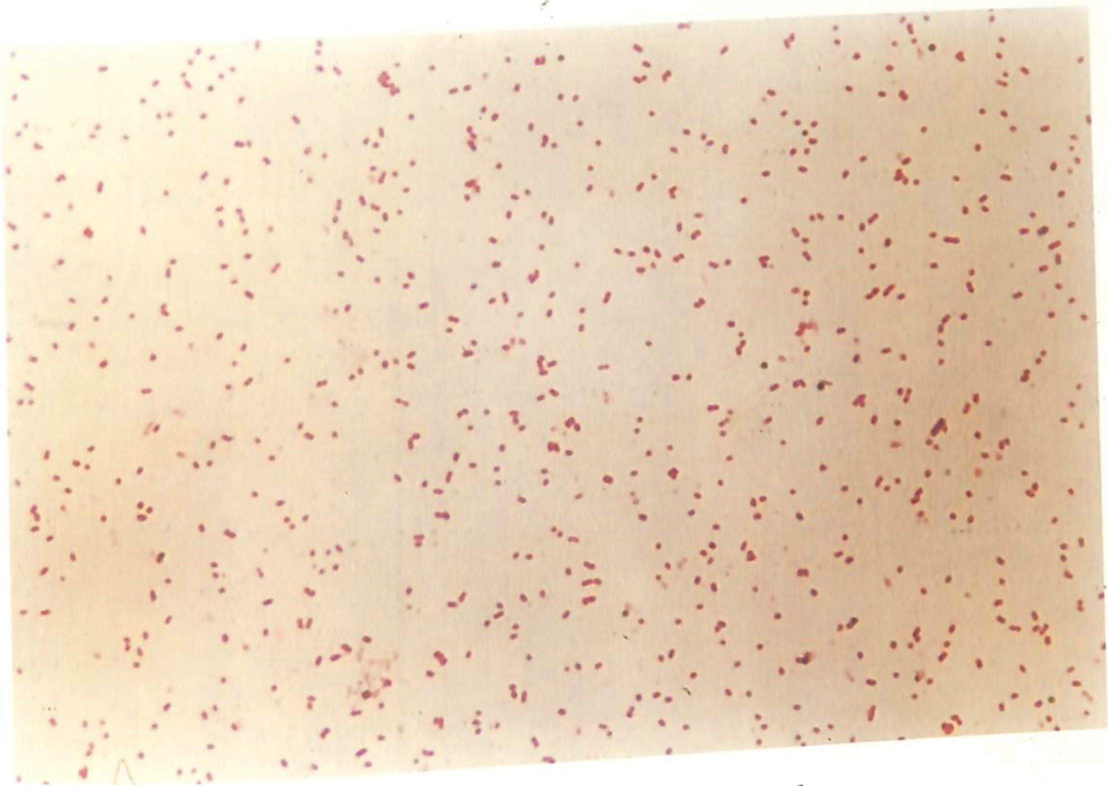


Fig. 5 Pure culture of *P. multocida*
(Gram's stain 1000 x)

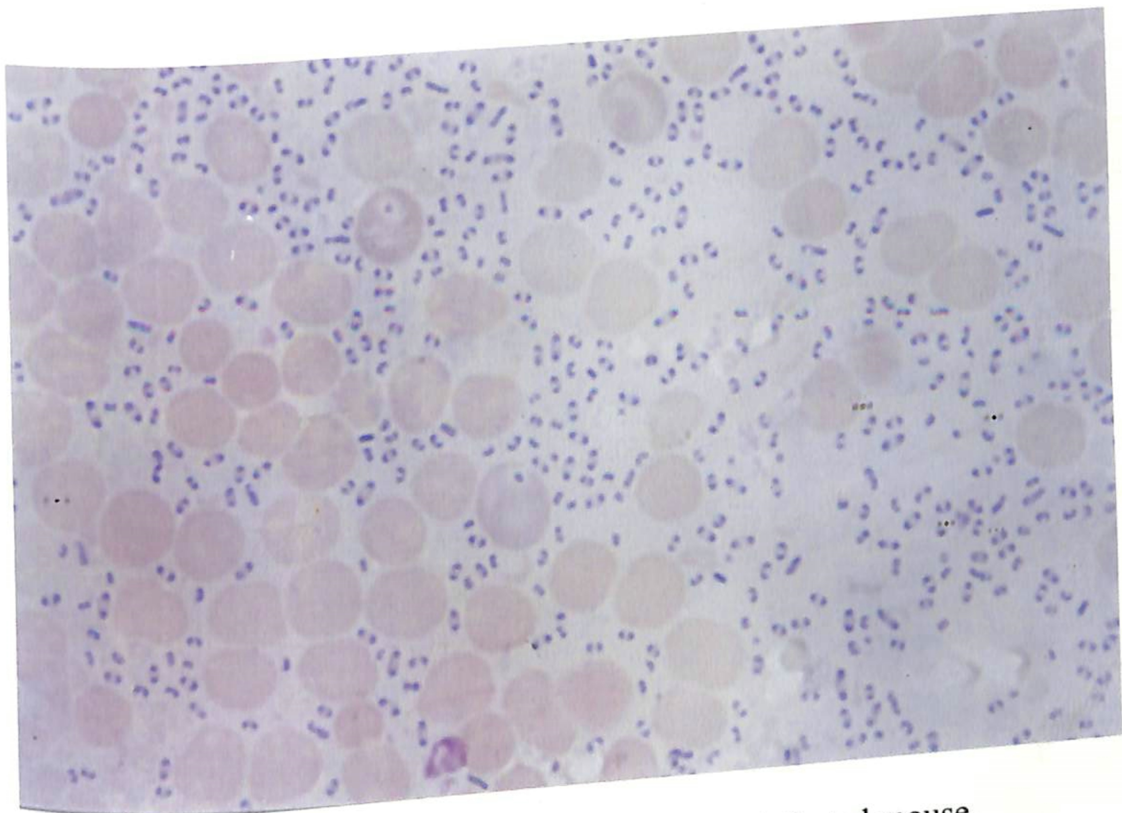


Fig. 6 Blood smear from exprimentally infected mouse.
(Leishman's stain 1000 x)

4.6 STORAGE OF ISOLATES

The *P. multocida* isolates, stored at -70ºC in defibrinated ovine/bovine blood, were revived once a month. All the isolates stored in this manner could be revived in blood agar and were found to be pure. Isolates were found to be viable for a period ranging from six months to one year.

4.7 SEROTYPING OF THE ISOLATES

All the isolates were sent to Indian Veterinary Research Institute, Izatnagar (UP), India for serotyping. Serotyping results are available only for nine isolates. Eight isolates from ducks (DP 1 to 8) and one isolate from fowl were serotyped (Table 5).

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

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

All the 25 isolates from ducks, the fowl isolate FP1 and the standard reference strain LKO when subjected to specific amplification by PM-PCR were found to be PM-PCR positive. Agarose gel (1.5 per cent) electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (pBR 322 DNA/*Alu* I digest) in 1 x TAE buffer. Analysis of the electrophoresed gel under UV transilluminator revealed the presence of a 460 base pair (bp) band in all the 25 isolates as well as the reference strain LKO and the fowl isolate FP1 (Fig. 7). In the negative control no amplification product was detected. Similar type of results were obtained with all types of preparations such as bacterial culture lysate, direct bacterial colony, genomic DNA and boiled extracts from morbid materials subjected to PCR amplification.

When PCR was performed with template DNA prepared from blood smears from suspected cases of pasteurellosis, a 460 bp product was obtained, although the intensity of the band was less compared to those derived from template DNA prepared from bacterial cultures.

Table 5. Serotyping of *P. multocida* isolates

S.No	Lab code	Species from which isolated	Serotype
1	DP 1	Duck	A:1
2	DP 2	Duck	A:1
3	DP 3	Duck	A:1
4	DP 4	Duck	A:1
5	DP 5	Duck	A:1
6	DP 6	Duck	A:1
7	DP 7	Duck	A:1
8	DP 8	Duck	A:1
9	FP 1	Fowl	A:1

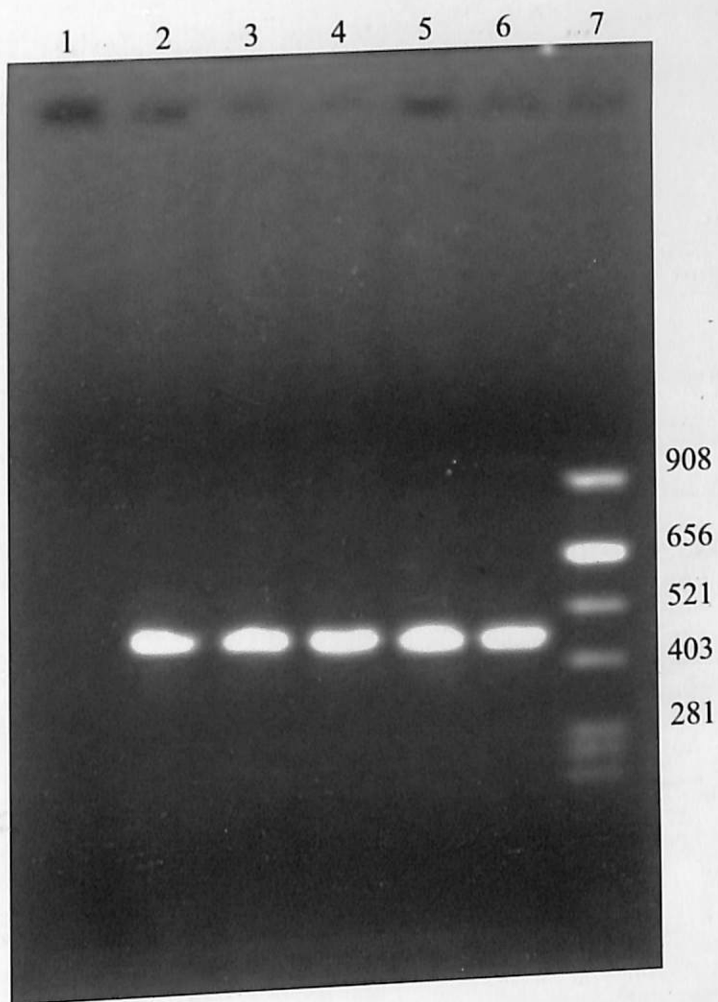


Fig. 7 Detection of *P. multocida* by PM-PCR

Lane 1 Negative control

Lane 2-4 DP 1, DP 12 & DP 25

Lane 5-6 FP 1 & LKO

Lane 7 pBR 322 DNA/*Alu* I digest marker

4.8.1.1 Specificity of the Primers

No amplification product was detected when primers KMTISP6 and KMTIT7 were used to amplify the DNA prepared from *Streptococcus zooepidemicus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Leptospira* serogroup canicola serovar *canicola*.

4.8.2 PCR-Restriction Fragment Length Polymorphism (RFLP)

When the PCR product (460bp) amplified by using primer pairs KMTISP6 and KMTIT7, was digested with restriction enzyme *Hae* III, two bands of molecular sizes 317 bp and 143 bp were observed. No polymorphism was observed, as all the 25 duck isolates, the reference strain LKO and the fowl isolate FP1 showed similar restriction patterns (Fig. 8).

4.9 RESTRICTION ENDONUCLEASE ANALYSIS OF GENOMIC DNA.

The genomic DNA isolated from all the twenty five duck isolates of *P. multocida* as well as the fowl isolates FP1 and LKO were subjected to REA with restriction enzymes *Hpa* II and *Hha* I.

Three different banding patterns were observed among the 25 duck isolates of *P. multocida* and the fowl isolates LKO and FP1, when they were subjected to restriction enzyme analysis with *Hpa* II enzyme. Isolates DP 1 to DP 20, except DP 6, FP 1 and LKO could be grouped into profile I. Profile II consisted of a single isolate viz., DP 6. Five isolates DP 21 to 25 were grouped into profile III (Fig. 9).

Restriction analysis of genomic DNA of all isolates with *Hha* I, also yielded three profiles. Isolates DP 20 and 25 were placed in profile I, DP 6, which had a unique profile was grouped in profile II , while rest of the isolates, including the fowl isolates LKO and FP 1 were categorized as profile III (Fig. 10)

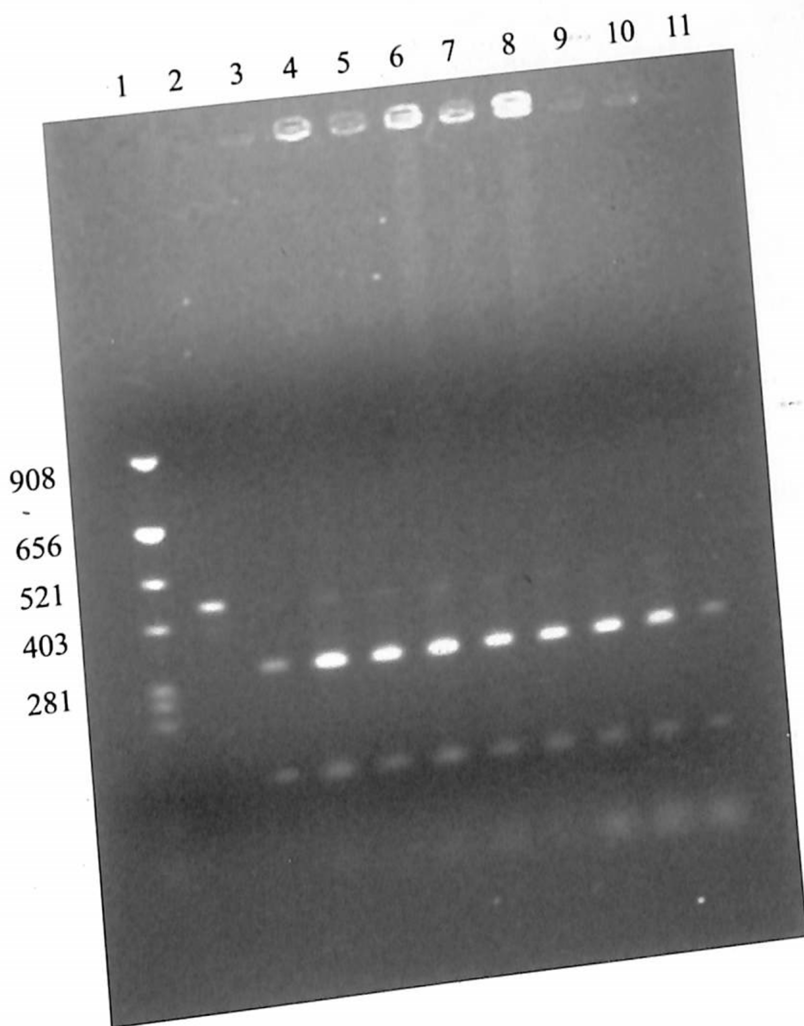


Fig. 8 PCR-RFLP of 460 bp amplified PCR product digested with *Hae* III

Lane 1 pBR 322 DNA/*Alu* I digest marker

Lane 2 Undigested 460 bp PCR product

Lane 3-9 DP 1, DP 6, DP 10, DP 14, DP 18, DP 20 & DP 25

Lane 10 FP 1

Lane 11 LKO

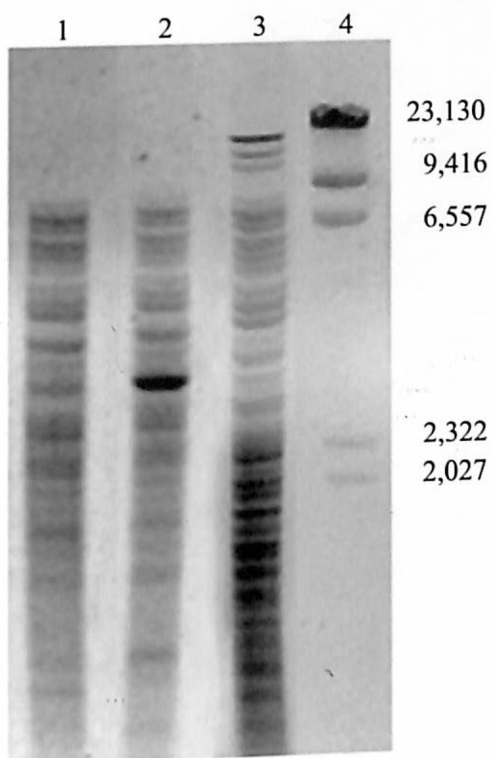


Fig. 9 REA profiles of genomic DNA of *P.multocida* digested with *Hpa* II

Lane 1 Profile I

Lane 2 Profile II

Lane 3 Profile III

Lane 4 Lambda DNA/ *Hind* III digest

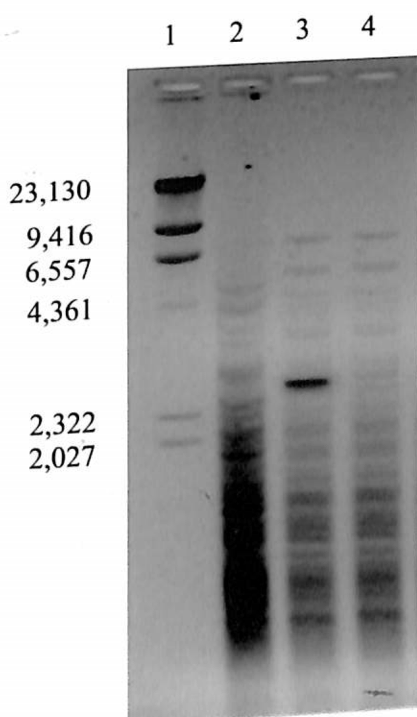


Fig. 10 REA profiles of genomic DNA of *P.multocida* digested with *Hha* I

Lane 1 Lambda DNA/ *Hind* III digest

Lane 2 Profile I

Lane 3 Profile II

Lane 4 Profile III

4.10 PLASMID PROFILE OF *P. multocida* ISOLATES

The plasmid profiles of the duck isolates of *P. multocida* have been analysed. Among the 25 isolates from ducks three duck isolates DP 8, 12 and 18 lacked plasmids. Rest of the duck isolates could be grouped into two plasmid profiles (Fig. 11). Isolate DP 6 which harboured four plasmids (46.2 kbp, 10.9 kbp, 6.5 kb and 0.8 kbp) was categorized as profile I while the rest of the isolates, each of which carried a single high molecular weight plasmid (46.2kbp) were grouped in profile II. Of the fowl isolates, FP1 carried a single high molecular weight plasmid (46.2 kbp) while LKO did not harbour any.

4.11 REP – PCR

All the 25 duck isolates of *P. multocida* as well as the fowl isolates LKO and FP1, which were found to be positive by PM-PCR, were subjected to REP-PCR, using the primer pairs REP-1 and REP-2. Analysis of the banding patterns of REP fragments indicated a total of 13 bands, ranging in size from 4.0 kbp to 330 bp. The REP profile of all the 25 duck isolates and the fowl isolate FP 1 appeared to be identical. The reference strain LKO showed an extra band of size about 800 bp (Fig. 12)

4.12 WHOLE CELL PROTEIN PROFILE BY SDS-PAGE

Whole cell proteins of four representative (DP1, DP6, DP12 and DP25) isolates of *P. multocida* from ducks, fowl isolate FP1 and LKO and a bovine strain P:52 were extracted by the method described by Ireland *et al.* (1991) and subjected to the discontinuous system of SDS-PAGE along with the standard protein marker (Fig.13).

The standard protein marker (Genei, Bangalore) yielded five bands ranging from 97.4 kDa to 18.4 kDa. Examination of the whole cell protein profiles of the duck isolates of *P. multocida* as well as the fowl isolates and the bovine strain revealed the presence of 20 to 26 visible protein bands. Their

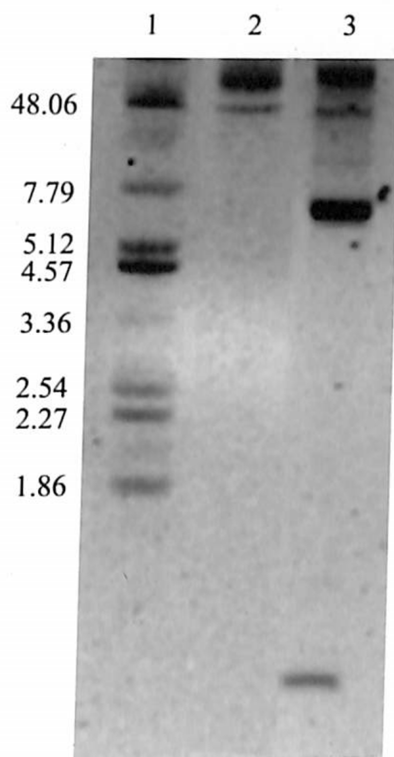


Fig. 11 Plasmid profiles of *P.multocida*

Lane 1 *E.coli* V517

Lane 2 Profile I

Lane 3 Profile II

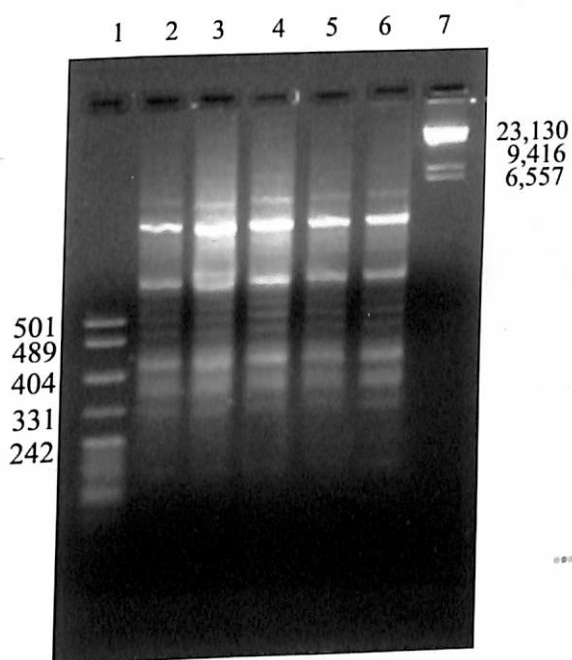


Fig. 12 REP-PCR profiles of *P.multocida*

Lane 1 pUC 19 DNA/*Msp* I digest marker

Lane 2-3 FP 1 & LKO

Lane 4-6 DP 1, DP 16 & DP 25

Lane 7 Lambda DNA/*Hind* III digest marker

molecular weights ranged from 102 kDa to 19 kDa, as determined by the calibration curve between Rf values and molecular weights of the standard protein markers. Five intensely stained major bands could be located, at 85.90, 56.75 , 52.23 , 45.09 and 35.97 kDa. No staining occurred in the 20-26 kDa region. The bovine strain B:2 showed an intense band around 32 kDa.

4.13 ANALYSIS OF OMPs OF *P. multocida*

4.13.1 Protein Concentration of Outer Membrane Proteins (OMPs)

The outer membrane proteins of four duck isolates of *P. multocida* DP 1, DP 6, DP 12 and DP 25, fowl isolates FP 1and LKO and one bovine strain P:52 were extracted by the process of sonication and ultra centrifugation as per the method described by Davis and Donachie (1996)

The protein concentration of the OMPs were estimated by method of Lowry *et al.* (1951). The outer membrane protein concentration of the four representative duck isolates of *P. multocida*, viz. DP 1, DP 6, DP 12 and DP 25, the fowl isolates FP 1and LKO and the bovine strain B:2 were found to be 2.6 mg/ml, 3.1 mg/ml, 4.16 mg/ml, 2.86 mg/ml, 3.46mg/ml, 3.75 mg/ml and 4.05 mg/ml respectively.

4.13.2 Determination of Optimal Conditions for Analysis of Outer Membrane Proteins of *P. multocida*

At 50ºC and 60ºC proteins with approximate molecular weights of 156 kDa and 124 kDa and 26 kDa were intensely stained. At 70ºC a thick, intensely staining band could be observed around 37 kDa. This protein was also seen at OMPs treated at 80ºC, 90ºC and 100ºC. However, the thickness of band was higher at samples treated at 100ºC. The high molecular weight proteins seen at 50ºC and 60ºC could not be observed at 80ºC, 90ºC and 100ºC. Samples treated at 70ºC showed a band at 156 kDa region but the 124 kDa protein seen at 50ºC and 60ºC was not observed (Fig 14).

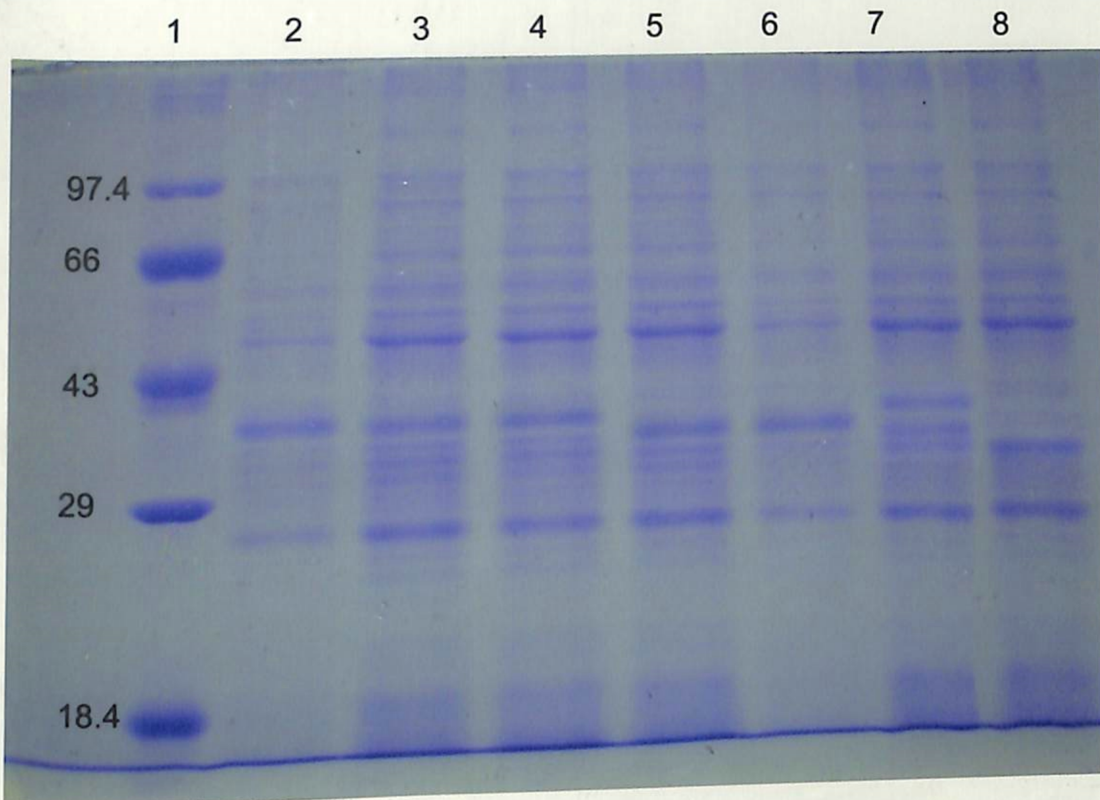


Fig. 13 Whole cell protein profiles of *P. multocida*
Lane 1 Medium range protein mol. wt. marker
Lane 2-8 FP1, DP 1, DP 6, DP 12, DP 25, LKO & B: 2

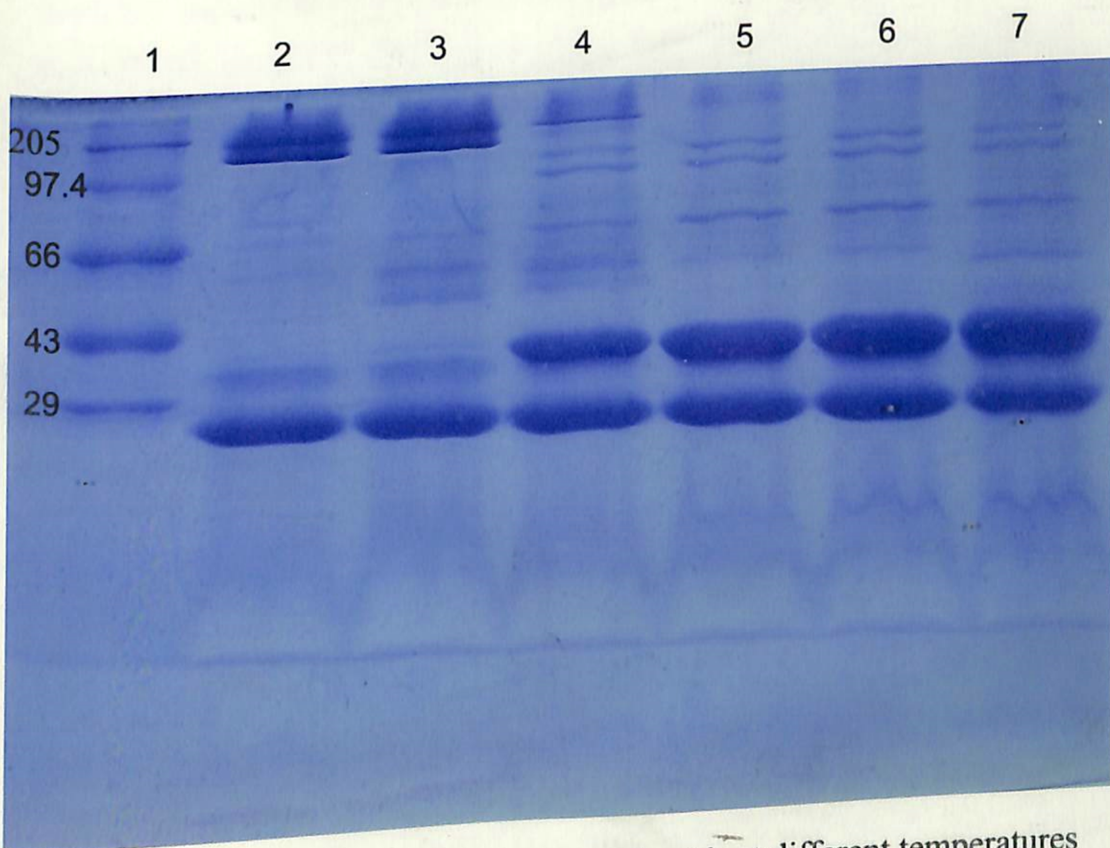


Fig. 14 Resolution of OMPs of *P. multocida* at different temperatures
Lane 1 Broad range protein mol. wt. marker
Lane 2-7 OMPs subjected to 50, 60, 70, 80, 90 & 100°C

4.13.3 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS- PAGE) Profiles of OMP Rich Extract

The outer membrane protein (OMP) extracts and standard protein marker were subjected to SDS-PAGE.

The standard protein marker yielded five bands ranging in molecular weights from 97.4 kDa to 18.4 kDa. The OMP profiles of all the four duck isolates and the fowl isolates FP 1 and LKO were almost identical. There were about 10 protein bands with approximate molecular weight ranging from 19.02 to 91.84 kDa as determined by the calibration curve between Rf values and molecular weights of the standard protein markers. Molecular weights of the 10 different polypeptide bands were 91.84, 89.74, 79.43, 62.09, 51.05, 44.0, 37.15, 31.33, 26.36 and 19.02 kDa. Two protein bands with approximate molecular weight of 37.15 kDa and 26.36 kDa were thicker than others, suggesting that they were the major outer membrane proteins. Isolates DP 25, FP 1 and the bovine strain B:2 showed an extra band at 100.2kDa. The bovine strain B:2 showed two major OMPs around 32 kDa and 24.86kDa (Fig. 15).

4.14 DETECTION OF ANTIGENIC OMPs OF *P. multocida*

4.14.1 Agar Gel Immunodiffusion Test

On viewing the gel against diffuse light three precipitin lines, one thick and two faint bands, could be seen. Serum from rabbit number two in which precipitin lines could be seen upto 1:32 dilution was used for immunoblotting.

4.14.2 Western Blotting

Western blotting using rabbit hyperimmune sera showed a 37.15 kDa OMP. Based on the thickness of the band this was found to be the major antigenic protein in all the three OMPs of isolates, DP1, DP 25 and FP1 (Fig. 16).

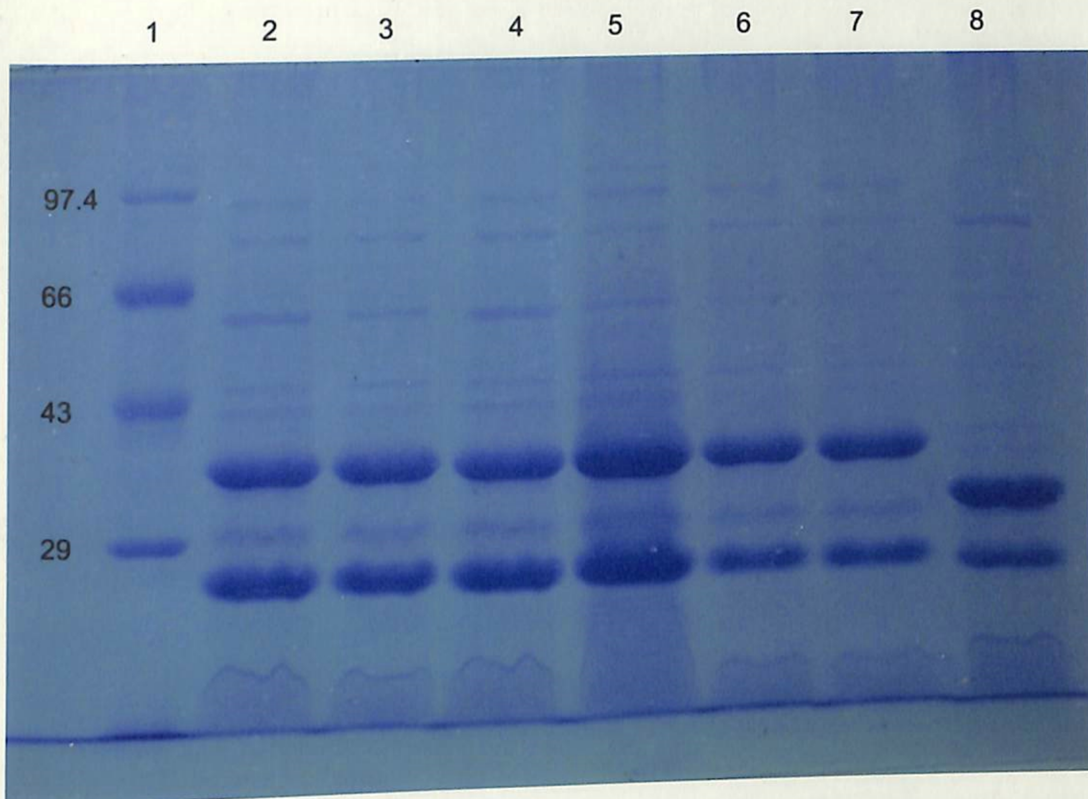


Fig. 15 SDS-PAGE analysis of OMPs of *P. multocida*
Lane 1 Medium range protein mol. wt. marker
Lane 2-8 LKO, DP 1, DP 6, DP 12, DP 25, FP 1 & B:2

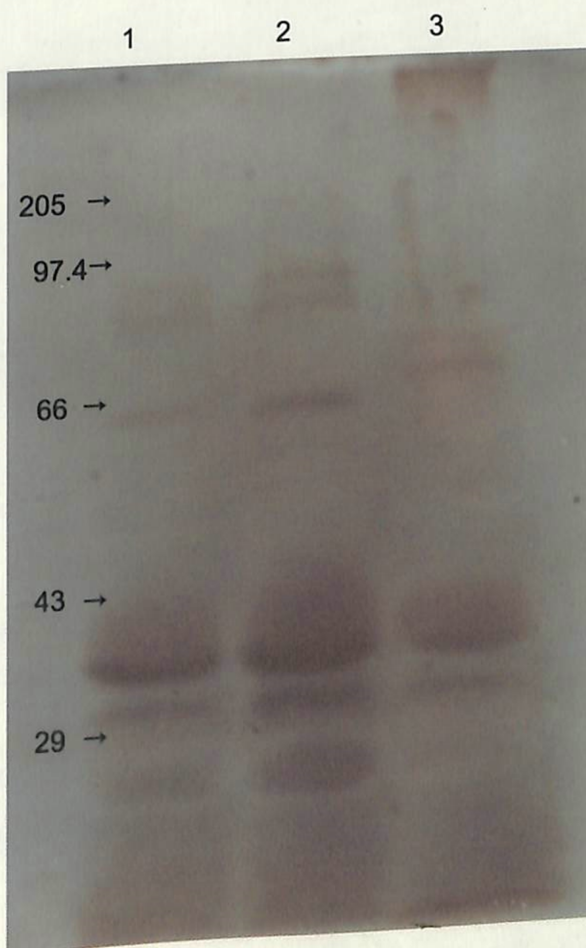


Fig. 16 Immunoblots of *P. multocida*
Lane 1-3 DP 1, DP 25 & FP 1

Apart from this two other proteins with molecular weights 31.33 kDa and 26.36 kDa, which stained less intensely than the previous one, could also be considered as major antigens.

In addition to these three major antigens, protein with a molecular weight of 62.09 kDa was also found in immunoblots of DP1 and DP25, which was absent in FP1.

Immunoblot of DP 25 also revealed two more proteins having molecular weights of approximately 91.84 kDa and 89.74 kDa.

Immunoblot of FP1 showed a faint band at 73.2 kDa, which could also be considered as a minor antigen of this isolate.

4.15 AMPLIFICATION OF O*mpH* GENE BY PCR

The primer pairs OmpH 1 and OmpH 2, designed to amplify the *OmpH* gene of *P. multocida* successfully amplified the *OmpH* gene of all the 25 isolates from ducks, fowl isolate FP1 and the reference strain LKO. It also amplified the *P. multocida* serotypes A:3 and bovine strain B:2 (Fig. 17). The amplified product had a size of approximately 1000bp.

4.15.1 Specificity of the Primers

Primer pairs OmpH 1 and OmpH 2 did not amplify the DNA prepared from unrelated bacterial species such as *Streptococcus zooepidemicus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Leptospira* serogroup *canicola* serovar *canicola*.

4.15.2 Restriction Enzyme Analysis of Amplified PCR Product

The amplified PCR product having an approximately size of 1000 bp was digested with restriction enzymes *Dra* I and *Hinf* I.

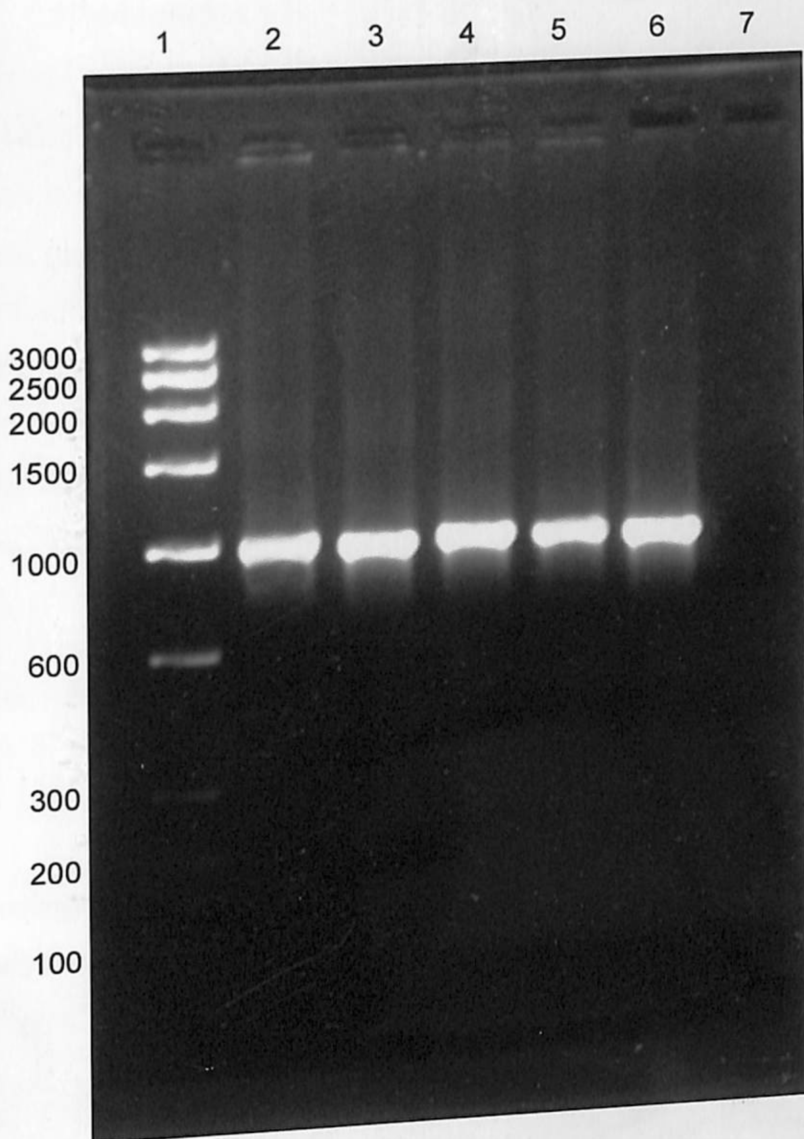


Fig. 17 Amplification of *OmpH* gene of *P. multocida*

Lane 1 Low range DNA marker
Lane 2-3 LKO & FP 1
Lane 4 DP 1
Lane 5 A: 3
Lane 6 B: 2
Lane 7 negative control

Electrophoresis of the restricted PCR products was carried out in eight per cent polyacrylamide gels along with undigested amplified product and pBR 322/*Hinf* I digest molecular size marker. Analysis of *Hinf* I digested PCR product revealed fragments of sizes 821, 107 and 72 base pairs (Fig. 18).While *Dra* I digestion generated four fragments of sizes 346, 314, 209 and 131 base pairs. (Fig 19). Similar digestion patterns were obtained for all the 25 isolates from ducks as well as the fowl isolate FP 1 and the reference strain LKO. Though, the restriction analysis of amplified products for serotypes A:3 and B:2 with *Hinf* I showed a similar pattern, a variation was observed in the A:1 serotype. Digestion of amplified product with *Dra* I from serotypes A:1, A:3 and B:2 showed unique profiles (Fig. 20).

4.16 ANALYSIS OF THE RESTRICTION ENZYME DIGESTED PCR PRODUCTS FROM INACTIVATED FOWL CHOLERA VACCINE

Amplification of *OmpH gene* from template DNA prepared from the inactivated FC vaccine generated a product of about 1000 bp. The amplicons from the vaccine and the duck isolate DP 1 (used for comparison) were digested with restriction endonucleases *Dra* I and *Hinf* I.

Electrophoresis of the restricted amplified products was carried out on eight per cent polyacrylamide gels, along with undigested PCR product and pBR322/*Alu* I digest molecular size marker. On comparison of the fragments obtained by *Hinf* I digestion showed the presence of nine bands ranging in size from (900-72 bp) in the restricted amplified product of vaccine, of which three were having the same size as that of DP1.

Analysis of the fragments obtained by *Dra* I digestion revealed the presence of eight bands (ranging in size from 900-131 bp) in the restricted amplified product of vaccine, of which four were of the same size as that of DP 1 (Fig. 21).

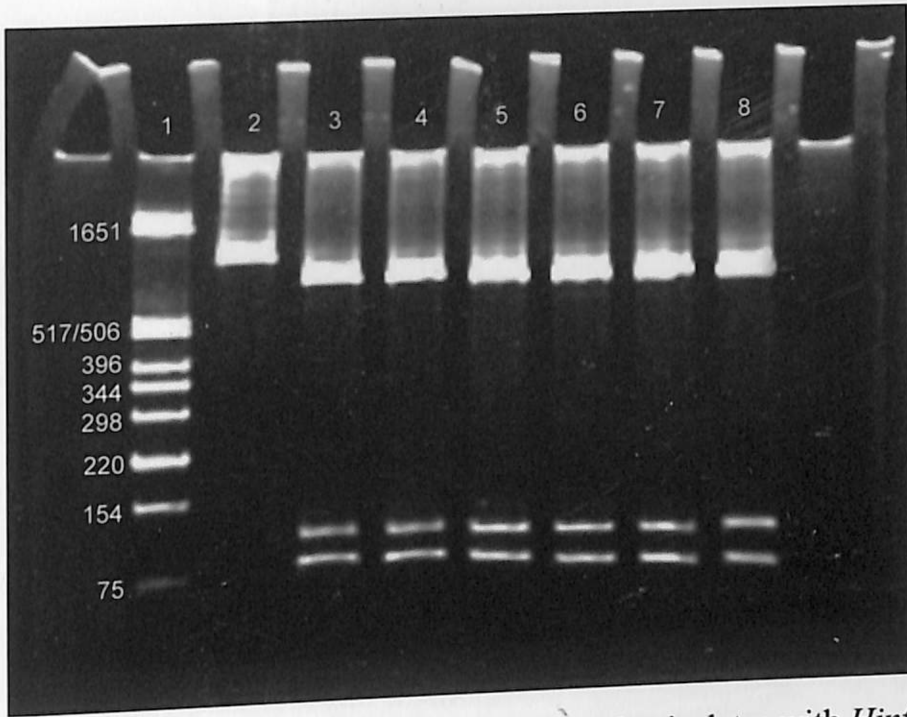


Fig. 18 REA of OmpH-PCR product of avian isolates with *Hinf* I

Lane 1 pBR 322 DNA/*Hinf* I digest marker

Lane 2 Undigested OmpH-PCR product

Lane 3-8 LKO, FP 1, DP 1, DP 8, DP 15 & DP 25

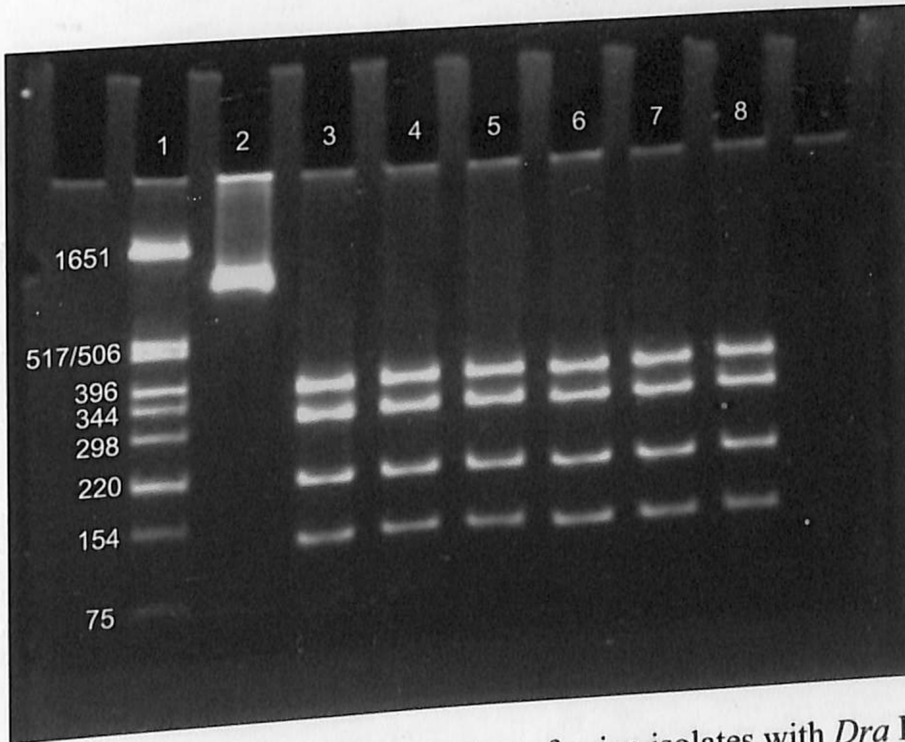


Fig. 19 REA of OmpH-PCR product of avian isolates with *Dra* I

Lane 1 pBR 322 DNA/*Hinf* I digest marker

Lane 2 Undigested OmpH-PCR product

Lane 3-8 LKO, FP 1, DP 1, DP 8, DP 15 & DP 25

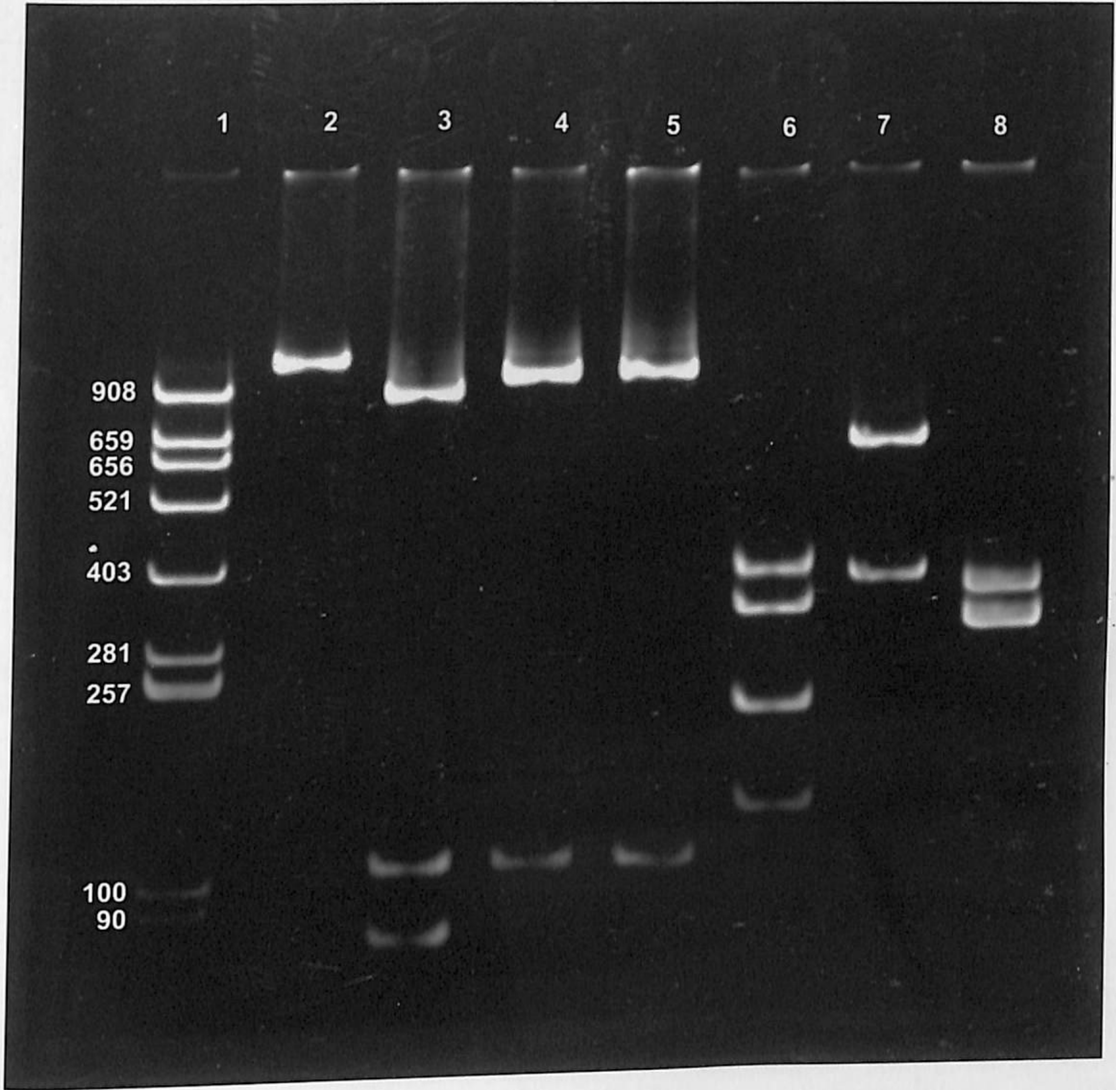


Fig. 20 Restriction enzyme profiles of OmpH-PCR products (serotypes A:1, A:3 & B:2) with *Hinf* I & *Dra* I

Lane 1 pBR 322 DNA/*Alu* I digest marker
Lane 2 Undigested OmpH-PCR product
Lane 3-5 A:1, A:3 & B:2 (*Hinf* I digest)
Lane 6-8 A:1, A:3 & B:2 (*Dra* I digest)

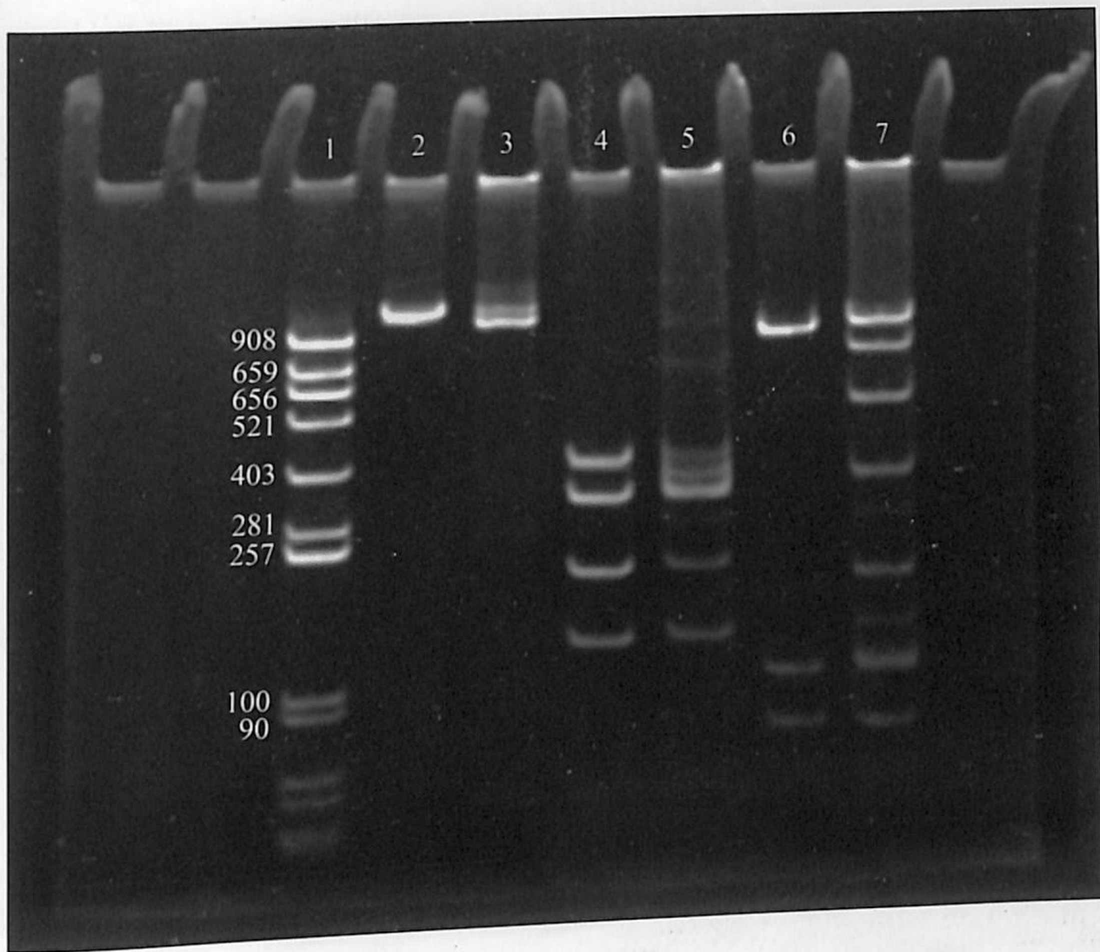


Fig. 21 REA of OmpH-PCR products from inactivated FC vaccine

Lane 1 pBR 322 DNA/*Alu* I digest marker
Lane 2 Undigested OmpH-PCR product from DP 1
Lane 3 Undigested OmpH-PCR products from vaccine
Lane 4 OmpH-PCR product of DP 1 digested with *Dra* I
Lane 5 OmpH-PCR products of vaccine digested with *Dra* I
Lane 6 OmpH-PCR product of DP I digested with *Hinf* I
Lane 7 OmpH-PCR products of vaccine digested with *Hinf* I

4.17 SEQUENCING

The PCR product with an approximately molecular size of 1000 bp was sequenced by Sanger's dideoxy chain termination method.

The amplified product was electroeluted from the gel and used for sequencing. The eluted product was further checked by electrophoresis for the presence of any non-specific bands or oligonucleotide primers. Sequencing revealed the presence of 994 base pairs and is shown in figure 22. The sequence has been submitted to the GenBank and has been assigned the accession No AY606823.

Sequence similarity searches were performed with Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI). The sequence had 98 per cent identity with *P. multocida* strain X-73 outer membrane protein (*OmpH*) gene (Accession No U50907). The result of the alignment is shown in figure 23.

Fig. 17 Sequence ***of OmpH gene*** **of DP1**

gcgtttcattcaaagcatctcatgatttaggcgaaggcttaagcgcattagcttatacagaacttcgttttagtaaaaatgtacccgtgcaagtaaaagaccaacaagg
tgaagtagtacgtgagtatgaggttgagaaacttggtaacaatgttcacgtaaaacgtctttatgcgggtttcgcgtatgaaggtttaggtacattaaca ttcggtaacc
aattaactatcggtgatgatgttggtctatctgactatacctatttcaacagtggtattaataacctcctttctagcggtgaaaaagcaattaactttaaatctgcagaatt
caatggtttcacatttggtggtgcgtatgtcttctctgctgatgctgacaaacaagcattacgtgatggtcgcggtttcgttgtagcaggtttatacaacagaaaaatgg
gtgatgttggttttgcattcgaagccggttatagccaaaaatatgtgaaacaagaagttgaacaagcacaagcaccaaaagtatttaaagatgaaaaagagaaagct
ttcatggtgggtgctgagttatcatatgctggtttagcgcttggtgttgactacgcacaatctaaagtgactaacgtagatggtaaaaaacgtgctcttgaagtgggttt
aaattatgaccttaacgacagagcgaaagtttacacagacttcatctgggaaaaagaaggtcc taaaggtgatgttacaagaaaccgtactgtcgctgtaggttttgg
ttacaaacttcacaaacaagtggaaacttttgttgaagcagcttggggtagagagaaagactctgatggtgtaacaacaaaaaacaacgtagtaggtacaggtttac
gcgtacacttctaatttttgttagaatctgaaaaaagccagtgttaaacactggctttttattgggttttatttgttttacttacaataaattaggattttgaa agtcgttacgc
ggtcat- 994bp

Fig 23. Results of sequence alignment

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.6 [Apr-09-2003]

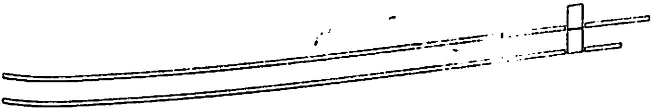
Sequence 1 U50907
Sequence 2 AY606823

NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score = 1838 bits (956), Expect = 0.0
Identities = 987/1000 (98%), Gaps = 6/1000 (0%)
 Strand = Plus / Plus


Query: 561   gcgtttcattcaaagcatctcatgatttaggcgaaggcttaagcgcattagcttatacag 620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1     gcgtttcattcaaagcatctcatgatttaggcgaaggcttaagcgcattagcttatacag 60


Query: 621   aacttcgttttagtaaaaatgtacccgtgcaagtaaaagaccaacaaggtgaagtagtac 680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 61    aacttcgttttagtaaaaatgtacccgtgcaagtaaaagaccaacaaggtgaagtagtac 120


Query: 681   gtgagtatgaggttgagaaacttggtaacaatgttcacgtaaaacgtctttatgcgggtt 740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 121   gtgagtatgaggttgagaaacttggtaacaatgttcacgtaaaacgtctttatgcgggtt 180


Query: 741   tcgcgtatgaaggtttaggtacattaacattcggtaaccaattaactatcggtgatgatg 800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 181   tcgcgtatgaaggtttaggtacattaacattcggtaaccaattaactatcggtgatgatg 240


Query: 801   ttggtctatctgactatacctatttcaacagtggtattaataacctcctttctagcggtg 860
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 241   ttggtctatctgactatacctatttcaacagtggtattaataacctcctttctagcggtg 300


Query: 861   aaaaagcaattaactttaaatctgcagaattcaatggtttcacatttggtggtgcgtatg 920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 301   aaaaagcaattaactttaaatctgcagaattcaatggtttcacatttggtggtgcgtatg 360


Query: 921   tcttctctgctgatgctgacaaacaagcattacgtgatggtcgcggtttcgttgtagcag 980
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 361   tcttctctgctgatgctgacaaacaagcattacgtgatggtcgcggtttcgttgtagcag 420


Query: 981   gtttatacaacagaaaaatgggtgatgttggttttgcattcgaagccggttatagccaaa 1040
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct: 421   gtttatacaacagaaaaatgggtgatgttggttttgcattcgaagccggttatagccaaa 480

Query: 1041 aatatgtgaaacaagaagttgaacagaatccgccagcagcacaaaaagtgtttaaagatg 1100
             |||||||||||||||||| |||||            ||  ||||| |||||| ||||||||||
Sbjct: 481   aatatgtgaaacaagaagtagaaca------agcacaagcaccaaaagtatttaaagatg 534

Query: 1101 aaaaagagaaagctttcatggtgggtgctgagttatcatatgctggtttagcgcttggtg 1160
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 535   aaaaagagaaagctttcatggtgggtgctgagttatcatatgctggtttagcgcttggtg 594

Query: 1161 ttgactacgcacaatctaaagtgactaacgtagatggtaaaaaacgtgctcttgaagtgg 1220
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 595   ttgactacgcacaatctaaagtgactaacgtagatggtaaaaaacgtgctcttgaagtgg 654

Query: 1221 gtttaaattatgaccttaacgacagagcgaaagtttacacagacttcatctgggaaaaag 1280
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 655   gtttaaattatgaccttaacgacagagcgaaagtttacacagacttcatctgggaaaaag 714

Query: 1281 aaggtcctaaaggtgatgttacaagaaaccgtactgtcgctgtaggttttggttacaaac 1340
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 715   aaggtcctaaaggtgatgttacaagaaaccgtactgtcgctgtaggttttggttacaaac 774

Query: 1341 ttcacaaacaagtggaaactttgttgaagcagcttggggtagagagaaagactctgatg 1400
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 775   ttcacaaacaagtggaaactttgttgaagcagcttggggtagagagaaagactctgatg 834

Query: 1401 gtgtaacaacaaaaaacaacgtagtaggtacaggtttacgcgtacacttctaatttttgt 1460
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 835   gtgtaacaacaaaaaacaacgtagtaggtacaggtttacgcgtacacttctaatttttgt 894

Query: 1461 tagaatctgaaaaaagccagtgttaaacactggcttttattgggtttatttgttttac 1520
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 895   tagaatctgaaaaaagccagtgttaaacactggcttttattgggtttatttgttttac 954

Query: 1521 ttacaataaattaggatttgaaagtcgttacgcggtcat 1560
             |||||||||||||||||||||||||||||||||||||||
Sbjct: 955   ttacaataaattaggatttgaaagtcgttacgcggtcat 994

Score = 50.7 bits (26), Expect = 0.010
Identities = 28/29 (96%)
 Strand = Plus / Minus
```



```
Query: 1471 aaaaagccagtgttaaacactggctttt 1499
             |||||||||||||| ||||||||||||||
Sbjct: 933  aaaaagccagtgtttaacactggctttt 905

CPU time:     0.03 user secs.     0.00 sys. secs     0.03 total secs.

Lambda     K      H
```

```
  1.33    0.621     1.12

Gapped
Lambda      K       H
  1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 3
Number of Sequences: 0
Number of extensions: 3
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 1604
length of database: 10,875,341,743
effective HSP length: 25
effective length of query: 1579
effective length of database: 10,875,341,718
effective search space: 17172164572722
effective search space used: 17172164572722
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Discussion

5. DISCUSSION

Avian pasteurellosis is a septicaemic disease of chicken, ducks and turkeys, caused by avian strains of *P. multocida*. In Kerala, there are over 11.87 lakhs of ducks (Anon., 1996). Among the various infectious diseases, which take a heavy toll, pasteurellosis is a major concern to the poultry industry, resulting in huge economic loss. Identification and characterization of *P. multocida* by conventional culture methods have been supported by modern molecular biology techniques, which are based on the genetic information of the organism. Molecular approaches such as amplification of specific nucleic acid sequences of the genome has allowed bacterial detection and disease diagnosis, easy, fast and reliable, as compared to the labourious and time consuming conventional techniques.

5.1 POST MORTEM LESIONS

In most cases the gross lesions comprised of epicardial petechiae, enlargement, congestion and pin point white multiple necrotic foci of the liver, enlargement and congestion of spleen, haemorrhagic tracheitis and diffuse haemorrhages in internal organs like proventriculus, lungs and intestines. Similar findings have been reported by several workers (Panda *et al.*,1981; Kulkarni *et al.*, 1988 and Glisson *et al.*,1989).

Smears prepared from the heart blood, liver and spleen when stained by Leishman's stain revealed bipolar organisms suggestive of *Pasteurella*. Chandran *et al.* (1995) autopsied 50 Japanese quails and demonstrated bipolar organisms morphologically indistinguishable from *P. multocida*, in heart blood and liver impression smears.

5.2 ISOLATION

Primary isolation of the organism was done in five per cent ovine/bovine blood agar. Both the types of blood agar were equally useful for primary isolation of *P. multocida*. Several workers have used ovine blood agar for primary isolation of *P. multocida.* Zhao *et al.* (1992) used five per cent ovine blood agar 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) found bovine blood agar to be useful in 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) and Rajalakshmi (2001).

5.3 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 is probably due to the high amount of capsular material. Similar observations have been made by Mutters *et al.* (1989).

All the twenty-five isolate from ducks (DP1 to DP25) as well as the fowl isolates (FPI) and the reference strains LKO were Gram-negative, coccobacillary, non-motile and catalase and oxidase positive. None of the isolates grew on Mac Conkey's agar. These results are in agreement with those of Carter (1984) and Kawamoto *et al.* (1990).

The second stage biochemical reactions used for characterization of *P. multocida* (Barrow and Feltham, 1993) were almost identical for the 25 duck

isolates of *P. multocida* as well as the fowl isolates FP1 and LKO. Variations were observed only in the fermentation of the sugars.

Similar findings have been reported by Fegan *et al.* (1995); Blackall *et al.* (1997) and Mehrotra and Bhargava (1999).

All the isolates uniformly fermented glucose, galactose, mannose and sucrose and did not utilize lactose. Rimler and Rhoades (1989) have identified these sugars as well as fructose as the sugars most commonly fermented by *P. multocida*.

The isolates differed in their ability to ferment, inositol, maltose, salicin and mannitol. These sugars were fermented by 11, 29, 18 and 88 per cent of the isolates respectively. Several workers have reported such variations in sugar fermentation. (Mohan *et al.*, 1994; Rajini *et al.*, 1995 and Rajalakshmi, 2001). However several others (Chandran *et al.*, 1995 and Fegan *et al.*, 1995) have reported fermentation of mannitol by all the isolates of *P. multocida* tested.

Positive reactions to indole and ornithine decarboxylase have been described as the most useful biochemical indicators in the identification of *P. multocida. Riemerella anatipestifer* can be readily distinguished from *P. multocida* based on inability of the former to produce indole, non-fermentation of glucose, lactose, sucrose, maltose and a negative ornithine decarboxylase reaction (OIE, 2000).

On the basis of morphological, cultural and biochemical characteristics all the duck isolates as well as the fowl isolate FP1 were identified as *P. multocida*.

5.4 BIOTYPING

Separation of isolates of *P. multocida* into subgroups or biotypes is based upon variation in biochemical characteristics. This 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 25 duck isolates of *P. multocida* as well as the fowl isolate FP1 and LKO could be grouped into ten biovars A-J (Table 4), 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) reported that action on dulcitol and sorbitol were of taxonomic significance in dividing the taxon *P. multocida* into the following three subsp. The sorbitol positive and dulcitol positive variety becomes subsp. *gallicida*; negative for both, subsp. *septica* and positive for sorbitol but negative for dulcitol becomes subsp. *multocida*.

Based on this criteria the 25 duck isolates and the two fowl isolates of *P. multocida* could be grouped into two subsp. viz., *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. Biovars D (DP 4) and E (DP 5) (Table 4) were classified as *P. multocida* subsp. *septica*. while the rest, were identified as *P. multocida* subsp. *multocida.*

These findings are in accordance with those of Blackall *et al.* (1995) who have classified four biovars A, B, C and D into two subsp. Biovars A, B and C were classified as *P. multocida* subsp. *multocida* and biovar D as *P. multocida* subsp. *septica.*

Majority of the 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 (Schneider, 1948; Smith, 1958; Walser and Davis, 1975; Heddleston, 1976; Blackall *et al.*, 1995 and Muhairwa *et al.*, 2000).

None of the isolates tested could be biotyped as *P. multocida* subsp. *gallicida*. Similar results have been reported by Mohan *et al.* (1994); Blackall *et al.* (1995); Loubinoux *et al.* (1999) and Rajalakshmi (2001).

5.5 ANTIBIOGRAM

A variety of chemotherapeutic agents have been used in the treatment of fowl cholera. Since there is often a wide variation in the responsiveness of *P. multocida* to these agents, *in vitro* drug sensitivity testing is essential for the selection of an appropriate drug in a given situation.

All the duck isolates and fowl isolates of *P. multocida* used in the present study were subjected to antibiotic sensitivity testing. Enrofloxacin, pefloxacin and chloramphenicol appeared to be the most effective drugs as all the isolates tested were found to be sensitive to these antibacterials.

Enrofloxacin was reported to have the highest sensitivity (Abeynayake *et al.*, 1993) while Balakrishnan (1998); Devi *et al.* (2000) and Rajalakshmi (2001) found that pefloxacin was the most effective drug for isolates of *P. multocida* from ducks.

With regard to the sensitivity to chloramphenicol the results of the present study are in agreement with those of Kawamoto *et al.* (1990); Rammanath and Gopal (1993) and Rajini *et al.* (1995) who have also found all *P. multocida* of isolates from cases FC to be highly sensitive to it. However Kulkarni *et al.* (1988) found all the isolates of *P. multocida* from chicken to be resistant to this drug.

Seven isolates representing 25.92 per cent of the isolates tested were resistant to co-trimoxazole. Moderate sensitivity to co-trimoxazole has been reported by several workers. (Sambyal *et al.,* 1988; Bhaumik and Dutta, 1995 and

Rajini *et al.,* 1995). This present study indicates a shift in sensitivity pattern of drugs once considered to be the drug of choice for FC.

All the isolates tested were resistant to metronidazole, while only 22.2 per cent of the isolates tested were sensitive to furazolidone. Similar observations have been made by Balakrishnan (1998).

5.6 PATHOGENICITY STUDIES IN MICE

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

Sambyal *et al*. (1988) Jayakumar (1998) observed that *P. multocida* of duck origin killed mice within 24h and 12 h post inoculation respectively.

Congestion of spleen and liver was observed in the internal organs of mice after experimental infection. Bipolar shaped organisms could be demonstrated in the heart blood and impression smears of spleen and liver by Lesihman's staining. The organism could be isolated from the heart blood and visceral organs.

Collins and Woolcock (1976) observed an overwhelming increase in the number of *P. multocida* in visceral organs associated with death in mice, following experimental infection. Similar observations have also been made by Balakrishnan (1998) and Rajalakshmi (2001).

5.7 SEROTYPING

Pasteurella multocida is characterized serologically by identification of capsular antigens by passive haemagglutination (Carter, 1955) and somatic antigens by agar gel diffusion tests (Heddleston *et al.*, 1972). Five capsular serotypes A, B, D, E and F and 16 somatic serotypes have been recognized.

In the present study all the 25 duck isolates as well as the fowl isolate FPI were sent to the Division of Bacteriology and Mycology, Indian Veterinary

Research Institute, Izatnagar, Uttar Pradesh, for serotyping. Of these serotyping results for only nine isolates are available. All of them have been serotyped as A:1.

Choi *et al.* (1989) have reported that serotypes A:1, A:3 and A:4 were responsible for avian pasteurellosis in the United States.

Serotype A:3 had been implicated as the single reason for fowl cholera in chicken and ducks in Zimbabwe.(Mohan *et al.,*1994).

Using capsular PCR typing Davis *et al.* (2003) characterized one hundred avian *P. multocida* isolates from England and Wales and found that 68 per cent of them were capsular type A.

Outbreaks of FC in ducks due to serotype A:1 have been reported in India. (Rammanath and Gopal, 1993 and Murugkar and Ghosh, 1995). The results of the present study are in agreement with these findings.

5.8 STORAGE OF ISOLATES

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

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

Amplification of all the *P. multocida* isolates by PCR using species specific PCR primer pairs, KMTISP6 and KMTIT7 generated product of approximately 460 bp size. These results are in accordance with those of Townsend *et al.* (1998a), Townsend *et al.* (2000) and Dutta *et al.* (2001).

The specificity of the primers was tested by performing PM-PCR on template DNA prepared from non-pasteurella organisms like *Streptococcus*

zooepidemicus, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Leptospira* serogroup *canicola* serovar *canicola*. None of these organisms gave an amplified product. These results are in agreement with those of Townsend *et al.* (1998a), who had reported that the same primer pairs amplified template DNA from all strains of *P. multocida* viz., three subspecies *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica,* as well as *P. canis* biovar 2, but had failed to amplify DNA from other members of *Pasteurellaceae* family, or unrelated bacteria. In India so far there is no report of *P. canis* biovar 2 and hence it might not interfere with the diagnosis of pasteurellosis by PM-PCR. Hunt *et al.* (2000) and Dutta *et al.* (2001) have also reported similar findings.

In the present study different preparations of template DNA were used for PCR amplification. These included purified genomic DNA, boiled culture lysates, boiled extracts from morbid materials and DNA extracts from blood smears.

Initially, purified bacterial genomic DNA and from boiled culture lysates were used in PM-PCR. Since these preparations gave identical results and since the time and effort needed to prepare boiled culture lysates were greatly reduced compared to preparation of purified genomic DNA, boiled culture lysates were routinely used as template DNA for PCR amplification.

Polymerase chain reaction was also carried out by colony touch method in which the bacterial colony was used directly in PCR master mix. The results obtained by this method were similar to those of PM-PCR with purified genomic DNA or boiled culture lysates and hence represent a rapid technique for identification of *P. multocida.* The use of boiled extracts from morbid materials as template DNA in PCR also gave clear results. This technique could be of immense use in cases were the carcasses might be putrified and unfit for routine bacteriological examination. Several workers have reported similar findings (Townsend *et al.*, 1998a; Blackall and Miflin, 2000 and Dutta *et al.*, 2001).

Performing PCR on template DNA prepared from blood smears greatly reduces the time required for a specific diagnosis. The PM-PCR assay using template DNA from blood smears gave clear-cut results, although the band intensity was less compared to those achieved by use of boiled culture lysates as template DNA. This technique will probably be more useful in animal pasteurellosis since blood smears are at times the only material sent by field veterinarians to diagnostic laboratories. The presence of artifacts resembling bipolar organisms often limits the accuracy of diagnosis based on microscopic examination of stained blood smears.

On the basis of the observations made by Towsend *et al.* (1998a), Blackall and Miflin (2000); Dutta *et al.* (2001) and Davis *et al.* (2003), and the results obtained in the present study it could be concluded that PM-PCR offers a rapid and specific method for diagnosis of pasteurellosis. The PCR-assay conducted on template DNA prepared from blood smears represents a novel and practical means of detection of pasteurellosis. Hunt *et al.* (2000) have argued that modifications to sample preparations have allowed PCR analysis to be performed on clinical specimens, dramatically reducing the time required for bacterial identification. Our results also indicate such conclusions.

5.9.1 PCR-Restriction Fragment Length Polymorphism (RFLP)

The approximately 460 bp product of PM-PCR assay was subjected PCR-RFLP using restriction endonuclease *Hae* III. This enzyme was chosen based on results obtained in Web cutter programme. The study was undertaken to detect polymorphism within the KMT1 gene. The digestion resulted in two fragments of molecular sizes 317 and 143 bp. The restriction pattern was identical for all the 25 isolates of *P. multocida* from duck as well as the fowl strains FP 1 and LKO. This suggested that using restriction enzyme *Hae* III no polymorphism could be detected within the KMTI gene.

Similar finding have been reported by Gautam *et al.* (2004) who had used restriction endonuclease *Bgl* II to demonstrate polymorphism within the capsular biosynthetic locus of *P. multocida* serogroup A. The 560 bp product when digested with *Bgl* II yielded three fragments of 220 bp, 185 bp and 155 bp in length. The study revealed that all strains of serogroup A, possessed the same genetic makeup for capsular biosynthetic loci.

5.10 RESTRICTION ENDONUCLEASE ANALYSIS OF GENOMIC DNA

The REA of genomic DNA is now an established technique for the study of molecular epidemology of bacterial infections. This method is a highly reproducible technique that is not influenced by inconsistent expression of phenotypic traits, which limit the sensitivity and specificity of conventional typing methods (Snipes *et al.*, 1989).

The use of direct nucleic acid analysis has been particularly valuable for strain differentiation and allows the monitoring of distribution of strains (Harel *et al.*, 1990).

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). Though several restriction enzymes have been used for DNA fingerprinting of *P. multocida* by REA, *Hpa* II and *Hha* I were found to be the most informative and yield an easily distinguishable profiles from a wide variety of serotypes (Wilson *et al.*, 1992; Blackall *et al.*, 1997 ; Diallo *et al.*, 1995 and Dutta *et al.*, 2003).

The genomic DNA of all the 25 duck isolates of *P. multocida* as well as the fowl isolates FP 1 and LKO, were subjected to REA with *Hpa* II could be grouped into three different profiles (Fig. 9).

Isolates DP 1 to DP 20, except DP 6, FP 1 and LKO could be grouped into profile I. Profile II consisted of a single isolate viz., DP 6. Five isolates DP 21 to 25 were grouped into profile III.

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 three profiles among the 25 duck isolates and two fowl isolates of *P. multocida*.

Restriction fragment length polymorphism generated by *Hpa* II allowed 39 avian strains of *P. multocida* to be placed into ten groups, of which three groups contained a single isolate in each and another group contained 11 isolates. The other six groups contained three to six isolates each (Diallo *et al.*, 1995). A similar observation was made in the present study also.

Using *Hpa* II, Dutta, (2001) observed six different REA profiles among the seven isolates of serotype A:1 from different species of hosts. Out of the three poultry isolates studied two different patterns emerged. However, the same enzyme generated only three profiles amongst the 27 isolates of *P. multocida* examined in the present study.

Restriction analysis of genomic DNA of all isolates with *Hha* I, also yielded three profiles. Isolates DP 20 and 25 were placed in profile I, DP 6, which had a unique profile was grouped in profile II , while rest of the isolates, including the fowl isolates LKO and FP 1 were categorized as profile III (Fig. 10)

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). Based on this study they concluded that restriction endonuclease *Hpa* II was more discriminatory than *Hha* I. In another study, five isolates of *P. multocida* serogroup D, four from pigs and one from sheep, were analysed by REA using *Hha* I and *Hpa* II. Each of these enzymes yielded three different profiles and was concluded that both the enzymes were equally discriminatory Dutta (2001). In the present study, both the enzymes, *Hpa* II and *Hha* I yielded three profiles each and hence both the enzymes were found to be equally useful to differentiate all the 27 avian isolates of *P. multocida.* .

5.11 PLASMID PROFILE OF *P. multocida* ISOLATES

The plasmid DNA of all the duck isolates as well as the fowl isolates were analysed on agarose gel electrophoresis. Of the twenty-five duck isolates, twenty-two (88 per cent) carried plasmids. Gunther *et al.* (1991) demonstrated that among 28 isolates of *P. multocida* from rabbits, 92 per cent carried plasmids. However, two other studies showed greater variation in the occurrence of plasmids in avian isolates and it varied from 24 percent (Price *et al.*, 1993) and 70.7 per cent to (Hirsh *et al.*, 1985).

The sizes of the plasmids detected in the present study ranged from 46.2 kbp to 0.8 kbp. Strains of *P. multocida* have been shown to harbour plasmids from 1.3 kbp. (Diallo *et al.*, 1995) to approximately 100 kbp (Hirsh *et al.*, 1989) in size.

The twenty-five duck isolates could be placed into two plasmid profiles. Profile II consisted of a single isolate, DP6, which carried four plasmids with molecular sizes of 46.2, 10.9, 6.5 and 0.8 kbp. Of the remaining twenty-four duck isolates three (DP 8, DP 12 and DP 18) did not harbour any plasmids, while the remaining 21 carried a single plasmid each of 46.2 kbp size. Amongst the fowl isolates, FP1 carried a single plasmid (46.2 kbp) while strain LKO did not harbour any.

Diallo *et al.* (1995) observed that of the 45 avian strains of *P. multocida* studied, twenty strains yielded no plasmids. The remaining twenty-five were placed into two plasmid profiles. Profile I consisted of seven isolates that carried a single plasmid each of 1.3 kbp while the remaining 18 were grouped into profile II that showed two plasmids each of 2.4 and 7.5 kbp

Of the four avian strains of *P. multocida* examined by Balakrishnan (1998) three of them were found to contain plasmids, ranging in size from 2.69 kbp to 7.07 kbp.

Similar results have also been reported by Shivshankara *et al.* (2000). They identified that 10 out of 12 isolates of *P. multocida* contained plasmids and they were placed into two groups, group one containing seven isolates, carrying a single plasmid and remaining isolates forming group II harbouring two plasmids each.

The isolates DP 8, DP 12 and DP 18 did not harbour any plasmids. However, they were found to be resistant to five, one and four antimicrobial agents respectively out of the 15 tested. Thus correlation between the presence of plasmids and antibiotic resistance could not be ascertained in the present study. Similar observations have been made by Diallo *et al.* (1995) who found that although only 55 per cent of the 45 avian isolates of *P. multocida* carried plasmids, all of them were uniformly resistant to streptomycin, trimethoprim and lincomycin.

Pathogenicity tests conducted in mice revealed that all the isolates, even those which lacked plasmids were pathogenic. All the duck as well as the fowl isolates killed weaned mice within 24h. This observation could not establish any correlation between the presence of plasmids and virulence. These results are in accordance with the observations made by Diallo *et al.* (1995); Balakrishnan (1998) and Shivshankara *et al.* (2000).

An interesting finding of the present study is that isolate DP6, showed a unique plasmid profile as well as a unique REA profile with both *Hpa* II and *Hha* I, compared to the rest of the isolates. A similar observation was made in the REA patterns of *P. multocida* serotype D isolates of porcine origin by Harel *et al.* (1990) using restriction enzyme *Eco R* I. They suggested that the unique set of restriction fragments generated, might have been contributed by the genomic as well as the plasmid DNA of the organism.

Rubies *et al.* (2002) have also reported a strong relationship between REA patterns and the presence of plasmids. Their results were based on plasmid profiles and REA patterns of 218 isolates of *P. multocida* from swine.

5.12 REP-PCR

Repetitive extragenic palindromic (REP) PCR has been shown to be a highly discriminatory fingerprinting method that differentiates strains of related bacteria. Analysis of the distribution of REP sequences in prokaryotic genomes forms the basis of a novel PCR-DNA fingerprinting technique known as REP-PCR.

REP-PCR fingerprints of *P. multocida* isolates causing haemorrhagic septicaemia showed a high degree of homogeneity while that of *P. multocida* isolates causing FC exhibited a marked heterogeneity (Townsend *et al*., 1997b). In the present study all the 25 duck isolates of *P. multocida* as well as the fowl isolates showed a similar REP-PCR profile.

These findings are similar to those observed by Townsend *et al.* (1998b) and Gunarwardana *et al.* (2000). They reported that isolates of *P. multocida* from FC (serotype A:1) and HS (serotype B:2) from Vietnam, showed only minimal variation with a single REP-profile for each of them. Interestingly the isolates used in the present study for which the serotyping results are available belong to serotype A:1.

5.13 PROTEIN PROFILES OF *P. multocida*

5.13.1 Whole Cell Protein Pofiles of *P. multocida*

The whole cell protein profiles of the four duck isolates of *P. multocida* viz., DP1, DP6, DP 12 and DP 25 and the fowl isolates FP 1 and LKO when analysed on SDS-PAGE revealed 20 to 26 protein bands, with molecular weights ranging from 102 kDa to 19 kDa.

These results are similar to the findings of Lee *et al*. (1991) who had reported the presence of 26 bands in SDS-PAGE of whole cell proteins of *P. multocida* isolates from chicken and turkeys. The molecular weights of the proteins ranged from 160 kDa to 14 kDa.

Kedrak and Opacka (2002) analysed the whole cell protein profiles of 18 avian isolates of *P. multocida*, serotypes 1 and 3, on 10 per cent polyacrylamide gels and found that they contained 18 to 31 clearly visible protein fractions ranging in molecular weights from 24 kDa to 140 kDa.

In the present investigation the protein profile of all the avian isolates of *P. multocida* were almost identical. Isolates DP 12 and DP 25 showed minor differences in the position of the approximately 37 kDa protein. These findings are in accordance with those of Ireland *et al.* (1991) who examined the protein profiles of *P. multocida*, serotype 1, isolated from cases of FC. The patterns obtained with Coomassie blue staining of whole cell proteins were similar. The major differences among isolates were seen in the 34 kDa to 38 kDa region.

5.13.2 Analysis of OMPs of *P. multocida*

5.13.2.1 *Optimal Conditions for Analysis of Outer Membrane Proteins of P. multocida.*

An attempt was made to determine the optimal conditions for analysis of OMPs. The OMPs of the duck isolate DP 1 was subjected to heat treatment

ranging from 50ºC to 100ºC and then analysed on 12.5 per cent SDS-PAGE. It appeared that heat seemed to have a significant effect on the molecular weight of the OMPs.

The major OMP of *P. multocida* with an approximate molecular mass of 37 kDa could not be seen in preparations heated to 50ºC and 60ºC. They could only be observed in preparations subjected to heat treatment of 70ºC or higher.

These findings are in accordance with those of Davis *et al.* (2003), who have reported that the major OMP of various capsular serotypes of avian *P. multocida*, viz., A, F, D and B, with a molecular mass ranging from 33.1 to 38.3 kDa, could not be seen in the gel unless heated to 60ºC or higher, prior to analysis on SDS-PAGE. They opined that this protein, which they termed as OmpH, was tightly associated with the peptidoglycan and was not released unless heated to a temperature of 60ºC or higher.

5.13.2.2 Outer Membrane Proteins of P. multocida

The outer membrane protein (OMP) of the representative duck isolates as well as the fowl isolates FP1 and LKO and the bovine isolate B:2 when analysed on SDS-PAGE revealed 10 protein bands with approximate molecular weights ranging from 19.02 to 91.84 kDa. Among these two OMPs with molecular weights of 37.15 kDa and 26.36 kDa were considered as the major OMPs, based on the staining intensity and thickness. However, the major OMPs of the bovine isolate B:2 were 32 kDa and 24.86 kDa.

Choi *et al.* (1989) reported that the major OMP of standard reference strain for *P. multocida* viz., X-73 strain, serotype A:1 had a molecular weight of approximately 38 kDa. Two other proteins with approximate molecular weights of 29 kDa and 45 kDa were also considered as major OMPs, although they stained less intensely compared to the 38 kDa protein. The isolate DP1, DP6 and FP1 have been serotyped as A:1 and the results of the present study are in agreement with those of Choi *et al.* (1989). The 44 kDa (band with less staining

intensity), 37.15 kDa and 26.36 kDa proteins observed in the present study might be similar to the 45 kDa, 38 kDa and 29 kDa protein reported by Choi *et al.* (1989).

Marandi and Mittal (1995) examined the OMPs of reference strains of five capsular serotypes of *P. multocida*. They found that the major OMPs when analysed by SDS-PAGE had a molecular mass of 37, 32, 38 and 36 kDa for the capsular serotypes A, B, E and F respectively. Capsular serotype D exhibited two OMP types, type I and type II with molecular weights of 32 and 37 kDa respectively. Our study reflects this picture since the four duck isolates and fowl isolate FP1 have been serotyped as A:1 and the major OMP is around 37 kDa.

Luo *et al.* (1997) have also reported that the major OMP of *P. multocida* serotype A:1, strain X-73, had a molecular mass of approximately 37 kDa.

Several other workers (Lu *et al.*, 1988 and Chevalier *et al.*, 1993) have also reported that a protein with a molecular mass of 37.5 kDa was the major OMP of *P. multocida*.

Confer *et al.* (1996) had observed that protein bands in the range of 100 to16 kDa could be observed in the OMPs extracted from *P. multocida* serotype A:3. The 35 to 36 kDa protein was considered to be the major OMP. Similar results have also been reported by Kedrak and Opacka (2002) in their study on OMPs extracted from poultry isolates of *P. multocida*, serotype A:3. Eleven proteins could be observed with molecular weights ranging from 104 kDa to 20 kDa. No staining occurred in the 19-26 kDa region. Similar findings have been reported by Ireland *et al.* (1991) who had observed the absence of protein bands in the 20-26 kDa region of the gel as it was consistent with the presence of lipopolysaccharide (LPS) and hence did not stain well with Coomassie blue.

Rajalakshmi (2001) identified two major OMPs with molecular weights of 27 and 32 to 33 kDa in the five isolates of *P. multocida* of avian origin.

However, in the present study the major OMP was found to have a molecular weight of approximately 37 kDa.

Davis *et al.* (2003) isolated OMPs from 100 avian *P. multocida* isolates belonging to various capsular serotypes and identified two major OMPs with molecular weights ranging from 36.9 to 37.9 kDa and 33.1 to 38.3 kDa respectively. The 36.9 to 37.9 kDa protein could be correlated with the 37.15 kDa protein seen in all the avian isolates used in the present study.

5.14 DETECTION OF ANTIGENIC OMPs OF *P. multocida*

5.14.1 Agar Gel Immunodiffusion Test

The presence of three precipitin lines in AGID test is probably due to the fact that the antiserum raised against whole cell protein reacted with multiple antigen fractions in the *P. multocida* sonicated antigen.

5.14.2 Western Blotting

By Western blotting using hyperimmune serum against fowl isolate FP1, raised in rabbits, a 37.15 kDa outer membrane protein was found to be the major antigenic protein, based on the thickness of the band, in all the three isolates viz., DP1, DP 25 and FP1, tested.

Two other proteins with molecular weights of 31.33 kDa and 26.36 kDa could also be considered as major antigens based on the staining intensity, although they stained less intensely compared to the 37.15 kDa protein. These findings are in accordance with the studies of Lu *et al.* (1991a) who identified a 37.5 kDa outer membrane protein of *P. multocida*, serotype A:3, as the major reactive antigen for a protective monoclonal antibody.

The results of the present study are also in agreement with those of Ireland *et al.* (1991) who observed strong reactions with proteins of molecular weights 26, 31 and 34 kDa, by Western blotting of OMPs of *P. multocida* serotype A:1 isolated from cases of fowl cholera.

Pati *et al.* (1996) have reported that the proteins with molecular weights of 30 kDa, 37 kDa and 44 kDa were the major immunogens of *P. multocida*. The antigens with molecular weights of 31.33 kDa and 37.15 kDa observed in the present study could probably be correlated with the 30 kDa and 37 kDa immunogens observed by Pati *et al.* (1996). However, the 44 kDa protein described by Pati *et al.* (1996) was not observed to be antigenic by Western blotting although this protein was observed in SDS-PAGE stained gel.

Chawak *et al.* (2001) reported that two OMPs with molecular weights of 17 kDa and 25.7 kDa were the major immunogens of avian strains of *P. multocida*. But in the present study no protein with a molecular weight of 17 kDa was found to be antigenic. The 25.7 kDa protein reported by Chawak *et al.* (2001) may be equated to the 26.36 kDa antigen identified in our study.

Western blotting revealed a 39 kDa protein to be a major immunogen of *P. multocida* X-73 serotype A:1 (Borrathybay *et al.*, 2003). This protein may be similar to the 37.15 kDa OMP identified as the major antigen in all the isolates examined in the present investigation.

Several studies have been carried out to identify the immunogenically important outer membrane proteins (OMPs) of *P. multocida*. Lu *et al.* (1991b) demonstrated that vaccination with OMPs protected rabbits against homologous challenge.

A monoclonal antibody (MAb) against a 37.5 kDa OMP protected both mice and rabbits against *P. multocida* infection (Lu *et al.*, 1991a).

Zhang *et al.* (1994) demonstrated that the polyclonal antibodies to a 35.5 kDa OMP protected chicken against avian pasteurellosis. Marandi and Mittal (1997) speculated the possible role played by 32 kDa porin-H specific protective MAbs, in the inhibition of colonization of *P. multocida* on the respiratory mucosal surface.

Further studies are required to ascertain the immunogenicity of the antigens detected by western blotting in the present investigation.

5.15 AMPLIFICATION OF *OmpH* GENE OF *Pasteurella multocida*

The oligonucleotides designed to amplify the *OmpH* gene, based on the sequence of *P. multocida* X-73 *OmpH* gene, successfully amplified the *OmpH* genes of all the duck isolates of *P. multocida* as well as the fowl strains FP 1 and LKO. This PCR assay also amplified the *OmpH* genes from *P. multocida* serotypes A:3 and B:2 . A single PCR product with a molecular size of approximately 1000 bp was obtained in each case. Similar results were reported by Luo *et al.* (1999) who had reported successful amplification of *OmpH* genes of the serotypes 1, 3 and 4 to 16 of *P. multocida* and the product had an approximate size of 1 kilo-base pairs.

5.15.1 Restriction Enzyme Analysis of Amplified Products of OmpH-PCR

Amplified products of OmpH-PCR were subjected to REA using the enzymes *Dra* I and *Hinf* I. These two endonucleases were chosen with the help of the Webcutter programme, on the published *OmpH* gene sequence and since these enzymes could generate clearly distinguishable fragments.

When the products were digested with restriction enzyme *Dra* I, four fragments of molecular sizes 346, 314, 209 and 131 were generated. (Fig. 19). The PCR-RFLP of all the 27 duck isolates from ducks and fowl yielded similar patterns.

Restriction digestion with *Hinf* I generated three fragments of molecular sizes 821, 107 and 72 bp. The patterns were identical for all the duck isolates and the fowl isolates FP 1 and LKO. (Fig. 18)

Thus the PCR-RFLP of OmpH-PCR products with the two restriction endonucleases viz., *Dra* I and *Hinf* I could not reveal any polymorphism or heterogeneity within or between the duck and fowl isolates of *P. multocida.* This

indicates a high level of homogeneity amongst the amplified region of the isolates.

The serotyping results so far available indicate that most of the duck isolates and fowl isolate (FP1) of *P. multocida* belong to serotype A:1. This further indicates the high level of homogeneity between the duck and the fowl isolates of *P. multocida* isolated from Kerala.

This finding could be of epidemiological significance, indicating that fowl isolates could readily infect ducks and vice-versa. The pathogenicity studies also seemed to support this finding.

Muhairwa *et al.* (2001a) showed that strains of *P. multocida* isolated from chicken and ducks in the same area were identical by phenotypic markers and ribotyping. They suggested that the isolates of chicken and ducks from the same area were closely related and have the potential to adapt to different hosts. The authors also stressed the importance of dogs acting as a transient carrier of *P. multocida* and transmitting the disease to other flocks by moving carcasses. A similar scenario is seen in the villages of Kerala where ducks and fowl are kept in close proximity and an ever-increasing population of stray dogs may help in the transmission of the disease between the species.

Restriction analysis of the amplified products of serotypes A:3 and B:2 were also carried out using the same restriction endonucleases *Dra* I and *Hinf.* Enzyme *Hinf* I generated patterns similar in A:3 and B:2 but distinct from A:1, while *Dra* I profiles were distinct from each other for the three serotypes (Fig 20). Thus, REA of amplified PCR products of OmpH-PCR with *Dra* I offers a novel technique for differentiation of various serotypes of *P. multocida*. However, further studies have to be carried out with all the different serotypes to know whether profiles unique to each serotype are obtained, before the technique can be put for routine use.

5.16 RESTRICTION ENZYME ANALYSIS OF AMPLIFIED PRODUCTS OF OmpH-PCR FROM INACTIVATED OIL ADJUVANT FOWL CHOLERA VACCINE.

Extraction of template DNA from inactivated oil adjuvant fowl cholera vaccine, using isopropanol was found to be a simple rapid and efficient method, for performing PCR.

Maas *et al.* (2003) used isopropyl myristate to extract New Castle disease (ND) virus from inactivated ND oil emulsified vaccine. They used the antigens extracted for quantification of haemagglutinin-neuraminidase (NH) and fusion (F) proteins of NDV in oil-adjuvanted vaccines.

Cholera Triple is a trivalent fowl cholera vaccine incorporating serotypes A:1, A:3 and A:4. The duck isolate DP 1 has been serotyped as A:1 by IVRI, Izatnagar. Similar sized restriction fragments generated by enzymes, viz., *Dra* I and *Hinf* I in both the duck isolate DP1 and the vaccine indicates that there is no polymorphism in the *OmpH* gene of DP 1 as compared to those of the vaccine.

Several studies have indicated that OmpH is an important immunogen of *P. multocida*. Native OmpH was shown to be able to induce protective immunity in chicken against homologous challenge. (Luo *et al*., 1999)

From the results of the present study it can be concluded that *OmpH* gene of the local duck isolate DP 1 and the A:1 serotype incorporated in the vaccine are similar. Thus the vaccine (Cholerin Triple) can be expected to confer immunity to ducks in Kerala against pasteurellosis.

The additional fragments observed in the restriction profile of *Dra* I and *Hinf* I on the OMPH-PCR product derived from the vaccine is probably attributable to the two other serotypes viz., A:3 and A:4 present in the vaccine. The PCR-RFLP studies with A:3 and B:2 serotypes supports this hypothesis.

5.17 SEQUENCING OF *OmpH* GENE

The PCR product with an approximately molecular size of 1000 bp, sequenced by Sanger's dideoxy chain termination method revealed a product of 994 base pairs. The sequence has been submitted to the GenBank and has been assigned the accession No AY606823. Sequence similarity searches were performed with BLAST provided by NCBI showed a 98 per cent identity with *P. multocida* strain X-73 outer membrane protein (*OmpH*) gene (Accession No U50907). These findings are in accordance with that of Luo *et al.* (1999) who analyzed the DNA sequence of *OmpH* genes of *P. multocida* serotypes 4-16. The PCR products were purified and directly sequenced. Their study revealed a high degree of homology among different serotypes.

The present investigation indicates that serotype A:1 is prevalent in Kerala and there is little variation between the isolates from ducks and chicken. However, continuous monitoring of the field situation for emergence of new serotypes is a must for effective control of the disease. This situation necessitates the serotyping of the field isolates at the earliest. The PCR-RFLP of OmpH-PCR product offers a simple effective tool in this endeavour. This technique has the potential to identify a serotype with a few days of the receipt of the sample. However, work has to be carried out with all serotypes to determine profiles unique to each of them before the technique can be put to routine use.

A 37.15 kDa protein has been identified as the major OMP of avian isolates of *P. multocida*. Correlation between capsule content and the amounts of major OMPs produced has been reported (Borrathybay *et al*., 2003). Hence selection of strain expressing high concentration of OMP (37.15 kDa) as vaccine candidate as well as formulation of media that favours enhanced capsule production may offer a practical solution towards the goal of an efficacious vaccine for pasteurellosis.

Summary

6. SUMMARY

Pasteurellosis and duck plague have been reported as the major cause of mortality in ducks in Kerala. While duck plague has been controlled to some extent by the use of vaccines, pasteurellosis continues to be a major threat to duck farming in the state. Although FC has also been reported in Kerala, its incidence is far less when compared duck pasteurellosis. Work has been carried out on the nucleic acid, protein and plasmid profile of avian isolates of *P. multocida*. However, no effort has been made to study the molecular characteristics of the duck and chicken isolates of *P. multocida* to establish the homogeneity/ heterogeneity amongst them. The present study was undertaken with this objective.

A total of 25 isolates from ducks (DP 1- DP 25) and one from fowl (FP 1) have been employed in this study. They have been characterized as *P. multocida* by morphological, cultural and biochemical tests. A reference chicken strain of *P. multocida* (LKO) was used for comparison.

All the avian isolates were found to be pathogenic for mice. Pure cultures of all the twenty-five isolates as well as the fowl isolates FP1 and LKO were able to kill weaned mice within 24 h post inoculation.

Based on the variation in fermentation patterns of dulcitol, mannitol, sorbitol and trehalose the 27 isolates could be grouped into ten biovars.

Two biotypes were observed among the 27 isolates. Isolates DP 4 and DP 5 were classified as *P. multocida* subsp. *septica*, while the rest were biotyped as *P. multocida* subsp. *multocida*.

All the isolates were uniformly sensitive to enrofloxacin, chloramphenicol and pefloxacin. Seven isolates representing 25.92 per cent were resistant to co-trimoxazole.

Of the 27 isolates, nine (eight duck and one fowl), have been serotyped as A:1 by the Division of Bacteriology and Mycology, IVRI, Izatnagar.

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

The 27 avian isolates were confirmed as *P. multocida* using species-specific primer pairs KMT1SP6 and KMT1T7, where non-pasteurella organisms were used as negative controls. Similar results were obtained with various preparations of template DNA such as purified genomic DNA, boiled culture lysates and DNA obtained from morbid materials and blood smears. Among these template DNA prepared from blood smears represents an extremely rapid and novel method for diagnosis of pasteurella.

Restriction analysis of PM-PCR product of all the isolates with *Hae* III could not reveal any polymorphism within the KMT1 gene, while analysis of the genomic DNA of all the avian isolates with *Hpa* II and *Hha* I revealed polymorphic profiles. Both the enzymes were equally discrimatory as they could group all the isolates into three REA profiles. The fowl isolates had a profile that was common to majority of the duck isolates.

Among the 27 avian isolates examined, three duck isolates and the reference strain LKO did not reveal the presence of any plasmid. Rest of the isolates shared two plasmid profiles. The fowl isolate FP 1 shared a profile with majority of the duck isolates.

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

The outer membrane protein profiles of all the avian isolates were similar. Two protein fractions with molecular weights of 37.15 and 26.36 kDa were identified as the major OMPs by SDS-PAGE.

Western blotting revealed that 37.15-kDa protein is the major antigenic fraction of OMPs of avian isolates of *P. multocida* . In addition to this two other proteins with a molecular mass of 31.33 and 26.36 kDa were also found to be antigenic.

The primers designed from the published *OmpH* gene sequence of a chicken isolate of *P. multocida* successfully amplified the *OmpH* gene of all the avian isolates as well serotypes A:3 and B:2. The amplified product had an approximate molecular size of 1000 bp.

Digestion of the OmpH-PCR product with restriction endonucleases *Dra* I and *Hinf* I generated four and three fragments respectively. The profiles were similar for all the avian isolates of *P. multocida,* suggesting a high degree of homogeneity amongst the amplified region of the isolates.

Restriction analysis of the amplified products of serotypes A:1, A:3 and B:2 were also carried out using the same restriction endonucleases, *Dra* I and *Hinf.* Enzyme *Hinf* I generated similar patterns in A:3 and B:2 but distinct from A:1, while *Dra* I profiles were distinct from each other for the three serotypes. Thus, REA of amplified PCR products of OmpH-PCR with *Dra* I offer a novel technique for differentiation of various serotypes of *P. multocida*. However, further studies have to be carried out with all the different serotypes to know whether profiles unique to each serotype can be obtained, before the technique can be put for routine use.

The use of isopropanol to prepare template DNA from inactivated, oil adjuvanated fowl cholera vaccine represents a simple and efficient method for use in PCR.

The OmpH primers successfully amplified the template DNA prepared from the vaccine. Digestion of the PCR products with restriction enzymes *Dra I* and *Hinf I* generated patterns with many restriction fragments. This may be due to presence of three serotypes A:1, A:3 and A: 4 in the vaccine. However, similar

sized restriction fragments generated by enzymes *Dra* I and *Hinf* I were observed in both the restricted products of the duck isolate DP1 and the vaccine indicating that there is no polymorphism in the *OmpH* gene of DP 1 as compared to those of the vaccine. Thus the *OmpH* gene of A:1 serotype incorporated in the vaccine is similar to the *OmpH* gene of the local duck isolate DP 1. Therefore the vaccine can be expected to confer immunity to ducks in Kerala against pasteurellosis, as OmpH is one of the major antigenic proteins of *P. multocida*.

The OmpH-PCR product with an approximate molecular size of 1000 bp was sequenced by Sanger's dideoxy chain termination method. The size of the product was 994 base pairs. The sequence has been submitted to the GenBank and has been assigned the accession No AY606823. Sequence similarity searches were performed with Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI). The sequence had 98 per cent identity with *P. multocida* strain X-73 (serotype A:1) outer membrane protein (*OmpH*) gene.

References

REFERENCES

Abeynayake, P., Wijewardana, T.G. and Thalagoda, S.A. 1993. Antimicrobial susceptibility of *Pasteurella multocida* isolates. *Proceeding of International workshop on Pasteurellosis in production animals. Bali, Indonesia, 10-13 August, 1992*. pp. 193-196

Alberts, J.O. and Graham, R. 1948. Fowl cholera in turkeys. *North Am Vet*. **29**: 24-26

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

Anon. 1996. *XV Quinquennial livestock census – 1996*. Directorate of Animal Husbandry, Government of Kerala. Tiruvananthapuram. p. 56

Anon. 1998. *Final report ICAR scheme on Development of cell culture duck plague vaccine for simultaneous vaccination with duck pasteurellosis.* Department of Microbiology, College of Veterinary and Animal Sciences, Kerala Agricultural University, Thrissur. p. 93

Anon. 2001. *Annual Report of NATP on Productivity Enhancement of Ducks.* Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Kerala Agricultural University, Thrissur, p.60

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

Barenkamp, S.J., Munson,R.S. and Granoff, D.M. 1981. Subtyping isolates of *Haemophilus influenzae* type B by outer membrane protein profiles. *J. Infect. Dis.* **143** : 668-676

Barrow, C.I. and Feltham, R.K.A. 1993. *Cowan and Steel's manual for identification of medical bacteria.* Third Edn. Cambridge University Press. p. 331

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

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

Bhaumik, A. and Dutta, S. 1995. Studies on bacteriological parameters of *Pasteurella multocida* isolated from Kakhi Campbell ducklings in Tripura. *Indian Vet. J.* **72**:1092-1093

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

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

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

Blackall, P.J., Pahoff, J.L., Marks, D., Fegan, N. and Morrow, C.J. 1995. Characterisation of *Pasteurella multocida* isolated from fowl cholera outbreaks on turkey farms. *Aust. Vet. J.* **72**: 135-138

Borkataki, P., Baxi, K.K., Sambyal, D.S. and Sodhi, S.S. 1987. *In vitro* sensitivity of *Pasteurella multocida* field strains to antimicrobial agents. *J. Res.* PAU, Ludhiana **24**: 307-310

Borrathybay, E., Sawada, T., Kataoka, Y., Okiyama, E., Kawamoto, E. and Amao, H. 2003. Capsule thickness and amounts of a 39 KDa capsular protein of avian *Pasteurella multocida* type A strains correlated with their pathogenicity for chickens. *Vet. Microbiol*. **97**: 215-227

Bowles, R.E., Pahoff, J.L., Smith, B.N. and Blackall, P.J. 2000. Ribotype diversity of porcine *Pasteurella multocida* from Australia. *Aust. Vet. J.* **78**: 630-635

Brogden, K.A. and Rimler, R.B. 1983. Lysates of turkey – grown *Pasteurella multocida* : effects of solubilising agents on the immunologic properties of membrane vesicles. *Am. J. Vet. Res.* **44**: 428-432

Brown, T.A. 1990. *Gene cloning*. Second edition. Library of Congress cataloguing. p. 564

Brown, P.D. and Levett, P.N. 1997. Differentiation of Leptospira species and serovars by PCR-restriction endonuclease analysis, arbitrarily primed PCR and low-stringency PCR. *J. Med. Microbiol*. **46**: 173-181

Buchanan, R.E., Holt, J.G. and Lessel, E. F. 1966. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida. Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

Burrows, T.W. and Gillett, W.A. 1966. Cited by Smith, G.K. and Phillips, J.E. 1990. Buxton, A. and Fraser, G. 1977. *Animal Microbiology*. Vol. 1. Blackwell Scientific Publication, Oxford. pp. 121-130

Buxton, A. and Fraser, G. 1977. *Animal Microbiology*. Vol.1. Blackwell Scientific Publication, Oxford, p. 586

Carpenter, T.E., Snipes, K.P., Kasten, R.W., Hird, D.W. and Hirsh, D.C. 1991. Molecular epidemiology of *Pasteurella multocida* in turkeys. *Am. J. Vet. Res.* **52**: 1345-1349

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

Carter, 1963. Cited by Rimler, R.B. and Rhoades, K.R 1989. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida*. *Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

Carter, G.R. 1967. Pasteurellosis. *Pasteurella multocida and Pasteurella haemolytica. Advances in Veterinary Science*, Vol. 2 (eds. Brandly, C.A. and Cornelius, C.) Academic Press, New York, pp. 321-367

Carter, G.R. 1981. Prokaryotes. *A handbook on habitats, isolation and identification of bacteria* (eds. Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Selegal, H.G.) Springer-Verlag, New York, pp. 1383-1391

Carter, G.R. 1984. In Bergey's Manual of Systematic Bacteriology. Vol. 1 (eds Krieg, N.R and Holt, J.G). Williams and Wilkins. Co., Baltimore. London. p 555.

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

Carter, G.R. and Rundell, S.W. 1975. Identification of type A strains of *Pasteurella multocida* using staphylococcal hyaluronidase. *Vet. Rec.* **96**: 343

Chabert 1782. Cited by Rhoades, K.R. and Rimler, R.B. 1991. Pasteurellosis. *Disease of Poultry*. (eds. Calnek, B.W., Barnes, H.J., Bareand, C.W., Reid, W.M. and Yoder, H.W.) Ninth edition. Iowa State University Press, Ames, Iowa, pp 145-152

Chandran, N.D.J., Prabakar, T.G., Albert, A., David, B.P. and Venkatesan, R.A. 1995. Pasteurellosis in Japanese quail (*Coturnix coturnix japonica*). *Indian Vet. J.* **72**: 876-877

Chaslus-Dancla, E., Lesage, M.C., Leroy-Setrin, S., Martel, J.L., Coudent, P. and Lafont, J.P. 1996. Validation of random amplified polymorphic DNA assays by ribotyping as tools for epidemiological surveys of *Pasteurella* from animals. *Vet. Microbiol*. **52**: 91-102

Chawak, M.M., Verma, K.C. and Kataria, J.M. 2001. Characterization and immunizing properties of outer membrane proteins of *Pasteurella multocida* grown in iron sufficient and iron restricted conditions. *Indian J. Comp. Microbiol. Immunol. Infect. Dis*. **22**: 36-42

Chevalier, G., Duclohier, H., Thomas, D., Shechter, E. and Wroblewski, H. 1993. Purification and characterization of protein H, the major porin of *Pasteurella multocida*. *J. Bacteriol.* **175**: 266-276

Choi, K., Maheswaran, S.K. and Felice, L.J. 1989. Characterization of outer membrane protein-enriched extracts of *Pasteurella multocida* isolated from turkeys. *Am. J. Vet. Res.* **50**: 676-683

Choi, K., Maheswaran, S.K., Felice, L.J. and Molitor, T.W. 1991. Relationship between the iron–regulated outer membrane proteins and the outer membrane proteins of *in vivo* grown *Pasteurella multocida. Vet. Microbiol.* **28**: 75-92

Christensen, J.P. and Bisgaard, M. 2000. Fowl cholera. *Rev. Sci. Tech.* **19**: 626-637

Christensen, J.P., Dietz, H.H. and Bisgaard, M. 1998. Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from backyard poultry and from two outbreaks of avian cholera in avifauna in Denmark. *Avian Pathol.* **27**: 378-381

Christensen, J.P., Olsen, J.E. and Bisgaard, M. 1993. Ribotypes of *Salmonella enterica* serovar *Gallinarum* biovars *gallinarum* and *pullorum*. *Avian Pathol*. **22**: 725-738

Christiansen, K., Carpenter, T.E., Snipes, K.P., Hird, D.W. and Ghazikhanian, G.Y. 1992. Restriction endonuclease analysis of *Pasteurella multocida* isolates from three California turkey premises. *Avian Dis.* **36**: 272-281

Chung, J.Y., Wilkie. I., Boyce, J.D., Townsend, K.M., Frost, A.J., Ghoddusi, M. and Adler, B. 2001. Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. *Infect. Immun*. **69**: 2487-2492

*Collins, F.M. and Woolcock, J.B. 1976. Immune response to *Pasteurella multocida* in the mouse. *J. Reticuloendothelial. Soc*. **19**: 311-321

Confer, A.W., Nutt, S.H., Dabo, S.M. and Panciera, R.J. 1996. Antibody responses of cattle to outer membrane proteins of *Pasteurella multocida* A:3. *Am. J. Vet. Res*. **57**: 1453-1457

Cowan, S.T. 1974. *Cowan and Steel's "Manual for the identification of medical bacteria*. Second edition. Cambridge University Press, Cambridge. p. 364

Curtis, P.E. 1979. Observations on avian pasteurellosis in Britain. *Vet. Rec.* **104**: 471-474

Curtis, P.E. and Ollerhead, G.E. 1981. Investigation to determine whether healthy chickens and turkeys are oral carriers of *Pastuerella multocida*. *Vet. Rec*. **108**: 206-207

Dabo, S.M., Confer, A.W. and Murphy, G.L.1997. Outer membrane proteins of bovine *Pasteurella multocida* serogroup A isolates. *Vet. Microbiol.* **54**: 167-183

Dabo, S.M., Confer, A.W. and Quijano, R.A. 2003. Molecular and immunological characterization of *Pasteurella multocida* serotype A:3 OmpA: evidence of its role in *P. multocida* interaction with extracellular matrix molecules. *Microbiol. Pathogenesis* **35**: 147-157

Dahl, C., Permin, A., Christensen, J.P., Bisgaard, M., Muhaiwra, A.P., Petersen, K., Poulsen, J.S and Jensen, A.L. (2002) The effect of concurrent infections with *Pasteurella multocida* and *Ascaridia galli* on free range chickens. *Vet. Microbiol*. **4**:313-324

Das, M.S. 1958. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida. Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

Das, S.C. and Bhagwan, P.S.K. 1997. Isolation and characterization of *Pasteurella multocida* from ovine pneumonia. *Indian J. Anim. Sci*. **67**: 29-30

Davies, R.L. and Donachie, W. 1996. Intra-specific diversity and host specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. *Microbiology*. **142**: 1895-1907

Davis, R.L., Ali, Q., Parton, R., Coote, J.G., Gibbs, H.A and Freer, J.H 1991. Optimal conditions for the analysis of *Pasteurella haemolytica*

lipopoylsaccharide by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *FEMS Microbiol. Lett*. **90**:23-28

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

Davies, R.L., Parton, R., Coote, J.G., Gibbs, H.A. and Frees, J.H. 1992. Outer-membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype A:1 under different growth conditions. *J. Gen. Microbiol*. **138**: 909-922

*Dawkins, H.J.S. (1989) Large DNA separation using field alternation agar gel electrophoresis. *J. Chromat*. **492**: 615-639

De Alwis, M.C.L. 1973. Cited by De Alwis, 1999.

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

Devi, V.R., Rao,T.S., Aruna, P. and Sujani, G. 2000. Incidence of duck cholera in Krishna district, Andhra Pradesh – a field report. *Indian Vet. J.* **79**: 535-536

Dhillon, S.S., Sambyal, D.S. and Kwatra, M.S. 1989. The *in vitro* drug sensitivity of *Pasteurella multocida* of poultry origin. *Indian J. Comp. Microbiol. Immunol. Infect. Dis*. **10**: 171-174

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

Donahue, J.M. and Olson, L.D. 1972. Characteristics of isolates of *Pasteurella multocida* obtained on consecutive or different years from the same turkey farms. *Avian Dis.* **16**: 529-534

Dorsey, T.A. 1963. Studies on fowl cholera. I. A biochemical study of avian *Pasteurella multocida* strains. *Avian Dis.* **7:** 386-392

*Dougherty, E. 1953 Disease problems confronting the duck industry. *Proc. Annu. Meet. Am. Vet. Med. Assoc*. pp 359-365.

Dutta. T.K. 2001. Molecular characterization of Indian isolates of *P. multocida* causing haemorrhagic septicaemia. PhD thesis submitted to Deemed University IVRI, Izatnagar. p. 86

Dutta, T.K., Gautam, R., Singh, V.P. and Kumar, A.A. 2003. Molecular characterization of *Pasteurella multocida* by REA and RAPD-PCR. *Indian J. Anim. Sci.* **73**: 579-582

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

Dwivedi, P.N. and Sodhi, S.S. 1989. Morphological, cultural and biochemical characterization of *Pasteurella multocida*. III. Isolated from poultry. *Poult. Advisor*. **21**: 69-71

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

*El-Ghali, A., Hussain, A and Mohamed, S.A. 1997. An outbreak of fowl cholera in a poultry farm in Atbara Town. *Sudan J. Vet. Res.* **15**: 25-29

Faddoul, G.P., Fellows, G.W and Baird, J. 1967 Pasteurellosis in wild birds in Massachusetts. *Avian Dis*. **11**:413-418

*Food and Agricultural Organization. 1987. *Animal Health Year book – 1986*. Rome, Italy. p 184

Fegan, N., Blackall, P.J. and Pahoff, J.L. 1995 Phenotypic characterization of *Pasteurella multocida* isolates from Australian poultry. *Vet Microbiol* **47**:281-286.

Freifelder, D. 1970. *Isolation of extrachromosomal DNA from bacteria. Methods in Enzymology* Vol XXI. (eds. Grossman, L. and Moldare, K.). Academic Press Inc., New York. pp 153-163

Gardner, I.A., Kasten, R., Eamens, G.J., Snipes, K.P. and Anderson, R.J. 1994. Molecular fingerprinting of *Pasteurella multocida* associated with progressive atrophic rhinitis in swine herds. *J. Vet. Diagn. Invest*. **6**:442-447

Gatto, N.T., Dabo, S.M., Hancock, R.E. and Confer, A.W. 2002. Characterization of and immune responses in mice to purified OmpA-equivalent outer membrane proteins of *Pasteurella multocida* serotype A:3 (Omp28). *Vet. Microbiol*. **87**: 221-235

Gautam, K., Kumar, A.A., Singh, V.P., Singh, V.P., Dutta, T.K. and Shivachandra, S.B. 2004. Specific identification of *Pasteurella multocida* serogroup A isolates by PCR assay. *Res. Vet. Sci*. **76**: 179-85

Glisson, J.R., Cheng, I.H.N., Rowland, G.N. and Stewart, R.G. 1989. *Pasteurella multocida* infection in Japanese quail (*Coturnix coturnix japonica*) – case report. *Avian Dis.* **32**: 820-822

Goering, R.V. 1993. Molecular epidemiology of nosocomial infection : analysis of chromosomal restriction fragment patterns by pulse-field gel electrophoresis. *Infect. Control Hosp. Epidemiol*. **14** : 595-600

Goto, Y., Nakura, R., Nasu, T., Sawada, T. and Shinjo, T. 2001. Isolation of *Pasteurella multocida* during an outbreak of infectious septicaemia in Japanese quails (*Coturnix coturnix Japonica*) *J. Vet. Med.Sci*. **63**:1055-1056

Grimont, F. and Grimont, P.A.D. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Anim. Inst. Pasteur Micorbiol*. **137**: 165-175

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

Gunther, R., Manning, P.J., Bouma, J.E., Delong, D. and Cook, D.B. 1991. Partial characterization of plasmids from rabbit isolates of *Pasteurella multocida* . *Lab. Anim. Sci*. **41**: 423-426

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

Hansen, L.M. and Hirsh, D.C. 1989. Serum resistance is correlated with encapsidation of avian strains of *Pasteurella multocida*. *Vet. Microbiol*. **27**: 177-184

Harel, J., Cote, S. and Jacques, M. 1990. Restriction endonuclease analysis of porcine *Pasteurella multocida* isolates from Quebec. *Can. J. Vet. Res.* **54**: 422-426

Heddleston, K.L. 1962. Studies on pasteurellosis. V. Two immunogenic types of *Pasteurella multocida* associated with fowl cholera. *Avian Dis*. **6**: 315-321

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

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

Heinemann, M.B., Gracia, J.F., Nunes, C.M., Gregori, F., Higa, Z.M.M., Vasconcellos, S.A. and Richtzenhain, L.J. 2000. Detection and differentiation of Leptospira spp. serovars in bovine serum by polymerase chain reaction and restriction fragment length polymorphism. *Vet. Microbiol.* **73**: 261-267

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

Hirose, K., Tamura, K. and Watanabe, H. 2003. Screening method for *Salmonella enteria* serovar Typhi and Serovar Paratyphi A with reduced susceptibility to fluroquinolones by PCR Restriction Fragment length Polymorphism. *Microbiol. Immunol*. **47**: 161-165

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

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

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

Holmes, B. 1998. Actinobacillus, Pasteurella and Eikenella. *Topley and Wilson's Microbiology and Microbial infections*. Vol. 3. (eds. Balows, A. and Duerdern, B.I.). Ninth edition. Oxford University press Inc., New York, pp. 1198-1209

*Holmes, D.S. and Quigley, M.A. 1981. A rapid boiling method for preparation of bacterial plasmids. *Anal. Biochem.* **114**:193-197

Hong, Y., Liu, T., Hofacre, C., Maier, M., White, D.G., Ayers, S., Wang, L and Maurer, J.J. 2003. A restriction fragment length polymorphism-based polymerse chain reaction as an alternative to serotyping for identifying Salmonella serotypes. *Avian Dis*. **47**: 387-395

Huber, B.S., Allred, D.V., Carmen, J.C., Frame, D.D., Whiting, D.G., Cryan, J.R., Olson, T.R., Jackson, P.J., Hill, K., Laker, M.T. and Robinson, R.A. 2002. Random amplified polymorphic DNA and Amplified fragment length polymorphism analyses of *P. multocida* isolates from fatal fowl cholera infections. *J. Clin. Microbiol.* **40**: 2163-2168

Hudson, 1954. Cited by Rimler, R.B. and Rhoades, K.R 1989. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida*. *Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

Hulton, C.S.J., Higgins, C.F. and Sharp, P.M. 1991. ERIC sequences: a novel family of repetitive elements in genomes of *Escherichia coli,*

Salmonella typhimurium and other enterobacteria. *Mol. Microbiol*. **5**: 825-834

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

Ireland, L., Adler, B. and Milner, A.R. 1991. Proteins and antigens of *Pasteurella multocida* serotype 1 from fowl cholera. *Vet. Microbiol.* **27**: 175-185

Jap, B.K. and Walian, P.J. 1990. Biophysics of the structure and function of porins. *Rev. Biophys*. **23**:367-403

Jayakumar, P.S. 1998. Comparative efficacy of different vaccines against pasteurellosis in ducks. M.V.Sc. thesis submitted to Kerala Agricultural University, Thrissur. p 126

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

Jonas, M. Morishita, T.Y. Angrick, E.J. and Jahja, T. 2001. Characterisation of nine *Pasteurella multocida* isolates from avian cholera outbreaks in Indonesia. *Avian Dis.* **45**: 34-42

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

Kasten, R.W., Carpenter, T.E., Snipes, K.P. and Hirsh, D.C. 1997. Detection of *Pasteurella multocida* specific DNA in turkey flocks by use of the polymerase chain reaction. *Avian Dis*. **41**: 676-682

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

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

Kedrak, A. and Opacka, B.B. 2002. Analysis of protein patterns of *Pasteurella multocida* strains isolated from poultry. *Bull. Vet. Inst. Pulawy*. **46**: 149-155

Kennett, L., Muniandy, N. and Mukkur, T.K.S. 1993. Comparative protective potential of non-living intact cells and purified outer membrane and associated proteins of *Pasteurella multocida* type 6: B grown under iron-regulated conditions. *Proceeding of International workshop on Pasteurellosis in production animals. Bali, Indonesia, 10-13 August, 1992*. pp. 144-148

Kielstein, P., Wuthe, H.H., Angon, O., Muttes, R. and Arrens, P. 2001. Phenotypic and genetic characterization of NAD-dependent Pasteurellaceae from respiratory tract of pigs and their possible pathogenetic importance. *Vet. Microbiol*. **81**: 243-255

Kim, J. and Nagaraja, K.V. 1990. DNA finger printing for differentiation of field isolates from reference vaccine strains of *Pasteurella multocida* in turkeys. *Am. J. Vet. Res.* **51**: 207-210

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

Kodjo, A., Villard, L., Bijet, C., Martel, J.L., Sanchis, R., Borges, E., Gautheir, D., Maurin, F. and Richard, Y. 1999. Pulse-field gel electrophoresis is more efficient than ribotyping and random amplified polymorphic DNA analysis in discrimination of *Pasteurella haemolytica* strains. *J. Clin. Microbiol*. **37**: 380-385

Kulkarni, D.D., Karpe, A.G., Bannalikar, A.S. and Kulkarni, G.B. 1988. Report of fowl cholera in six days old chicks. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **9**: 237-238

Kuhnert, P., Boerlin, P., Emler, S., Krawinkler, M. and Frey, J. 2000. Phylogentic analysis of *Pasteurella multocida* subspecies and molecular identification of feline *Pasteurella multocida* subsp. *septica* by 16s rRNA gene sequencing. *Int. J. Med. Microbiol*. **290** : 599-604

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

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685

Langenberg, W., Rauws, E.A. and Widjojokusumo, A. 1986. Identification of *Campylobacter pyloridis* isolates by restriction endonuclease analysis. *J.Clin. Microbiol*. **16**: 126-134.

Lee, M.D., Wooley, K.E., Brown, T. and Glisson, J.R. 1991. A survey of potential virulence markers from avian strains of *Pasteurella multocida*. *Vet. Microbiol*. **26**: 213-225

Lee, M.D., Burch, F.T., Maurer, J.J., Henk, A. and Thayer, S. 2000a. DNA finger printing of plasmid-containing serotype A: 3,4 *Pasteurella multocida* isolated from cases of fowl cholera in chickens and turkeys. *Avian Dis*. **44**: 201-204

Lee, C.W., Wilkie, I.W., Townsend, K.M. and Frost, A.J. 2000b. The demonstration of *Pasteurella multocida* in the alimentary tract of chickens after experimental oral infection. *Vet. Microbiol*. **72**:47-55

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

Lignieres, 1900. Cited by Rhoades, K.R. and Rimler, R.B. 1991. *Pasteurellosis. Disease of Poultry.* (eds. Calnek, B.W., Barnes, H.J., Bareand, C.W., Reid, W.M. and Yoder, H.W.) Ninth edition. Iowa State University Press, Ames, Iowa, pp 145-152

Loosemore, S.M., Yang, Y.P., Coleman, D.C., Shortreed, J.M., England, D.M. and Klein, M.M. 1997. Outer membrane protein D 15 is conserved among *Haemophilus influenzae* species and may represent a universal protective antigen against invasive disease. *Infect. Immun*. **65**: 3701-3707

Loubinoux, J., Lozhiewski, A., Lion, C., Gavin, D. Weber, M. and LeFaou, A.E. 1999. Value of enterobacterial repetitive intergenic consensus PCR for study of *Pasteurella multocida* strains isolated from mouths of dogs. *J. Clin. Microbiol.* **37**: 2488-2492

Lowry, D.H., Rosenbrough, N.I., Farr, A.L. and Randell, R.S. 1951. Protein measurement with Folin-phenol reagent. *J. Biol. Chem.* **193**: 263-275

Lu, Y.S., Afendis, S.J. and Pakes, S.P. 1988. Identification of immunogenic outer membrane proteins of *Pasteurella multocida* 3:A in rabbits. *Infect. Immun*. **56**: 1532-1537

Lu, Y.S., Lai, W.C., Pakes, S.P. and Nie, L.C. 1991a. A monoclonal antibody against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against pasteurellosis. *Infect. Immun.* **59**: 172-180

Lu, Y.S., Lai, W.C., Pakes, S.P. and Stefanu, C. 1991b. The outer membrane of *Pasteurella multocida* 3:A protects rabbits against homologous challenge. *Infect. Immun.* **59**: 4517-4523172-180

Lugtenberg, B., Boxtel, R.V., Evenberg, D., Jong, M.D., Strom, P. and Frik, J. 1986. Biochemical and immounological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect. Immun*. **52**:175-182.

Lugtenberg, B., Boxtel, R.V. and Jong, M.D. 1984. Atrophic rhinitis in swine: correlation of *Pasteurella multocida* pathogenicity with membrane protein and lipopolysaccharide patterns. *Infect. Immun*. **46**:48-54

Luo, Y., Glisson, J.R., Jackwood, M.W., Hancock, R.E., Bains, M., Cheng, I.N. and Wang, C. 1997. Cloning and characterization of the major outer membrane protein gene (*OmpH*) of *Pasteurella multocida* X-73. *J. Bacteriol.* **179**: 7856-7864

Luo, Y., Zeng, Q., Glisson, J.R., Jackwood, M.W., Cheng, N and Wang, C. 1999. Sequence analysis of *Pasteurella multocida* major outer membrane protein (OmpH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. *Vaccine*. **17**: 821-831

Maas, R.A., Komen, M., Diepen, M., Oei, H.L. and Claassen, I.J.T.M. 2003. Correlation of haemagglutinin-neuraminidase and fusion protein content with protective antibody response after immunization with inactivated Newcastle disease vaccines. *Vaccine* **21**: 3137-3142

Mahasreshti, P.J., Murphy, G.L., Wyckoff, J.H., Farmer, S., Hancock, R.E. and Confer, A.W. 1997. Purification and partial characterization of the OmpA Family of proteins of *Pasteurella haemolytica*. *Infect. Immun*. **65**: 211-218

Maillet, 1836. Cited by Rhoades, K.R. and Rimler, R.B. 1991. *Pasteurellosis. Disease of Poultry.* (eds. Calnek, B.W., Barnes, H.J., Bareand, C.W., Reid, W.M. and Yoder, H.W.) Ninth edition. Iowa State University Press, Ames, Iowa, pp 145-152

Mannheim, W. 1984. Pasteurellaceae. *Bergey's Manual of Systematic Bacteriology,* Vol 1 (eds. Kreig, N.R. and Holt, J.G.), Williams and Wilkins, Co., Baltimore, London, pp 559-569.

Mannheim, W. and Carter, G.R, 1984. Cited in "In Bergey's Manual of Systematic Bacteriology" Vol 1 (Kreig, N.R. and Holt, J.G. Eds.), Williams and Wilkins, Co., Baltimore, London, 1st edn. Pp 550-558

Marandi, M.V. and Mittal, K.R. 1995. Identification and characterization of outer membrane proteins of *Pasteurella multocida* serotype D by using monoclonal antibodies. *J. Clin. Microbiol*. **33**: 952-957

Marandi, M.V. and Mittal, K.R. 1996. Characterisation of an outer membrane protein of *Pasteurella multocida* belonging to the Omp A family. *Vet. Microbiol.* **53**: 303-314

Marandi, M.V. and Mittal, K.R. 1997. Role of outer membrane protein H (OmpH) and OmpA specific monoclonal antibodies from hybridoma tumours in protection of mice against *Pasteurella multocida*. *Infect. Immun*. **65**: 4502-4508

Mariana, S. and Hirst, R. 2000. The immunogenicity of *Pasteurella multocida* isolated from poultry in Indonesia. *Vet. Microbiol*. **72**: 27-36

Marshall, R.B., Wilton, B.E. and Robinson, A.J. 1981. Identification of leptospira serovars by restriction-endonuclease analysis. *J. Med. Microbiol*. **14**: 163-166

Matsumoto, M. and Strain, J.G. 1993. Pathogenicity of *Pasteurella multocida* : Its variable nature demonstrated by *in vivo* passages. *Avian Dis*. **37**: 781-785

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

May, B.J., Zhang, Q., Li, L.L., Paustian, M.L., Whittam, T.S. and Kapur, V. 2001. Complete genomic sequence of *Pasteurella multicida*. Pm 70. *Proc. Natl. Acad. Sci. USA.* **98**: 3460-3465

Mbuthia, P.G., Christensen, H., Boyee, M., Petersen, K.M.D., Bisgaard, M., Nyaga, P.N. and Olsen, J.E. 2001. Specific detection of *Pasteurella multocida* in chickens with fowl cholera and in pig lung tissues using fluorescent rRNA *in situ* hybridization. *J. Clin. Microbiol.* **39**: 2627-2633

Mehrotra, P.K. and Bhargava, S. 1999. A note on the characterization and antibiotic sensitivity of *Pasteurella* isolates from domestic animals. *Indian. J. Comp. Micrbiol. Immunol. Infect. Dis*. 20 : 144-146

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

Mitchison, M., Wei, L., Kwang, J., Wilkie, I. and Adler, B. 2000. Overexpression and immunogenicity of Oma87 outer membrane proteins of *Pasteurella multocida*. *Vet. Microbiol*. **72**: 91-96

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

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

Moreno, A.M., Baccaro, M.R., Ferreira, A.J.P. and Pestana de Castro, A.F. 2003. Use of Singh-enzyme Amplified Fragment Length Polymorphism for typing *Pasteurella multocida* subsp. *multocida* isolates from pigs. *J. Clin. Microbiol*. **41**: 1743-1746

Morishita, T.Y., Lowenstine, L.J., Hirsh, D.C. and Brooks, D.L. 1996a. *Pasteurella multocida* in psittacines prevalence, pathology and characterization of isolates. *Avian Dis.* **40**: 900-907

Morishita, T.Y., Lowenstine, L.J., Hirsh, D.C. and Brooks, D.L. 1996b. *Pasteurella multocida* in raptors: prevalence and characterization. *Avian Dis.* **40**: 908-918

Muhairwa, A.P., Christensen, J.P. and Bisgaard, M. 2000. Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. *Avian Path*. **29**: 133-142

Muhairwa, A.P., Christensen, J.P. and Bisgaard, M. 2001a. Relationships among Pasteurellaceae isolated from free ranging chickens and their animal contacts as determined by quantitative phenotyping, ribotyping and REA typing. *Vet. Microbiol*. **78**: 119-137

Muhairwa, A.P., Mtambo, M.M.A., Christensen, J.P. and Bisgaard, M. 2001b. Occurrence of *Pasteurella multocida* and related species in village free – ranging chickens and their animal contacts in Tanzania. *Vet. Microbiol.* **78**: 139-153

Mulbagal, A.N., Kulkarni, W.B. and Paranjape, V.L. 1972. Some observations on pasteurellosis in ducks. *Indian Vet. J*. **49**:544-546

Murphy, F.A.,Gibbs, E.P.J., Horzinet, M.C. and Studdert, M.J. 1999. *Veterinary Virology*. Third edition. Academic Press, San Diego, p. 629

Murugkar, H.V. and Ghosh, S.S. 1995. *Pasteurella multocida* serotype A: 1 in ducks in Tripura. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **16**: 109-111

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

Mutters, R., Mannheim, W. and Bisgaard, M. 1989. Taxonomy of group. *Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 3-34

Namioka, S. and Murata, M. 1961. Serological studies on *Pasteurella multocida* II. Characteristics of the somatic (o) antigen of the organism. *Cornell Vet*. 51: 522-528

*Neter, E. and Dryja, D.M. 1981. Use of pencillin and nafillin discs as an aid for identification of *Pasteurella multocida*. *J. Med*. **12**: 285-287

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

Owen, R.J. 1989. Chromosomal DNA finger printing – a new method of species and strain identification applicable to microbial pathogens. *J. Med. Microbiol*. 30: 89-99

Pal, A., Srivastava, S.K. and Singh, V.P. 2002. Heat modifiability of outer membrane proteins of *Pasteurella multocida* srotype B:2. *Indian J. Exp. Biol.* **40**: 106-108

Panda, S.N., Misra, B., Das, B.C. and Nayak, B.C. 1981. An outbreak of avian pasteurellosis in Orissa. *Indian Vet. J.* **58**: 418-420

Pande, A., Suryanarayana, V.V.S. and Singh, V.I. 1997. Nucleic acid probe – an approach in diagnosis of fowl cholera. *Indian Vet. J.* **74**:550-552

*Papova, T.P. and Tzvetkov, Y.M. 2002. Fowl cholera in pheasants (*Phasianus colchicus*) - etiological investigation and effect of therapy with thiamphenicol. *Bulgarian J. Vet. Med.* **5**: 23-28

*Parveen, Z., Nasir, A.A., Tasleem, K and Shah, A. 2003. Fowl cholera in a breeder flock. *Pakistan Vet. J.* **23**: 209-210

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

Pati, U.S., Srivastava, S.K., Roy, S.C. and More, T. 1996. Immunogenecity of outer membrane protein of *Pasteurella multocida* in buffalo calves. *Vet. Microbiol.* **52**: 301-311

Peterson, R.P., Deeb, B.J. and Digiacoma, F.R. 1997. Detection of antibodies to *Pasteurella multocida* by capture enzyme immunoassay using a monoclonal antibody against p37 antigen. *J. Clin. Microbiol*. **35**: 208-212

Prevost, G., Jaulhae, B. and Piemont, Y. 1992. DNA fingerprinting by pulse-field gel electrophoresis is more efficient that ribotyping in distinguishing among methicillin-resistant *Staphylococcus aureus* isolates. *J. Clin. Microbiol*. **30**: 967-973

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

Rajini, R., Rao, A.S., Dhanalakshmi, K. and Sarma, B.J.R. 1995. Studies on avian pasteurellosis in Andhra Pradesh. *Indian Vet. J.* **72**: 115-118

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

Ramdani and Adler, B. 1993. Serotype and genus-specific protein antigens of *Pasteurella multocida* and their role in immunity to infection. *Proceeding of International workshop on Pasteurellosis in production animals. Bali, Indonesia, 10-13 August, 1992*. pp. 55-59

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

Rao, G. 1964. Cited by Rammanath, K,R. and Gopal, T. 1993.

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

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

Rhoades, K.R. and Rimler, R.B. 1991. Pasteurellosis. *Pasteurellosis. Disease of Poultry.* (eds. Calnek, B.W., Barnes, H.J., Bareand, C.W., Reid, W.M.

and oder, H.W.) Ninth edition. Iowa State University Press, Ames, Iowa, pp 145-152

Rimler, R.B. 1978. Cited by Rimler, R.B. and Rhoades, K.R. 1989. *Pasteurella multocida* . *Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

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

Roberts, 1947. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida. Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

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

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

Roy, S., Das, A.B., Ghosh, A.N. and Biswas, T. 1994. Purification, pore-forming ability, and antigenic relatedness of major outer membrane protein of *Shigella dysenteriae* type 1. *Infect. Immun*. **62**: 4333-4338

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

Ruffolo, C.G. and Adler, B. 1996. Cloning, sequence, expression and protective capacity of the *Oma 87* gene encloding the *Pasteurella multocida*. 87-Kda outer membrane antigen. *Infect. Immun*. **64**: 3161-3167

Rundell, 1975. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida. Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

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

Sambyal, D.S., Soni, G.L., Sodhi, S.S. and Baxi, K.K. 1988. Characterisation of *Pasteurella multocida* (serotype I) from an outbreak of fowl cholera in ducks. *Indian J. Anim. Sci.* **58**: 1059-1060

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

Schneider, L. 1948. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella multocida. Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

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

Singh, R. and Goel, M.C. 2002. Isolation and purification of outer membrane proteins of *Pasteurella multocida*. *Indian J. Anim. Sci*. **72**: 625-626

Smith, J.E. 1958. Studies on Pasteurella septica II. Some cultural and biochemical properties of strains from different host species. *J. Comp. Pathol. Therap*. 69 : 315-323

Smith, G.R. and Phillips, J.E. 1990. Pasteurella. *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*. Vol.2. (eds. Parkar, M.T. and Duedven, B.I.). Eighth edition. Edward Arnold, Melbourne, Auckland, pp. 335-351

Snipes, K.P., Hiroch, D.C., Kasten, R.W., Hanson, L.M., Hird, S.W., Carpenter, T.E. and McCapes, K.H. 1989. Use of an rRNA probe and restriction endonuclease analysis to fingerprint *Pasteurella multocida* isolated from turkeys and wild life. *J. Clin. Microbiol.* **27**: 1847-1853

Snipes, K.P., Hirsh, D.C., Kasten, R.W., Carpenter, T.E., Hird, D.W. and Mc Capes, R.H. 1990. Differentiation of field isolates of *Pasteurella multocida* serotype 3,4 from live vaccine strains by genotypic characterization. *Avian Dis.* **34**: 419-424

Srilatha, C., Devi, V.R., Sujatha, K. and Ahmed, M.N. 2003. An outbreak of fowl cholera in Japanese quails. *Indian Vet. J.* **80**: 6-7

Srivastava, S.K. 1997. A Congo red binding assay for monitoring outer membrane protein production by *Pasteurella multocida* serotype 6:B. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **18**: 130-135

Srivastava, S.K. 1998. Outer membrane protein of *Pasteurella multocida* serotype B:2 is immunogenic and antiphagolytic. *Indian J. Exp. Biol.* **36**: 530-532

Srivastava, S.K. 1999. Immunogenecity of protein fractions of *Pasteurella multocida* in animals. *Indian J. Anim. Sci.* **69**: 653-656

Stainisich, V.A. 1988. Cited in *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*. (8th edn. Vol. 1. Edward Arnold London, Melbourne Auckland. p. 172

Stull, T.L., LiPuma, J.J. and Edlind, T.D. 1988. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J. Infect. Dis*. **157**: 280-286

Sulong, A.K. and Maheswaran, S.K. 1976. Studies on *Pasteurella multocida*. IV. Immunofluorescence detection of the organism in spleen and lungs of turkeys vaccinated with live oral vaccines. *Vet. Microbiol.* **1**: 3-14

Syuto, B. and Matsumoto, M. 1982. Purification of a protective antigen from a saline extract of *Pasteurella multocida*. *Infect. Immun.* **37**: 1218-1226

*Talha, A.F., Hussain, S.M., Chowdhury, M.M., Bari, E.H., Islam, H.R. and Das, P.M. 2001. Poultry diseases occurring in Mymemsingh district of Bangladesh. *Bangladesh Vet. J.* **18**:20-23

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulse-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol*. **33**: 2233-2239.

Thiermann, A.B., Handsaker, A.L. and Moseley, S.L. 1985. New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis. *J. Clin. Microbiol*. **21**: 585-592

Tomer, P., Chatruvedi, G.C., Minakshi, P., Malik, P. and Monga, D.P. 2002. Comparative analysis of the outer membrane protein profiles of isolates of the *Pasteurella multocida* (B:2) associated with haemorrhagic septicaemia. *Vet. Res. Commun*. **26**: 513-522

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

Toussaint, 1879. Cited by Rimler, R.B. and Rhoades, K.R 1989. *Pasteurella and Pasteurellosis*. (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 37-73

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad.Sci. USA.* **76**: 4350-4354

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

Townsend, K.M., Dawkins, H.J.S. and Papadimitriou, J.M. 1997a. Analysis of haemorrhagic septicaemia-causing isolates of *Pasteurella multocida* by ribotyping and field alternation gel electrophoresis (FAGE). *Vet. Microbiol*. **57**: 383-395

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

Townsend, K.M., Dawkins, H.J.S., Papadimitriou, J.M., Adamson, M.R., Johnson, R.B. and Spencer, T.L. 1993. Large DNA restriction fragment length polymorphism (RFLP) analysis of *Pasteurella multocida* isolates. *Proceeding of International workshop on Pasteurellosis in production animals. Bali, Indonesia, 10-13 August, 1992*. pp. 60-63

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

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

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

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

Walser, M.M. and Davis, R.B. 1975. *In vitro* characterization of field isolates of *Pasteurella multocida* from Georgia turkeys. *Avian Dis.* **19**: 525-532

Waltman, W.D. and Horne, A.M. 1993. Characteristics of fowl cholera diagnosed in Georgia, 1989-91. *Avian Dis.* **37**: 616-621

Wang, C and Glisson, G.R. 1994. Identification of common antigens of serotype 1 and serotype 3 *Pasteurella multocida* in poultry expressed *in vivo*. *Avian. Dis*. **38**: 334-340

Wang, T.F., Fang, H. and Chin, C.C. 1984. A preliminary report on the diagnosis of fowl cholera using indirect fluorescent antibody technique. *Chinese J. Vet. med*. **10**: 12-14.

Warner, 1996. Cited by De Alwis, 1999.

Welsh, J. and McClelland. 1990. Finger printing genomes using PCR with arbitrary primers. *Nucleic Acid. Res.* **18**: 7217-7218

*Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Jingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. **18**: 6531-6535

Wilson, M.A., Duncan, R.M., Nordholm, G.E. and Berlowski, B.M. 1995. Serotypes and DNA fingerprint profiles of *Pasteurella multocida* isolated from raptors. *Avian Dis.* **39**: 94-99

Wilson, M.A., Morgan, M.J. and Barger, G.E. 1993. Comparison of DNA fingerprinting and serotyping for identification of avian *Pasteurella multocida* isolates. *J. Clin. Microbiol.* **31**: 255-259

Wilson, M.A., Rimler, R.B. and Hoffman, L.J. 1992. Comparison of DNA fingerprints and somatic serotypes of serogroup B and E *Pasteurella multocida* isolates. *J. Clin. Microbiol.* **30**: 1518-1524

Zhang, H., Ainsworth, A.J. and Montgomery, R.D. 1994. Use of a 35.5 kDa cell membrane composition of *Pasteurella multocida* and an anti-idiotype antibody to induce protective immunity in Leghorn chickens. *Vet. Immunol. Immunopathol*. **41**:89-100

Zhao, G., Pijoan, C., Murtaugh, M.P. and Molitor, T.W. 1992. Use of restriction endonuclease analysis and ribotyping to study epidemiology of *Pasteurella multocida* in closed swine herds. *Infect. Immun.* **60**: 1401-1405

* Originals not consulted.

MOLECULAR CHARACTERIZATION OF *Pasteurella multocida* ISOLATED FROM DUCKS IN KERALA

P.X.ANTONY

Abstract of the thesis submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

2004

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR – 680 651

KERALA, INDIA

ABSTRACT

Twenty-five isolates from ducks and one from fowl were characterized as *P. multocida* using standard bacteriological procedures. A reference chicken strain (LKO) obtained from IVRI was used for comparison. All the avian isolates were found to be pathogenic for mice. Variation in fermentation patterns of dulcitol, mannitol, sorbitol and trehalose allowed the 27 isolates to be grouped into ten biovars. Two biotypes *P. multocida* subsp. *septica*, and *P. multocida* subsp. *multocida* were observed among the avian examined. All the isolates were uniformly sensitive to enrofloxacin, chloramphenicol and pefloxacin. Eight isolates from ducks and one from fowl have been serotyped as A:1. A species-specific PCR assay was used to confirm the identity of the isolates. Performing PCR on template DNA prepared from blood smears was found to be an extremely rapid method of diagnosis for pasteurella.

No polymorphism within the KMT1 gene could be demonstrated by restriction analysis of PM-PCR product with *Hae* III. Restriction endonucleases analysis of genomic DNA of all the avian isolates with *Hpa* II and *Hha* I revealed three profiles each. The fowl isolates had a profile that was common to majority of the duck isolates.

Eighty-eight per cent of the isolates carried plasmids. Two plasmid profiles were observed among the isolates examined. All the avian isolates showed a single REP-PCR profile indicating a high level of homogeneity among them.

The outer membrane protein profiles of all the avian isolates were similar. Two protein fractions with molecular weights of 37.15 and 26.36 kDa were identified as the major OMPs by SDS-PAGE.

A 37.15-kDa protein was identified the major antigenic fraction of OMPs of avian isolates as of *P. multocida* by Western blotting. Two other proteins with a molecular mass of 31.33 and 26.36 kDa were also found to be antigenic using this technique.

The *OmpH* gene of all the avian isolates as well serotypes A:3 and B:2 were amplified using primers designed based on published *OmpH* gene sequence of a chicken isolate of *P. multocida.* The amplified product digested with restriction endonucleases *Dra* I and *Hinf* I generated four and three fragments respectively. The similarity of the profiles for all the avian isolates of *P. multocida,* suggested a high degree of homogeneity amongst the amplified region.

Restriction analysis of the OmpH-PCR products of serotypes A:1, A:3 and B:2 with *Dra* I generated profiles which were distinct from each other for the three serotypes. This technique can be helpful for differentiation of various serotypes of *P. multocida*. Studies on the OmpH gene(s) of inactivated fowl cholera vaccine revealed similarity with the amplified region of the local duck isolate of *P. multocida*. Since this gene codes for the major antigenic fraction of *P. multocida* the vaccine can be expected to confer immunity to ducks in Kerala against pasteurellosis.

The sequence the OmpH-PCR product has been submitted to the GenBank and has been assigned the accession No AY606823. The sequence showed 98 per cent identity with *P. multocida* strain X-73 (serotype A:1) outer membrane protein (*OmpH*) gene.