NUCLEIC ACID AND PROTEIN PROFILE OF *Pasteurella multocida* OF AVIAN ORIGIN

By

S. RAJALAKSHMI @ RADABAI

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences

Kerala Agricultural University

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR - 680651

KERALA

2001

DECLARATION

I hereby declare that this thesis entitled "**NUCLEIC ACID AND PROTEIN PROFILE OF *Pasteurella multocida* OF AVIAN ORIGIN**", is a bonafide record of research work done by me during the course of research and that the 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.

31.10.2001
Mannuthy

S. Rajalakshmi

S. Rajalakshmi @Radabai

CERTIFICATE

Certified that thesis entitled **"NUCLEIC ACID AND PROTEIN PROFILE OF *Pasteurella multocida* OF AVIAN ORIGIN"**, is a record of research work done independently by **S. Rajalakshmi @Radabai,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

31/10/01

Mannuthy
31-10-'01

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

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Miss. S. Rajalakshmi @Radabai,** a candidate for the degree of Master of Veterinary Science in Microbiology, agree that this thesis entitled **"NUCLEIC ACID AND PROTEIN PROFILE OF *Pasteurella multocida* OF AVIAN ORIGIN",** may be submitted by **Miss. S. Rajalakshmi @Radabai,** in partial fulfilment of the requirement for the degree.

31/10/01

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 i/c,
Department of Microbiology,
College of Veterinary and
Animal Sciences,
Mannuthy.
(Member)

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

Dr. K. Narayanankutty,
Senior Scientist,
AICRP on Poultry for eggs,
Centre for Advanced Studies in Poultry Science,
College of Veterinary and Animal Sciences,
Mannuthy
(Member)

22/11/2001

External Examiner
DR. R. Madhu Sooda Rao Pillai
Prof. & HOD.
Dept. of Microbiology, Pondicherry.

ACKNOWLEDGEMENT

"What shall I render unto my LORD of all His benefits towards me?"
"I will offer to Thee the sacrifice of thanks giving and call upon the name of the LORD"

I extol the kind co-operation and parental affection offered to me by outstanding mentor, Dr. G. Krishnan Nair, Associate professor, Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy from the initiation of the work to the ship-shaping of the manuscript. His meticulous guidance, benign disposition and personal attention to the works are noteworthy.

I deem it my privilege in expressing my heartfelt gratitude and thanks to Dr. V. Jayaprakasan, Associate professor and Head, Department of Microbiology for his affectionate guidance and constructive criticisms during the course of work. But for his sumptuous suggestions, tremendous patience and co-operation during the scrupulous navigation of thesis-writing, this work would not have been the light of the day.

I owe a special word of thanks, with great fondness to my minor guide, Dr. M. Mini, Assistant professor, Department of Microbiology, for her timely advices, incessant help and strong support in times of difficulties.

I humbly place on record my respect and gratitude to Dr.K. Narayanankutty, Senior Scientist, Department of Poultry Science, member of Advisory Committee, for his expert advice, valuable suggestions and ardent encouragement during my work.

I am cordially obliged to Dr. Koshy John, Assistant Professor, Department of Microbiology for the help and encouragement rendered by him.

I am indebted to Dr. Aravindakshanan, Assistant Professor, Department of Animal Breeding and Genetics and Dr. Sisilamma George, Associate Professor and Head, Department of Biochemistry, for their pleasant co-operation and indispensable help for the completion of my work.

I am at a loss of words to express my special thanks to Dr. Nazeem, Dr. Girija, Deepa, Babu, Achuthan and Reema in the Department of Plant Biotechnology, College of Horticulture, for their professional guidance and valuable discussions. I express my diction of thanks to Dr. T.G. Prabhakar, VRC, Chennai for his well-timed assistance and perspicuous guidance during the tenure of work.

I take great pleasure in thanking Dr. Nandakumar, Officer in charge, University Sheep Farm and the farm workers for the help rendered in the collection of sheep blood required for my work.

With deep sense of gratitude, I place my earnest regards and indebtedness to my beloved Undergraduate teachers for their constant encouragement and motivation.

With immense pleasure, I owe a flow of thanks to Mr.Ranjit and Mrs.Vijayalakshmi (Research Associates) for their sharing of professional experiences, memorable concern and whose warm friendship provided a cordial environment.

I remember with gratitude the help rendered by Mr. Chandrashekaran, and Mrs. Sreeja, Central Instrumentation Lab, for their technical assistance.

I specially thank my Departmental senior colleagues, Dr. Mohan, Dr. Tressa, Dr.Pradeep, Dr. Priya, Dr. Binu T.V., Dr. Binu. K. Mani, Dr. Saji and Dr. Baiju for the help and encouragement. With great pleasure, I am thankful to my colleagues Dr. Sreeja R, Nair, Dr. Jaison George and also to my junior colleagues Dr. Chintu and Dr. Manju for their kind co-operation. My sincere thanks are also to my other senior colleagues Dr. Geetha, Dr. Srividhya, Dr. Sindhu, Dr. Bindya, Dr. Princy, Dr. Roney, Dr. Jayashree, Dr. Senthil (Surgery), Dr. Senthil (Pharm.) Dr. Bhaskar and Dr. Gopinath. Thanks are to Shiny, Simi, and Bindu also.

Thanks are due to Mrs. Kumari, Lathika, Suja, and Chandramathi, for their help and co-operation in the Department.

I wish to acknowledge the co-operation and help rendered by the staff and students of the Department of Pathology in the collection of samples.

Special thanks to Dr. Thirupathy and Dr. Radha for their timely support academically and personally.

The invaluable help rendered by my colleagues, Sangeetha, Vijayakumar, Rajkumar, Kantharaj, Sabiha, Binsy and Lakshmi are acknowledgeable.

I sincerely acknowledge the help rendered by Dr. Antony, Dr. Tressa, Manigandan, Mariya, Vimala, Marutham and Manoj for furnishing the relevant literatures regarding the work.

My bountiful thanks are due to my friends Abi, Hari, Ravi, Kalyan, Venkatesh and other batch mates of 94' RAGACOVAS for their warm friendship. My special and heartfelt gratitude to my friends Arul Anand, Arun, Lydia and

Manigandan for their mental support, incessant encouragement, and for lending an air of self-confidence in attaining this goal.

I am grateful to the former Dean, Dr. Sulochana and Dr. Muraleedharan Nayar, Dean in charge, COVAS, Mannuthy, for providing the necessary facilities for the work.

I am indebted to Kerala Agricultural University for the scholarship provided during the period of study.

I am forever fondly beholded to my APPA and AMMA whose perennial source of love, affection inspiration and encouragement enabled a strenuous task remain easy throughout. Lack of words to express my thanks to my sister Dhana for her over- whelming love, affection and encouragement. I am also indebted to Venkatesh anna for his financial support and motivation for this accomplishment.

Special thanks are due to M/s Delta Computers, Mannuthy for the patient, prompt and diligent preparation of the manuscript.

Above all I bow before the God Almighty for all His blessings which helped me to fulfil this endeavour.

S. Rajalakshmi

Rajalakshmi @Radabai.S

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Dedicated to

APPA & AMMA

Introduction

1. INTRODUCTION

Poultry rearing has metamorphosed into an industry contributing significantly to the GDP besides providing the much-needed protein. India's poultry sector contributes to atleast Rs.8,082 crores in terms of value of the annual agri-based produce (Chawla, 1998).

The 1996 livestock census indicated that the poultry population in Kerala was 26.95 millions, which included 25.65 millions of chicken, 1.19 millions of ducks and 0.11 million of other avian species (Anon, 1998).

Disease is one of the major hurdles in the development of poultry industry. Alterations in the existing germplasm to augment production and the introduction of exotic germplasm have all complicated the disease scenario. Apart from mortality, the production loss due to morbidity of the birds affects the poultry industry drastically. One such disease is Pasteurellosis (Fowl cholera), caused by *Pasteurella multocida*, an acute and contagious disease affecting domesticated and wild birds, which is of considerable economic importance to the poultry industry.

In 1986, the worldwide loss due to fowl cholera was estimated to be 200 million US dollars (Ratafia, 1988). Diagnosis of the disease depends on identification of the causative bacterium

Pasteurella multocida, from birds with signs and lesions consistent with the acute or peracute form of the disease.

Phenotypic characterization of the field isolates of *P. multocida*, helps to identify the isolates upto the species level. In addition, biotyping highlights the characteristics of the isolates for the subspeciation. *P. multocida* isolated from different places and different species of birds are shown to exhibit a wide variation in their phenotype, biotype and serotype with cross-infections between the different avian species.

The application of phenotypic characterization in identification of the field isolates of *P. multocida* obtained from different avian species had been emphasized on an epidemiological basis.

During outbreaks of pasteurellosis, administration of antibiotics remains the first measure of control of disease. But often, isolates exhibited difference in their antibiotic sensitivity patterns, which in turn, was directly responsible for the treatment failure.

Immunization is then sought as the next mode of approach. In place of bacterins, oil adjuvanted vaccines are currently in use for the disease prevention, incorporating a single seed bacterium of a particular biotype and serotype only. Though these vaccines are shown to be efficient in conferring protection against homologous infections, the degree of protection provided against

infection due to heterologous strains has been reported to be varying. Thus the need of the hour is to develop vaccines, effective even against heterologous challenge infections.

The whole cell protein profile of any bacterium discerned as major and minor polypeptides constitutes a major portion of the immunogenic moiety of the bacterium. The difference in the protein profiles between the strains can be attributed for their differences in the phenotype, biotype and serotype. The outer membrane, on the other hand, which contains lipopolysaccharides, several minor proteins and few major proteins, is characteristic to each of the strains and is also being viewed to contribute for its immunogenic / antigenic property.

Analysis of chromosomal DNA by Restriction Endonuclease Analysis (REA) is a method currently employed by which, the homogeneity / heterogeneity of the isolates is established. The study of Restriction Fragment Length Polymorphisms (RFLPs) is known to help in differentiating the individual strains of closely-related bacteria (Hunt *et al.*, 2000).

Thus, a detailed phenotypic and genotypic characterization of isolates of *P. multocida* was designed, aiming for the development of a cost-effective vaccine against avian pasteurellosis.

The present study was aimed at characterizing avian isolates of *Pasteurella multocida* based on,

1. Morphological, cultural and biochemical characters.
2. Antibiogram.
3. Whole cell protein profile.
4. Outer membrane protein profile.
5. Restriction Fragment Length Polymorphisms (RFLPs) of chromosomal DNA.

Review of Literature

2. REVIEW OF LITERATURE

Fowl cholera (avian pasteurellosis) is a commonly occurring acute septicaemic disease, often fatal and affecting all types of birds like chicken, ducks, turkeys, geese, psittacines, raptors, waterfowl and even the wild birds.

The disease is caused by a gram- negative facultatively anaerobic organism belonging to the genus Pasteurella. *Pasteurella multocida* subsp. *multocida* was identified to be the common cause of fowl cholera, although the subspecies *gallicida* and *septica* caused fowl cholera- like disease (Christensen and Bisgaard, 2000).

2.1 Nomenclature

Maillet (1836) named a disease in birds characterized by fever, anorexia, mucous discharge from the nostrils, diarrhoea, polypnoea, cyanosis with high mortality as fowl cholera.

Perroncito (1878) first isolated the causative organism of fowl cholera.

Topley and Wilson (1936) suggested *Pasteurella septica* as the suitable name to indicate the bacteria of septicaemia and also because the organism from different hosts behaved as if they belonged to a single species.

Rosenbusch and Merchant (1939) proposed the name *Pasteurella multocida*, which was later, listed as the type species of

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

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

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

2.2 Incidence of avian Pasteurellosis

2.2.1 Prevalence in countries outside India

The earliest report on the occurrence of avian pasteurellosis was by Androvandus in Italy in 1600. The disease occurred in Italy and France from 1770 to 1800 and Chabert in France, in the year 1780, first studied it (Mannheim and Carter, 1984).

Prevalence of turkey pasteurellosis in Britain (Curtis, 1979) and in Australia (Blackall *et al.*, 1995) have been reported.

Pasteurella multocida was isolated from quails, which suffered an acute condition with high mortality (Panigrahy and Glass, 1982). Glisson *et al.* (1989) reported cases of *P.multocida* from quails in Georgia and Bermudez *et al.* (1991) isolated the same organism from Bobwhite quails.

Sander and Glisson (1989) isolated *P.multocida* from chicken in the age group of 20 to 46 days in Georgia, while Waltman and Horne (1993) isolated *P.multocida* from chicken of four to 83 weeks age.

The first outbreak of fowl cholera in a wild duck (*Rosyibilled pochard*) in Japan was reported by Fujihara *et al.* (1986). Nakamine *et al.* (1992) isolated *P. multocida* subsp. *multocida*, from Muscovy ducks for the first time in Japan. A higher prevalence rate of fowl cholera in ducks, caused by *P.multocida* was recently reported by Muhairwa *et al.* (2001) in Tanzania.

Morishita *et al.* (1996a and b) reported the prevalence of fowl cholera in raptors and psittacine birds, isolating *P. multocida* from both groups of birds.

2.2.2 Prevalence in India

Pasteurella infection in birds has been considered to be a serious economic problem to the Indian poultry industry.

Outbreaks of fowl cholera in ducks and poultry have been reported from different parts of India (Sankaranarayanan and Banerjee, 1944), from Madras (Balasubramanian, 1960) and in the recent past from Kerala (Balakrishnan, 1998).

Pasteurella multocida was isolated from fowl cholera outbreaks in ducks from Andhra Pradesh and Bengal (Mohetada and Bhadury, 1947), sometime later again in Andhra Pradesh (Rao,

1964), Assam (Halder, 1972; Karim, 1987), Bombay (Mulbagal *et al.*, 1972), Srinagar (Sambyal *et al.*, 1988), West Bengal (Das *et al.*, 1991), Karnataka (Rammanath and Gopal, 1993), Tripura (Murugkar and Ghosh, 1995), Kerala (Jayakumar, 1998) and a recent outbreak in Andhra Pradesh (Devi *et al.*, 2000).

Pillai *et al.* (1993) isolated *P.anatipestifer* from fowl cholera outbreaks in ducks in Kerala.

P. multocida was isolated from the oedema fluid of wattles of the affected chicken in Orissa but not from the heart blood (Panda *et al.*,1981), from six days old chicks in Maharashtra (Kulkarni *et al.*, 1988) and from Andhra Pradesh (Rajini *et al.*, 1995).

Chandran *et al.* (1995) reported an outbreak of pasteurellosis in Japanese quails in Madras.

2.3 Isolation studies of *Pasteurella multocida*

Pasteur (1880) first isolated the causative organism of fowl cholera in pure cultures.

Namioka and Murata (1961) described a solid medium called Yeast Proteose Cystine (YPC) agar to demonstrate the colony morphology of *P.multocida*.

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

Nutrient agar containing five per cent sheep or bovine blood was used for the isolation of Pasteurella (Carter, 1967) but it was not satisfactory for the identification of colonial variants. He also found that tryptic soy blood agar containing five per cent bovine or sheep blood and Brucella agar containing two per cent haemolysed rabbit serum improved the growth of Pasteurella.

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

Carter (1981) reported that *P.multocida* grew best at a temperature of 37^{0}C aerobically or anaerobically with five per cent carbon dioxide on bovine or sheep blood agar.

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

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

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

Modified K-B medium composed of trytic soy agar with five per cent peptic sheep blood, 0.05 μg per ml of kanamycin and 2.5 μg per ml of bacitracin was used by Kawamoto *et al.* (1990) in the isolation of *P. multocida* from rabbits.

P. multocida was found to grow well in the temperature range of 12 to 43^{0}C with an optimal temperature of 37^{0}C (Smith and Phillips, 1990).

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

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

Lee *et al.* (2000) developed a new selective medium containing polymyxin B, crystal violet, thallous acetate, bacitracin and cycloheximide in ten per cent sheep blood dextrose starch agar which gave a high rate of isolation of *P. multocida* from chicken alimentary tract.

2.4 Phenotypic characterization of *P. multocida*

Phenotypic characterization of bacterial isolates comprises of observation of colonial morphology, staining reactions and other biochemical reactions, which form the basis for the taxonomy and identification of bacteria.

Cowan (1974) described the primary biochemical tests, for identifying *P. multocida* as tests for catalase and oxidase and oxidative or fermentative utilization of glucose. Growth on Mac Conkey's agar, haemolysis on blood agar and urease activity were included in the primary biochemical tests for the identification of *P. multocida* by Buxton and Fraser (1977). As per Mannheim (1984), the first stage tests for identification of *P. multocida* included test for beta haemolysis, growth on Mac Conkey's agar, indole production, urease activity, gas production from carbohydrates and acid production from lactose and mannitol.

The second stage of tests for further identification included production of indole and hydrogen sulphide, nitrate reduction, beta-galactosidase activity, ornithine decarboxylase activity, acid production from arabinose, lactose, maltose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose and xylose (Cowan, 1974).

Carter and Rundell (1975) described an identification method for type A mucoid strains of *P. multocida* using staphylococcal hyaluronidase.

Sambyal *et al.* (1988) characterized *P. multocida* (serotype I) from an outbreak of fowl cholera in ducks based on morphological and biochemical characteristics.

Phenotypic characterization of *P. multocida* isolates from poultry was performed using biochemical tests (Blackall *et al.*, 1995; Fegan *et al.*, 1995).

Blackall *et al.* (1997) reported the phenotypic characterization of indole-positive *P. multocida* isolates from pigs up to the subspecies level.

Muhairwa *et al.* (2001) employed the phenotypic characterization to identify the phenotypic similarities between isolates of *P. multocida* from chicken and duck strains.

2.5 Biotyping

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

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

Dorsey (1963) classified biochemically, 432 strains of *P. multocida* isolated from fowl cholera cases based on Bergey's

Manual of Systematic Bacteriology and the fermentation of xylose, arabinose and dulcitol.

Donahue and Olson (1972) determined the biochemical properties of turkey isolates of *P. multocida*, which varied in their ability to ferment arabinose, dulcitol, lactose and xylose.

Walser and Davis (1975) characterized 30 field isolates of *P. multocida* and found minimal variation in their biochemical patterns.

Lee *et al.* (1988) described a significant fermentation pattern of a vaccine strain of *P. multocida* with ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, N-acetylglucosamine, esculin and saccharose.

A homogeneity of biochemical characteristics among turkey isolates of *P.multocida* was identified by Snipes *et al.* (1990), paving the way for genotypic differentiation.

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

Mohan *et al.* (1994) studied the phenotypic characteristics of *P.multocida* isolates from Zimbabwe and reported

that all isolates irrespective of their origin fermented glucose and sucrose but did not ferment arabinose, dulcitol, inositol and salicin.

Rajini *et al.* (1995) reported fermentation of glucose, sucrose, fructose, galactose, and maltose by all the avian *P.multocida* isolates. Only ninety per cent of the isolates fermented sorbitol; while 63.3 per cent, 66.6 per cent and 53.3 per cent of isolates fermented mannitol, dulcitol and lactose respectively.

Chandran *et al.* (1995) described the fermentation of glucose, sucrose and mannitol without gas production by a quail isolate of *P.multocida*.

Blackall *et al.* (1995) grouped turkey *P.multocida* isolates into four groups (A, B, C, and D) based on biochemical profile. Biovar A fermented sorbitol and xylose, biovar B fermented sorbitol alone, biovar C fermented sorbitol, trehalose and xylose while biovar D fermented maltose, trehalose and xylose but not sorbitol.

Biovars of *P.multocida* isolated from poultry were differentiated based on fermentation of sorbitol, trehalose and xylose (Fegan *et al.*, 1995).

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

2.6 Antibiogram

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

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

Donahue and Olson (1972) studied that 90 per cent of the turkey *P.multocida* isolates were sensitive to chlortetracycline, chloromycetin, erythromycin, novobiocin, penicillin, polymyxin B, terramycin, furadantin, furoxone, kanamycin, neomycin and showed varied sensitivity to sulphathiazole. Moderate sensitivity to streptomycin and resistance to sulphadiazine and bacitracin were also observed.

Walser and Davis (1975) on the other hand, observed that a greater percentage of turkey *P.multocida* isolates were sensitive to sulphonamides.

Avian *P.multocida* isolates subjected to *in vitro* sensitivity tests revealed the sensitivity to tetracycline and resistance against streptomycin, furazolidone, chloramphenicol, erythromycin and triple sulphas (Panda *et al.*, 1981).

Sambyal *et al.* (1988) opined that the *P.multocida* (serotype 1) isolates of duck origin were highly sensitive to

chloramphenicol, nitrofurantoin and tetracycline. A moderate sensitivity to gentamicin and resistance to neomycin, ampicillin, trimoxazole, erythromycin and streptomycin were also observed.

Glisson *et al.* (1989) reported that *P.multocida* isolated from Japanese quail was sensitive to chlortetracycline only.

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

Lee *et al.* (1991) demonstrated little evidence of antibiotic resistance mediated by R- plasmids among isolates from fowl cholera.

Avian *Escherichia coli* and *P.multocida* showed *in vitro* susceptibility to danofloxacin followed by furaltadone, lincomycin, oxytetracyline, spectinomycin and trimethoprim sulphamethoxazole (Raemdonck *et al.*, 1992).

P.multocida isolated from cases of duck pasteurellosis showed sensitivity to chloramphenicol, chlortetracycline, oxytetracyline, cotrimoxazole, nalidixic acid, gentamicin, nitrofurantoin, streptomycin, kanamycin and neomycin (Rammanath and Gopal, 1993).

Pillai *et al.* (1993) found that the *P.anatipestifer* isolates were sensitive to chloramphenicol, ampicillin and flumequine.

Rajini *et al.* (1995) observed that the avian field isolates of *P.multocida* were highly sensitive to chloramphenicol (96.6 per cent), followed by doxycycline hydrochloride, chlortetracycline, nitrofurantoin, penicillin and cotrimoxazole with 80.0, 73.3, 73.3, 66.6 and 60.0 per cent of sensitivity respectively.

Murugkar and Ghosh (1995) reported that the *P.multocida* isolates of duck origin were highly sensitive to sulphadiazine at 300 microgram level and to trimethoprim, erythromycin, ampicillin and gentamicin at 10 microgram therapeutic levels.

Psittacine isolates of *P.multocida* were found to be sensitive to penicillinG, sulphisoxazole, gentamicin, erythromycin, tetracycline, trimethoprim and sulphamethoxazole, but were resistant to streptomycin (Morishita *et al.*, 1996b).

Arif and Champlin (1998) reported on the susceptibility of naturally occurring strains of avian *P.multocida* to novobiocin.

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

Devi *et al.* (2000) observed a high degree of sensitivity of duck *P.multocida* isolates to pefloxacin and gentamicin.

Avian *P.multocida* isolates from outbreaks in Indonesia were susceptible to ampicillin, trimethoprim and doxycycline (78 per cent), enrofloxacin (67 per cent) but were resistant to lincomycin and sulphadiazine (Jonas *et al.*, 2001).

2.7 Whole cell protein extraction

A majority of the bacterial genome is expressed and it is reasonable to examine the total protein profile of organisms in order to look at their genotypic relations.

Ireland *et al.* (1991) separated the cellular proteins from avian *P.multocida* isolates by treatment of the cell extract with lysozyme, followed by sonication.

Whole cell proteins were separated from sonicated *P.multocida* serotype B: 6 cells by precipitation at 45 per cent saturation of ammonium sulphate and the protein concentration was estimated by Lowry's method (Srivastava, 1997).

Srivastava (1998) reported that the whole cell proteins of *P.multocida* strain P52 when subjected to 45 per cent ammonium sulphate precipitation yielded a protein rich material which served as effective immunogen.

Ammonium sulphate perceptible protein (PSAP) of *P.multocida* serotype 6:B on fractionation using ion-exchange chromatography, yielded three different protein fractions. The whole

cell protein as well as the protein fractions provided a similar protection level against the challenge infection (Srivastava, 1999).

Srivastava (2000) extracted total proteins, outer membrane protein and lipopolysaccharide from sonicated *P. multocida* cells for testing the immunogenecity of each of the components.

2.7.1 Whole cell protein profile by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Lugtenberg *et al.* (1984) differentiated the porcine isolates of *P. multocida* by the presence of major protein in 34 to 38 kDa region, and named it as heavy ('H') protein.

Lugtenberg *et al.* (1986) identified the immunological and biochemical properties of the protein-H, extracted from the porcine isolates of *P. multocida*.

Ireland *et al.* (1991) demonstrated the major difference between SDS-PAGE profiles of avian *P. multocida* serotype 1 isolates in the position of one of the major proteins in the 34 to 38 kDa range.

Sonicated cells of *P.multocida* strain P52 on ammonium sulphate precipitation yielded a protein rich material comprising of atleast 14 polypeptides in the molecular weight range of 30 to 91 kDa. Of these, the 65 kDa protein was identified as the major band (Srivastava, 1998).

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

2.8 Outer membrane protein (OMP) extraction

The outer membrane of gram-negative bacteria contains lipopolysaccharides, several minor proteins and a limited number of major proteins. The composition of the outer membrane proteins (OMPs) appears to be rather conserved during evolution.

Barenkamp *et al.* (1981) extracted the OMP of *Haemophilus influenzae* type b by sonication followed by treatment with sodium lauryl sarcosinate and protein concentration was determined by Lowry's method.

Membrane vesicles from lysed suspensions of turkey *P.multocida* were treated with various solubilising agents to release cellular proteins, which contained cross protection factors (Brogden and Rimler, 1983).

Avian *P.multocida* cells were treated with proteinase K and phenylmethylsulphonylfluoride before passing through a French pressure cell-sarcosine treatment and on centrifugation the OMP could be recovered (Snipes *et al.*, 1988; Kennett *et al.*, 1993).

Truscott and Hirsh (1988) prepared OMP from avian *P.multocida* cells by passing the cell suspension through a French pressure cell, followed by treatment with sarcosine and SDS- mercaptoethanol solution.

Lu *et al.* (1988) extracted OMP from rabbit *P.multocida* by treating the cell extract with hyaluronidase, DNase and RNase, disrupting the cells by a French pressure cell and finally subjecting them to a sucrose gradient centrifugation.

Sodium lauryl sarcosinate-insoluble OMP-enriched fraction of turkey isolates of *P.multocida* was prepared by Choi *et al.* (1989).

Knights *et al.* (1990) separated the envelope proteins of *P.multocida* and *P.haemolytica* by treating the cell extract with lysozyme, DNaseI, RNase and further fractionation by sucrose density gradient centrifugation and extraction with Triton X-100.The protein concentration was estimated by Lowry's method.

Membranous proteins of *P.multocida* were collected by treating the cell extract with lysozyme, followed by sucrose density gradient centrifugation (Lee *et al.*, 1990).

Outer membrane protein- enriched extracts were separated by several workers from isolates of *P. multocida* of poultry (Choi-Kim *et al.*, 1991), swine (Zhao *et al.*, 1995) and cattle (Pati *et al.*, 1996) origin.

Marandi and Mittal (1996) separated the OMP fraction from French pressure cell lysates of *P.multocida* 656 by sarkosyl treatment.

Dabo *et al.* (1997) fractionated the OMP-enriched fraction of bovine *P.multocida* serogroupA isolates by treating the cell extract serially with sucrose, phenylmethylsulphonylfluoride, lysozyme, DNaseI and RNase, followed by sucrose density gradient centrifugation.

Srivastava (1997) extracted the OMP from *P.multocida* serotype 6: B isolates by sonication and sarkosyl treatment.

2.8.1 Outer membrane protein profile by SDS-PAGE

Fifty one isolates of *Haemophilus influenzae* type b were classified into nine categories based on their gradient gel pattern produced by four major peptides with molecular weight in the range of 25,000 to 40,000 Daltons (Da) and another peptide with molecular weight of 16,000Da (Barenkamp *et al.*, 1981).

Three protein bands in the 14,300 to 57,200 Da molecular weight range were found in the turkey-grown cell lysates of *P.multocida*, in addition to the protein bands obtained from broth-grown cell lysates (Brogden and Rimler, 1983).

Turkey strains of *P.multocida* grown in turkey plasma at 41^{0}C expressed OMP with molecular weights of 96,000Da, 84,000Da

and 80,000Da under conditions of iron limitation, which enhanced the OMP production (Snipes *et al.*, 1988).

Truscott and Hirsh (1988) demonstrated that the turkeys given antibodies specific for the 50 kDa OMP were protected against lethal challenge. The antiphagocytic activity was found only with the 50 kDa protein.

Lu *et al.* (1988) identified proteins of molecular weights 27 kDa, 37.5 kDa, 49.5 kDa, 58.7 kDa and 64.4 kDa as immunogens of *P. multocida* from rabbits among the 18 proteins found in OMP.

Extracts of avian strains of *P. multocida* grown *in vivo* expressed additional OMP in the 27 kDa, 48 kDa, 56 kDa, 60 kDa, 80 kDa and 94 kDa molecular weight regions, which were not detected in strains grown *in vitro*. The vaccine strains (CU and M9) expressed strain-specific OMP markers of 48 kDa and 45 kDa molecular weights (Choi *et al.*, 1989).

Lee *et al.* (1990) observed very little difference among the various avian *P. multocida* isolates except in the 31 kDa region. Variation in the molecular weight of proteins in this region was present for five of the ten mutant strains. One expressed two bands in the 50 kDa and 66 kDa range, which were more intensely staining than the vaccine strain (CU) or other mutants.

Knights *et al.* (1990) differentiated *P. multocida* serotype b isolates from other *P. multocida* serotypes by the presence of a major band of 33 kDa.

P. multocida grown in iron-restricted media as well as *in vivo* expressed major OMPs with molecular weights of 29 kDa, 34.5 kDa and 45 kDa. Iron restriction and growth *in vivo* induced expression of additional OMPs with molecular masses of 76 kDa, 84 kDa and 94 kDa (Choi-Kim *et al.*, 1991).

Lu *et al.* (1991) found that *P. multocida* OMP with an estimated molecular weight of 37.5 kDa was the target of a protective monoclonal antibody; using ELISA, whole cell radioimmunoprecipitation and immunoblot analyses.

Chevalier *et al.* (1993) purified and characterized protein –H that was identified as the major porin in *P. multocida* in the molecular weight range of 37 and 41.8 kDa on SDS-PAGE.

Kennet *et al.* (1993) demonstrated one major outer membrane protein with molecular mass of 35 kDa and three other faintly staining bands of 30, 23 and 17 kDa respectively. Additional intensely staining doublet bands (84 kDa) were present when isolates were grown in iron deplete and iron replete condition.

Zhao *et al.* (1995) compared iron regulated OMPs in the molecular weight range of 74 kDa to 109 kDa which were expressed by porcine *P. multocida* isolates. Avian isolates expressed OMPs of molecular masses of 74 kDa, 87 kDa and 99 kDa, whose antibodies cross-reacted with all the OMPs from porcine strains.

Pati *et al.* (1996) observed 10 major polypeptide bands in the molecular weight range of 25 to 88 kDa in the OMP, while the

sonicated supernatant revealed bands in the molecular weight range of 35 to 83 kDa.

A major heat modifiable OMP from *P. multocida* of molecular weight 28 kDa was obtained when the OMP preparation was solubilised at 60°C and a band of molecular mass of 37 kDa was seen when solubilised at 100°C. They were identified and characterized using a monoclonal antibody (Marandi and Mittal, 1996).

P. multocida outer membrane from sucrose density gradients and sarkosyl extractions revealed eight major OMPs of molecular weights 14, 19, 28, 29, 35, 36, 45 and 46 kDa of which two major OMPs (35 and 46 kDa at 100°C, 30 and 34 kDa at 37°C) demonstrated heat modifiability. Six patterns were observed amongst 11 of the isolates (Dabo *et al.*, 1997).

2.9 DNA isolation studies

By genotypic characterization, the genetic relatedness of the isolates could be ascertained at the species or subspecies level.

Whole cell DNA from avian *P. multocida* cultures was isolated and purified by sodium dodecyl sulphate- proteinase K treatment and precipitation with hexadecyl trimethyl ammonium bromide, followed by isopropanol. The final DNA concentration was estimated relative to the known concentrations of DNA electrophoresed in agarose gels (Snipes *et al.*, 1990; Carpenter *et al.*, 1991).

Kim and Nagaraja (1990) isolated chromosomal DNA from turkey isolates of *P. multocida* by phenol-chloroform treatment, followed by precipitation with ethanol.

P. multocida cells harvested in sodium chloride-EDTA buffer were treated with lysozyme, followed by sodium dodecyl sulphate and proteinase K, with suitable incubations. Following several phenol-chloroform extractions, purified DNA was dialysed against Tris-EDTA buffer (Harel *et al.*, 1990).

A rapid method for extracting DNA from *P. multocida* was developed wherein bacterial cell pellet was subjected to treatment with lysozyme-sodium dodecyl sulphate- ribonuclease and proteinase K. DNA was extracted with phenol-chloroform and precipitated with ethanol in presence of monovalent cations (Wilson *et al.*, 1992; Wilson *et al.*, 1993; Wilson *et al.*, 1995a; 1995b; 1995c).

Zhao *et al.* (1992) recovered the cell extract of *P. multocida* and suspended it in Tris EDTA- sucrose buffer containing lysozyme. Proteinase K- sarkosyl- ammonium acetate were added sequentially and the proteinase was inactivated by phenylmethylsulphonyl fluoride. DNA was precipitated with cold absolute ethanol, and its concentration was measured spectrophotometrically.

Blackall *et al.* (1995) extracted chromosomal DNA of avian *P. multocida* by a scaled down version of standard DNA

extraction methods and the concentration was determined in a spectrophotometer.

Morishita *et al.* (1996b) isolated and purified DNA from psittacine isolates by the cetyltrimethyl ammoniumbromide method. DNA concentration was determined using a DNA fluorometer.

Rimler (2000) compared the two techniques for isolation of chromosomal DNA, a DNA zol method and an enzyme lysis followed by a two-phase partition method. Not much difference was observed except for the rapidity and ease to perform the former method.

2.9.1 Restriction endonuclease analysis of chromosomal DNA

Restriction endonuclease analysis (REA) provides distinctive banding profiles capable of differentiating isolates belonging to the same serotype.

Snipes *et al.* (1990) performed REA of 55 serotype 3,4 isolates of *P. multocida* from turkeys to differentiate them from the vaccine strains. *Sma I* digestion revealed eight different restriction patterns and ribotyping using *Eco RI* or *Pst I* was used to confirm the results.

Kim and Nagaraja (1990) analysed and compared the genomic characteristics of serologically indistinguishable field isolates of *P. multocida* in turkeys and the reference (CU and M9) vaccine strains. Vaccine strains had similar patterns between them

when treated with *Bgl II*, with differences only in the migration pattern of the 900bp fragment. One of the field isolates had a typical pattern similar to that of the vaccine strain M9, while three isolates had patterns similar to that of CU strain. One isolate had differences in the 400 and 900bp fragment regions.

Seven of the 23 porcine isolates of *P. multocida* had unique fingerprints while rest of the isolates were grouped into six REA patterns. The plasmid bands generated by *Eco RI* digestion attributed to the major differences between the REA patterns (Harel *et al.*, 1990).

Eleven distinct ribotypes were identified using *Eco RI* with 28 out of 49 isolates of *P. multocida*. Ribotypes observed on *Pst I* digested DNA confirmed the results obtained with *Eco RI*. The different ribotypes were in the molecular weight range of 4.4 to 23.1 kbp (Carpenter *et al.*, 1991).

Wilson *et al.* (1992) recognized seven distinct DNA profiles within 16 bovine serogroup B isolates using *Hha I* endonuclease. Five unique DNA profiles were obtained on digesting 13 serogroup E isolates with *Hpa II*, while identical profiles were seen with *Hha I* digestion. Both enzymes generated profiles with most of the fragments between 23.1 and 4.4kb.

Christiansen *et al.* (1992) reported the importance of REA in identifying the interflock transmission of *P. multocida* with three flocks on the premises at the same time. *Sma I* REA type I

profile was evident from the majority of the isolates, although restriction pattern of some of the isolates were consistent with that of the vaccine strains.

Zhao *et al.* (1992) observed four different REA patterns of *P. multocida* isolates from swine among 149 serotype A strains and two other REA patterns among eight serotype D isolates in one herd (REA type I). Restriction patterns of isolates from another herd had identical DNA profiles (REA type II). Ribotype patterns of the two REA patterns differed in only one band.

Fourteen bovine *P. multocida* isolates generated three or four fragments on cleaving with *Not I*, but one isolate appeared to have no recognition site for *Not I* (Townsend *et al.*, 1993).

Twenty eight distinct DNA profiles were recognized (Wilson *et al.*, 1993) in 63 avian *P. multocida* field isolates, 13 attenuated vaccine strains and 12 somatic types of 16 reference strains using *Hha I*. Serotypically dissimilar isolates had identical profiles with *Hha I*.

Wilson *et al.* (1995b) differentiated 314 isolates of *P. multocida* from wild birds by REA using *Hpa II*. Four profiles were identified of which one was unique and the other three were previously reported. The DNA profiles of isolates from wild birds and raptors were found to be identical.

Diallo *et al.* (1995) employed *Eco RI, Hind III, Bgl II, Bam HI* and *Pst I* in the genomic analysis of Australian strains of

avian *P. multocida*. All enzymes produced complex patterns of at least 70 fragments but *Hpa II* gave clear patterns, which allowed the 39 strains tested to be divided into ten groups.

Eight different serotypes were identified among 21 isolates of *P. multocida* from raptors. Nineteen isolates had *Hha I* profiles identical to that of the reference strain X-73. Four *Hpa II* profiles were observed among 19 isolates with differences in the 6.6 kb and 9.4 kb regions only (Wilson *et al.*, 1995a).

Morishita *et al.* (1996a) identified various restriction site heterogeneities of *P. multocida* chromosomal DNA among the raptor isolates. DNA profile of isolates of somatic serotype 1 from diurnal raptors showed a genetic relation between the isolates and were similar to that found in a duck.

Rimler and Nordholm (1998) evaluated the use of seven restriction enzymes in DNA fingerprinting of *Rimerella anatipestifer*. Using *Hinf I* and *Dde I*, the profiles could be better resolved in the 2.3 to 6.6 kb molecular weight range. *Hinf I* produced readable patterns in 2.7 to 20 kb range, which served to distinguish various isolates. Serotype 1 revealed 17 different profiles and serotype 2 exhibited five profiles.

Hpa II and *Hha I* generated 17 and 15 profiles respectively among 30 strains of *P. multocida*. *Hpa II* only differentiated the outbreak clone from the closely related strains, which had identical biochemical properties. Ribotyping using *Hpa II*

and *Hha I* revealed 12 and 10 profiles respectively. Six out of 30 strains (20 per cent) had a cryptic plasmid of 3.4 kb (Christensen *et al.*, 1998).

Rimler (2000) identified 48 different profiles among 222 isolates of *P. multocida* from domestic and wild animals using *Hha I*. Two trailing bands at approximately 8.4 to 7.0 kb were noted in profiles of serotype B: 2 isolates. Combining the data from REA with *Hha I* and *Hpa II* used separately, 88 different groups could be distinguished among the same isolates.

Jonas *et al.* (2001) recognized eight distinct DNA profiles using *Eco RI* and seven distinct profiles with *Hind III* from the avian *P. multocida* isolates in Indonesia. Plasmid of size 2.3 kbp was present in three isolates and two vaccine strains.

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and Laxbro brand plastics were used in this study. All chemicals used were of molecular biology grade, obtained from Sisco Research Laboratories Private Limited (SRL), Mumbai and the readymade media were procured from Hi-Media Laboratories Private Limited, Mumbai unless otherwise mentioned.

3.1 Isolation of Pasteurella

3.1.1 Materials

Samples for the isolation of Pasteurella were collected from ailing or dead birds brought to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy for disease diagnosis.

3.1.1.1 Bacterial isolates

Pasteurella multocida isolates obtained from the clinical samples brought to the Department of Microbiology and a reference strain of the same organism (LKO) obtained from Indian Veterinary Research Institute, Izatnagar were employed in the study.

Nutrient agar supplemented with sterile defibrinated sheep blood at five per cent level was used for the isolation.

3.1.2 Method

Samples of heart blood, lung, liver and spleen collected from the birds died of suspected pasteurellosis were streaked onto blood agar plates and incubated at 37°C in a candle jar.

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

3.2 Identification

The bacterial isolates were identified based on morphology, colony characteristics, tests for catalase and oxidase, oxidative or fermentative utilization of glucose, growth on Mac Conkey's agar, haemolysis on blood agar, indole production, methyl red and Voges-Proskauer reactions, urease activity, H_2S production, nitrate reduction, citrate utilisation, gelatin liquefaction, beta-galactosidase activity, lysine and ornithine decarboxylase activities, production of acid or gas from carbohydrates (galactose, glucose, inositol, lactose, maltose, mannitol, melibiose, raffinose, salicin, and sucrose) as described by Cowan (1974).

3.3 Biotyping

3.3.1 Materials

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

peptone water containing 0.2 per cent phenol red as indicator to determine the acid production.

3.3.2 Method

Ten per cent stock solution of each sugar was prepared in sterile distilled water and sterilised by autoclaving at 110°C, 10lbs pressure for 20 minutes. The stock solution was diluted to one per cent solution in Andrade's peptone water prior to use. Three ml of each diluted solution was taken into five ml tubes and inoculated with the culture under study. The tubes were incubated at 37°C for a minimum of seven days and examined daily for acid production as indicated by a colour change.

3.4 Antibiogram

3.4.1 Materials

Mueller Hinton agar was used to study the antibiotic sensitivity pattern of the isolates. The following antibiotic discs with known concentrations as noted in micrograms (μg) or International Units (IU) per disc were used. (Hi-Media Laboratories Private Limited, Mumbai, India).

1. Ampicillin (A) - 10μg
2. Chloramphenicol (C) - 30μg
3. Cloxacillin (Cx) - 5μg
4. Cotrimoxazole (Co) - 25μg
5. Enrofloxacin (Ex) - 10μg

6. Erythromycin (E) - 15µg
7. Furazolidone (Fr) - 100µg
8. Gentamicin (G) - 30µg
9. Metronidazole (Mt) - 5µg
10. Nitrofurantoin (Nf) - 300µg
11. Penicillin G (P) - 10Units
12. Pefloxacin (Pf) - 5µg
13. Streptomycin (S) - 10µg
14. Tetracycline (T) - 10µg

3.4.2 Method

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

3.5 Maintenance of virulence of isolates

3.5.1 Materials

3.5.1.1 Mice

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

3.5.2 Method

Two mice per isolate were used for the revival. Each animal was injected with 0.1ml of inoculum containing $3x10^8$ organisms per ml of sterile saline. A control mouse was injected with 0.1 ml of sterile saline. All the animals were observed for signs of

infection. Blood smears were prepared from the dead mice and stained with Wright's stain. Reisolation of *P. multocida* from the heart blood, lung, liver and spleen of the dead mice was also carried out.

3.6 Storage of isolates

3.6.1 Materials

3.6.1.1 Nutrient broth

Dehydrated medium was used as per the manufacturer's instructions.

3.6.1.2 Glycerol broth

Rehydrated nutrient broth	-	70ml
Glycerol	-	30ml

Nutrient broth was autoclaved at 121^{0}C for 15 min. at 15lbs. pressure, glycerol was sterilised in hot air oven at 160^{0}C for 60 min and stored at 4^{0}C.

3.6.2 Method

The pure cultures of all the field isolates and the reference strain were maintained in the laboratory by preserving in glycerol broth at -20° C.

3.7 Whole cell protein extraction

3.7.1 Materials

3.7.1.1 Brain Heart Infusion Broth

Prepared as per manufacturer's recommendations and autoclaved at 121°C for 15 min. at 15 lbs pressure.

3.7.1.2 HEPES (N-2-hydroxyethyl piperazine N-2 ethane sulphonic acid) buffer, 10mM, pH 7.4 (Hi-Media)

HEPES buffer - 0.238g

Distilled water - 100ml

pH was adjusted to 7.4 and autoclaved at 121°C for 15 min at 15lbs pressure and stored at 4°C.

3.7.1.3 Saturated ammonium sulphate solution (ASS)

Ninety grams of ammonium sulphate was added to 100ml of sterile distilled water and maintained in a water bath at 56°C for 30 min. The pH of the solution was adjusted to 7.0 using ammonia solution just prior to use.

3.7.1.4 Phosphate buffered saline (PBS), pH 7.2

Sodium chloride - 8.0g

Potassium chloride - 0.2g

Disodium hydrogen phosphate dihydrate - 1.069g

Potassium dihydrogen phosphate - 0.2g

Distilled water to make upto - 1000ml

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

3.7.1.5 Barium chloride solution (10 per cent)

Ten grams of barium chloride was dissolved in 100ml of sterile distilled water and autoclaved at 121°C for 15 min at 15lbs pressure and stored at 4°C.

3.7.2 Method

The whole cell protein extraction was performed as per the method described by Srivastava (1997).

The bacterial culture was grown in brain heart infusion broth for 18 to 24h at 37°C and the cell pellet washed twice with HEPES buffer (10mM), pH 7.4 and resuspended in the same buffer.

The cell suspension was lysed at a rate of 60 cycles per min, four times, with a 30 second intermittent cooling between each burst, in a sonicator (Branson Sonifier 450). The unbroken cells and debris were removed by centrifugation at 1,700xg for 20 min. Ammonium sulphate solution was added slowly drop by drop to the cell lysate while mixing upto 45 per cent saturation. The solution was mixed for 30 min after the addition of the last drop of ASS and the solution was kept for precipitation at 4°C overnight.

The resultant precipitate was dialysed against PBS (pH 7.4) for 48h, to remove ammonium sulphate from the precipitated proteins. This was checked by adding ten per cent barium chloride to a small amount of acidified buffer. The protein was stored at -20°C after dispensing in sterile aliquotes.

3.7.2.1 Estimation of protein concentration

The protein concentration of the sample was determined by Lowry's method (Lowry *et al.*, 1951) using a bovine serum albumin standard curve.

3.8 Outer membrane protein extraction

3.8.1 Materials

3.8.1.1 Normal saline solution

Sodium chloride - 0.85 g

Distilled water - 100ml

Autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.8.1.2 HEPES buffer (10mM), pH 7.4 (3.7.1.2.)

3.8.1.3 Sodium lauryl sarcosinate (two per cent) (Sigma)

Two grams of sodium lauryl sarconisate was dissolved in 100ml of sterile 10mM HEPES buffer (pH 7.4) prior to use.

3.8.2 Method

The outer membrane protein (OMP)–enriched extract from Pasteurella was prepared as per the method described by Barenkamp *et al.* (1981).

The bacterial culture grown on brain heart infusion agar for 18 h at 37°C was harvested and washed twice with sterile normal saline and suspended in 10ml of 10mM HEPES buffer (pH 7.4).

The cell suspension was lysed at a rate of 60 cycles per min, four times, with a 30 second intermittent cooling between each burst, in a sonicator (Branson sonifier 450). Intact cells and debris were removed by centrifugation at 1,700xg for 20 min. The supernatant was centrifuged at 1,00,000x g for 60 min at 4°C. The pellet which contained total membrane was resuspended in two ml of two per cent sodium lauryl sarcosinate detergent and incubated at 26°C for one hour.

The insoluble outer membrane–enriched fraction was sedimented by centrifugation at 1,00,000xg for 60 min at 4°C, washed twice with distilled water and dialysed against distilled water for 48h. The OMP extract was then dispensed in sterile aliquotes and stored at -20°C.

3.8.2.1 Estimation of protein concentration

The protein content of the OMP-enriched extract of the isolates was determined by Lowry's method using a bovine serum albumin standard curve.

3.9 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of whole cell and outer membrane proteins

3.9.1 Materials

3.9.1.1 Acrylamide – Bisacrylamide stock (30 per cent)

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

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

3.9.1.2 Resolving gel (12.5 per cent)

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

3.9.1.3 Stacking gel (four per cent)

Acrylamide stock (30 per cent)	-	0.67ml
Tris hydrochloride (0.5M), pH 6.8	-	1.25ml
Sodium dodecyl sulphate (ten per cent)	-	0.05ml
Ammonium persulphate (ten per cent)	-	25μl
N, N, N', N'-tetramethyl ethylenediamine (TEMED)	-	2.5μl
Distilled water	-	3.0ml

3.9.1.4 Electrophoresis buffer

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

3.9.1.5 Sample buffer (2x)

0.5M Tris hyrochloride, pH 6.8	- 2.5ml
Glycerol	- 2.0ml
Sodium dodecyl sulphate (ten per cent)	- 4.0ml
2-mercaptoethanol	- 0.2ml
Bromophenol blue	- 0.2mg
Distilled water to make	- 10ml

3.9.1.6 Coomassie brilliant blue (Sigma) staining solution

Coomassie brilliant blue (R250)	- 0.5g
Methanol	- 80ml
Glacial acetic acid	- 14ml
Distilled water to make to	- 200ml

3.9.1.7 Destaining solution I

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

3.9.1.8 Destaining solution II

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

3.9.2 Method

The whole cell and outer membrane protein profiles were determined by discontinuous system of polyacrylamide gel electrophoresis as per Laemmli (1970).

Thirty ml of 10 per cent resolving gel was prepared for a plate of 14x15cm dimension. The solution was degassed followed by addition of ammonium persulphate, TEMED and poured in between the glass plates with a pipette and allowed to polymerise. A thin layer of distilled water was layered on top of the gel to make the surface smooth.

When polymerisation was complete, the distilled water layer was poured off and 10 ml of five per cent stacking gel was prepared and poured in between the glass plates with the comb in position and kept for polymerisation. Then the plates were fitted in the reservoir buffer, and the comb was removed and the samples were charged into each slot.

Twenty micrograms each of the samples were mixed with 10µl of sample buffer kept in boiling water at 100°C for two minutes and then the entire volume was loaded into the well. The molecular weight marker (Sigma) was loaded in one corner well as standard.

The electrophoresis was run at 100 volts until the marker dye reached the lower level of the stacking gel and then the voltage was changed to 150 volts and continued until the dye reached the bottom of the resolving gel.

Then the gel was removed from the glass plate, the stacking gel was discarded and the resolving gel was stained with Coomassie brilliant blue solution for four hours and destained with the destaining solution, until the gel background became clear.

3.9.2.1 Photography

The gel was viewed under the white light in the Alpha Imager (Alpha Innotech Corporation, USA) and photographed.

3.9.2.2 Estimation of molecular weight

The molecular weights of the different bacterial proteins were directly obtained by comparing the distance migrated by the different proteins with that of the standard marker proteins of known molecular weights.

A standard graph was plotted with the $\log_{10}$ 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 chromosomal DNA

3.10.1 Buffers and Reagents

The following buffers and reagents were prepared and used as per Sambrook *et al.* (1989) with slight modifications.

3.10.1.1 Luria Bertani (LB) medium

Bacto-tryptone	-	10.0g
Bacto yeast extract	-	5.0g
Sodium chloride	-	10.0g
Distilled water	-	1000ml

pH was adjusted to 7.4 and autoclaved at 121°C at 15 lbs pressure for 15 minutes and stored at 4°C.

3.10.1.2 Phosphate buffered saline (PBS), pH 7.2 (3.7.1.4)

3.10.1.3 Tris –EDTA (TE) buffer, pH 8.0

Tris chloride, pH 8.0	-	10mM
EDTA, pH 8.0	-	1mM
Distilled water	-	100ml

Autoclaved at 121°C, 15lbs pressure for 15 minutes and stored at 4°C.

3.10.1.4 Lysozyme (Sigma)

Ten milligrams of lysozyme was dissolved in one ml of sterile distilled water just before use.

3.10.1.5 Sodium dodecyl sulphate (ten per cent)

Ten milligrams of sodium dodecyl sulphate was dissolved in 100 ml of sterile distilled water and stored at room temperature.

3.10.1.6 Proteinase K (Genei Private Limited, Bangalore)

Ten milligrams of Proteinase K was dissolved in one ml of sterile distilled water. It was initially preincubated for one hour and stored at -20°C.

3.10.1.7 Ribonuclease (RNase)

Pancreatic RNase (SRL) was dissolved at a concentration of 10 mg per ml in TE buffer. The solution was heated to 100°C for 15 min and allowed to cool to room temperature slowly. It was then dispersed into one ml aliquotes and stored at -20°C.

3.10.1.8 Phenol chloroform isoamyl alcohol mixture (25:24:1 ratio) (Sigma-Aldrich, USA)

3.10.1.9 Chloroform isoamyl alcohol mixture

Chloroform and isoamyl alcohol mixture in the ratio of 24:1(v/v) was prepared and stored in closed container at room temperature.

3.10.1.10 Sodium acetate solution

5M sodium acetate	-	68.04g
Distilled water to make up to	-	100 ml

pH was adjusted to 5.2 with glacial acetic acid and autoclaved at 121°C for 15 min. at 15lbs pressure and stored at 4°C.

3.10.1.11 Isopropanol

3.10.1.12 Tris Acetate EDTA (TAE) buffer stock solution 50 x, pH 8.0

Tris base	-	242.0g
Glacial acetic acid	-	57.1ml
0.5M EDTA (pH 8.0)	-	100.0ml
Distilled water to make upto	-	1000ml

Autoclaved at 121°C for 15 min. at 15lbs. pressure. The stock solution was diluted to 1 x before use.

3.10.1.13 Gel loading buffer

Bromophenol Blue	-	0.25 per cent
Glycerol	-	60 ml
TAE buffer (1 x)	-	40 ml

3.10.1.14 Agarose (Sigma)

3.10.1.15 Ethidium bromide

Ethidium bromide at a concentration of ten milligrams per ml of sterile distilled water was prepared as stock solution. The solution was mixed using a magnetic stirrer for one to two hours. The solution was then dispensed in amber coloured bottle and stored at room temperature.

3.10.2 Chromosomal DNA isolation from Pasteurella cultures

The total DNA from the Pasteurella cultures was isolated according to the method described by Wilson *et al.* (1992) with modifications.

3.10.2.1 Growth and harvest of bacterial culture

Bacteria were grown in 10ml of LB medium overnight with frequent shaking for aeration. The growth of the culture was monitored by reading the optical density (OD) at 600nm at which wavelength one OD unit corresponded to about $0.8x10^9$ cells per ml.

Harvest of the bacterial cells was done by centrifuging two ml of the culture at 16,000xg for four minutes. The cell pellet obtained was washed twice with PBS (pH 7.2). The pellet was then resuspended in one ml of TE buffer.

3.10.2.2 Processing of cell extract

The suspended cells were treated with 150µl of freshly prepared lysozyme solution and the mixture was placed on ice for 15 min to lyse the cells.

Then, 10 µl of RNase solution was added and mixed for one minute. Twenty five microlitres of proteinase K solution was added followed by 40 µl of ten per cent sodium dodecyl sulphate solution and mixed until the suspension cleared. The resulting mixture was incubated at 37°C for one hour.

3.10.2.3 Purification of DNA

Equal volume of phenol chloroform isoamyl alcohol mixture (25:24:1 ratio) was added to the cell extract and mixed gently for five to ten minutes for deproteinization.

The mixture was centrifuged at 16,000xg for one minute and the upper aqueous phase containing DNA was transferred to another tube. The white coagulated mass of protein at the interphase between the aqueous and organic layers was left undisturbed.

The extraction step was repeated two more times with equal volume of chloroform isoamyl alcohol mixture to purify the nucleic acid.

Finally, the upper clear aqueous phase was treated with 40µl of 5M sodium acetate solution and mixed slowly.

3.10.2.4. Concentration of DNA

Ice-cold isopropanol was layered on top of the DNA sample causing molecules to precipitate at the interphase. The precipitated DNA was recovered by spooling using a glass rod into a fresh tube. The DNA sample was centrifuged at 16,000xg for 15 min and the supernatant was removed. The DNA pellet was vacuum dried for ten minutes and finally suspended in 75µl of TE buffer.

3.10.2.5. Measurement of DNA concentration

The purity and concentration of DNA were determined by spectrophotometry. About five µl of the suspended DNA was diluted to 1.5ml using sterile distilled water and the blank was prepared with five µl of TE buffer diluted to 1.5 ml. An absorbance of 1.0 at 260nm wavelength corresponded to 50µg of double stranded DNA per ml. Ratio between the readings at 260nm and 280nm (OD260/OD280) indicated the purity of DNA. The samples having a ratio of 1.8 were selected as pure DNA, which were used for further studies.

3.11 Restriction enzyme analysis of chromosomal DNA.

3.11.1 Restriction enzymes (RE)

Four restriction enzymes viz., *Not I*, *Eco RI*, *Hinf I* and *Hpa II* were used in the study and were obtained from Genei Laboratories Private Limited, Bangalore.

3.11.1.1 Not I (8 units / µl)

10x assay buffer (unique)

3.11.1.2 Eco RI (20 units / µl)

10x assay buffer A

3.11.1.3 Hinf I (10 units / µl)

10x assay buffer C

3.11.1.3 Hpa II (10 units /µl)

10x assay buffer L

3.11.2 Restriction digestion

The restriction digestion mixture was prepared as follows:

DNA sample	-	10 μg
Restriction enzyme	-	4 μl
10x RE assay buffer	-	8 μl
Sterile distilled water to make to	-	30 μl

The total reaction volume of 30μl was incubated at 37°C overnight. The restriction enzyme activity was then inactivated by heating at 65°C for 20min before electrophoresis.

3.11.3 Agarose gel electrophoresis (AGE)

This procedure was carried out as per the procedure of Meyers *et al.* (1976) with modifications.

Agarose (0.6g) was dissolved in 75ml of TAE buffer (1 x) by heating and then cooled to 50°C. Ethidium bromide was then added to a final concentration of 0.5μg/ml.

The clean, dry, gel platform edges were sealed with adhesive tape and the comb was kept in position before pouring the agarose solution into the glass plate for setting. A gap of half to one mm was provided between the plate and comb tip to form a seal of agarose within the well formed beneath the comb tip.

The agarose gel was allowed to solidify for 30 to 45 min. Then the tape was removed and the platform with the gel was placed within the tank filled with TAE (1 x) buffer until the gel was completely submerged. Thirty µl of DNA sample was added with five µl of loading buffer and then loaded into the respective slot.

Electrophoresis was carried out at 35mA for two hours or till the loading dye reached three-fourths of the gel.

3.11.3.1 Photography

The gel was viewed under the UV – transilluminator in the Alpha imager and photographed.

3.11.3.2 Molecular size estimation

The Lambda DNA *Hind III* digest was run along with the DNA samples. A standard graph was plotted with the $\log_{10}$ Kb values of the marker DNA on the Y-axis, and the mobility of the known fragments of the marker on the X-axis. The values of the distance migrated by the sample DNA were interpolated with the standard curve to determine the molecular sizes of enzyme digested fragments of the chromosomal DNA.

Results

4. RESULTS

4.1 Isolation

Ailing/dead birds brought to the Department of Microbiology for disease diagnosis were subjected to detailed post mortem examination. Of the birds examined, four dead birds (one quail and three ducks) with the history of sudden, high mortality in the flock and gross lesions suggestive of pasteurellosis were included in the present study.

The gross lesions comprised of epicardial petechiae, enlargement of liver with congestion and pin point, white, necrotic foci, congested spleen, haemorrhagic tracheitis and diffuse haemorrhages in internal organs like proventriculus, lungs and intestine (Fig. 1,2). The lesions were of an acute form in the quail, while the three ducks obtained from different places revealed a peracute form of the disease.

On examination of heart blood smear and impression smears from liver, lungs and spleen of all necropsied birds, after staining with Leishman's stain, bipolar organisms were observed in both types of smears (Fig 3), although the number of bipolar organisms was considerably less in the quail blood smear.

Bacterial isolation was done using the biomaterials collected from all the four birds. On culturing, the biomaterials collected from all the four birds yielded mucoid, convex, grayish-

white and non- haemolytic colonies suggestive of pasteurella on blood agar after 24 h incubation at 37^0 C in a candle jar. The isolates thus obtained were designated as follows:

QP1 - from Japanese quail (Kolancheri, Ernakulam district)
DP1 - from Muscovy duck (Niranam, Pathanamthitta district)
DP2 - from Muscovy duck (Mannuthy, Thrissur district)
DP3 - from Muscovy duck (Chembukavu, Thrissur district)

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

4.2 Identification of the organism

4.2.1 First stage

All the isolates were gram negative, non-motile, coccobacilli (Fig.4). They grew aerobically and anaerobically, did not grow on Mac Conkey's agar and showed no haemolysis on blood agar, but were catalase and oxidase positive and fermented glucose. The same type of reactions were given by the reference strain (LKO) also (Table 1).

4.2.2 Second stage

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

and lysine decarboxylase negative. The two duck isolates (DP1 and DP3) and the LKO strain were ornithine decarboxylase positive while quail isolate (QP1) and the other duck isolate (DP2) showed a negative reaction. With regard to the fermentation of sugars, all isolates including the LKO strain utilized galactose, glucose, mannose and sucrose but did not ferment inositol, lactose, maltose, melibiose, raffinose and salicin (Table 2).

4.3 Biotyping

Fermentation of sugars like arabinose, dulcitol, sorbitol, trehalose and xylose by *P. multocida* isolates were studied to biotype them. Two biotypes were observed among the four field isolates. The quail isolate (QP1) fermented arabinose and xylose but not dulcitol, sorbitol and trehalose and hence classified under the subspecies *septica*. The other three duck isolates (DP1, DP2 and DP3) and the reference strain (LKO) fermented arabinose, sorbitol and xylose but not dulcitol and trehalose, and so included under the subspecies *multocida* (Table 3).

4.4 Antibiogram

The antibiograms of the four isolates and reference strain were determined, based on their susceptibility and resistance to various antibiotics/antibacterials as presented in Table 4. Four different antibiotic sensitivity patterns were exhibited by the four field isolates when 14 antibiotics/antibacterials were used.

All the isolates were uniformly sensitive to chloramphenicol, cotrimoxazole, enrofloxacin, pefloxacin and tetracycline but resistant to ampicillin, furazolidone, metronidazole and streptomycin. In addition, the isolate QP1 was resistant to cloxacillin, erythromycin, nitrfurantoin, and penicillin G. The isolates DP1 and DP2 were resistant to cloxacillin and penicillin G, while the DP3 isolate was sensitive to the two antibiotics. Also, the DP2 and DP3 isolates showed a resistance to gentamicin, while QP1, DP1 and LKO strain were quite sensitive to it.

4.5 Maintenance of virulence of isolates

The pure cultures of the four field isolates and reference strain were revived in mice once in two months. The inoculum containing $0.3x10^8$ organisms per 0.1 ml of normal saline of each of the four isolates and reference strain, killed mice within 10 to 12 h of infection.

The gross lesions observed in the internal organs of the dead mice were petechiae in the liver, pericardium and congestion of lungs and spleen. Blood smear and tissue impression smears collected from the dead mice, on staining with Leishman's stain, revealed the presence of bipolars (Fig. 5, 6). Reisolation of *P. multocida* was also done from the heart blood, lungs, liver and spleen.

4.6 Storage of the isolates

Al the isolates were successfully preserved viable upto 12 months in glycerol broth at – 20°C.

4.7 Whole cell protein profile by SDS-PAGE

4.7.1 Whole cell protein extraction and estimation of concentration

Whole cell proteins of all the four field isolates (QP1, DP1, DP2 and DP3) and the reference strain (LKO) were extracted by sonication and ammonium sulphate precipitation as described by Srivastava (1997).

The protein concentrations of QP1, DP1, DP2, DP3 and LKO as estimated by Lowry's method were found to be 3.5mg/ml, 4.2mg/ml, 3.37mg/ml, 3.25mg/ml and 4.3mg/ml respectively.

4.7.2 SDS-PAGE of whole cell proteins

The whole cell protein extracts of the different isolates, the reference strain and the standard protein marker were subjected to the discontinuous system of SDS-PAGE.

The standard protein marker (Genei, Bangalore) yielded nine bands ranging in the molecular weights from 205kDa to 3 kDa. Based on the position of the bands of the standard, the molecular weights of the four isolates and the reference strain were calculated directly by placing the stained gel in the Alpha Imager (Alpha Innotech Corporation, USA).

The number of bands that could be resolved by auto-query in the five isolates and their molecular weights are shown in Table 5 and the bands represented diagramatically in Fig.7a (Fig. 7).

The isolates QP1, DP1, DP2, DP3 and LKO produced 8,7,11,8 and 8 bands respectively. Two protein bands of which, one in the molecular weight region of 36 to 38 kDa and the other in the 10 to 11 kDa region were presented by all the four field isolates and the reference strain. Intensely- staining major bands in all the isolates ranged in the molecular weight of around 36 to 38 kDa and 29 to 32 kDa regions.

Sharing of the bands between the different field isolates and the reference strain (LKO) were observed in the following positions; between the LKO, QP1 and DP3 strains (at 87.8kDa); between LKO and QP1 isolates (at 16.3 to 16.6 kDa); between LKO and DP1 isolates (at 20.5 to 20.9 kDa and 10.9 kDa) and between LKO and DP3 isolates (at 71.3 kDa).

The isolates QP1 and DP2 shared a band in the molecular weight range of 42.0 to 42.5 kDa; DP1 and DP3 shared one band in the 2.5 to 2.6 kDa range and the DP2 and DP3 isolates shared one band in the 19.5 kDa position.

Unique protein bands with molecular weights at 62.1 kDa and 22.0 kDa were presented by the QP1 isolate; at 197.4 kDa, 81.2 kDa and 56.6 kDa molecular weight positions by the DP1 isolate; at 94.9 kDa, 77.1 kDa, 53.2 kDa, 25.5 kDa, 15.6 kDa and 4.6 kDa positions by the DP2 isolate; at 47.1 kDa position by DP3 isolate and at 51.7 kDa region by the LKO strain.

4.8 Outer membrane protein (OMP) profile by SDS-PAGE

4.8.1 OMP extraction and estimation of concentration

The OMPs of all the four field isolates and the reference strain were extracted by the process of sonication and ultracentrifugation as per the method described by Barenkamp *et al.* (1981).

The protein concentrations of the filed isolates (QP1, DP1, DP2 and DP3) and the reference strain were estimated by Lowry's method to be 1.5mg/ml, 2.8mg/ml, 3.0mg/ml, 1.65mg/ml and 1.6mg/ml respectively.

4.8.2 SDS-PAGE of OMP

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

The standard protein marker yielded nine bands ranging in molecular weights from 205 kDa to 3 kDa. Based on the position of the bands of the standard, the molecular weights of the four field isolates and reference strain were calculated directly by placing the stained gel in the Alpha Imager.

The bands resolved by auto-query in the five isolates and their molecular weights are shown in Table 6 and the bands represented diagrammatically in Fig.8a (Fig. 8).

The isolates QP1, DP1, DP2, DP3 and LKO produced 11, 13, 9, 9 and 13 bands respectively. Polypeptide bands of molecular weights around 87 kDa and 27 kDa were common to all the field isolates and the reference strain. Intensely-staining major bands could be located in the 32 to 33 kDa and 27 kDa regions in all the five isolates. The other major bands in 43 to 45 kDa molecular weight range were variable in their staining character on the gel. The protein subunits as calculated in the 66 to 67 kDa molecular weight range in all the isolates were also not clearly visible in the stained gel.

The QP1, DP1 and DP3 isolates showed approximately similar protein bands in the molecular weight range of 69.4 to 69.5 kDa.

The reference strain (LKO) shared two protein bands with DP1 isolate (at 91 kDa, 74 kDa and 44 kDa positions); one band in common with DP2 isolate (at 70.0 kDa position) and one band with DP3 isolate (at 6.2 to 6.5 kDa).

The QP1 and DP2 isolates shared protein bands at the 93.1 kDa, 45.1 kDa to 45.6 kDa range and 11.9 kDa positions.

Unique protein bands were observed at 42.3 kDa in the QP1 isolate; at 4.0 kDa, 10.6 kDa, 5.1 kDa and 3.1 kDa molecular weight regions in the DP1 isolate; at 4.9 kDa in the DP2 isolate; at 82.3 kDa in the DP3 isolate and at 20.6 kDa, 16.7 kDa and 9.9 kDa in the LKO strain.

4.9 Restriction endonuclease analysis of chromosomal DNA

4.9.1 Extraction of DNA and estimation of concentration

The DNA from the four field isolates (QP1, DP1, DP2 and DP3) and the reference strain (LKO) was extracted as per the method described by Wilson *et al.* (1992) with modifications.

Briefly, the cell pellet was lysed, deproteinised and then subjected to several phenol-chloroform extractions followed by precipitation of the DNA with isopropanol. The DNA suspended in the TE buffer was used for further studies.

The DNA concentrations per microlitre quantity of the five isolates (QP1, DP1, DP2, LKO and DP3) as estimated by spectrophotometry were found to be 1.84μg, 1.99 μg, 1.82 μg, 1.95μg and 1.82 μg respectively.

4.9.2 Restriction endonuclease analysis (REA)

The isolates (QP1, DP1, DP2 and DP3) and the reference strain (LKO) were subjected to restriction enzyme digestion using the enzymes; *Not I, Eco RI, Hinf I,* and *Hpa II*. After suitable incubations, the digests were electrophoresed in agarose gels. The different restriction fragments generated by the different enzymes were analysed for their molecular sizes in comparison with those of the standard DNA marker, by auto-query in the Alpha Imager.

4.9.2.1 REA using Not I

Not I enzyme did not digest any of the five DNA samples. The QP1, DP1, DP2, LKO strain and DP3 isolates showed the single undigested DNA above the 23.1 kbp molecular size level of the marker DNA, diagrammatically shown in Fig.9a (Fig. 9).

4.9.2.2 REA using Eco RI

On digestion with *Eco RI* enzyme, the QP1, DP1, DP2, LKO and DP3 isolates yielded 15, 7, 10, 13 and 16 fragments respectively and the molecular sizes are listed in Table 7 and the fragments represented diagrammatically in Fig.10

The molecular sizes of the restriction fragments of the isolates ranged from 19.5 to 4.6 kbp (QP1), 19.3 to 0.2 kbp (DP1), 19.7 to 0.2 kbp (DP2 and LKO) and 20.5 to 0.2kbp (DP3).

The QP1, DP1, DP2 and LKO strain shared a band in the 0.6 kbp region. The DP1, DP2, DP3 and LKO shared a fragment in the 0.2 kbp region, while the isolates QP1, DP2, DP3 and LKO shared a fragment in the 0.8 kbp region.

The reference strain (LKO) shared bands with the DP2 isolate alone at the 19.7 kbp region; with QP1 and DP2 isolates at 0.4 kbp region; with DP3 alone at the 11.1 kbp and 0.3 kbp regions and with QP1 and DP3 at 0.5 kbp region.

The QP1 isolate shared bands in common with the DP1 isolate in the 19.3 to 19.5 kbp range and 1.1 kbp region, and with the DP3 isolate in the 17.5kbp, 14.0 kbp, 12.2 kbp and 0.7 kbp regions.

Unique fragments were shown by the QP1isolate at 15.8 kbp, 10.6 kbp, 5.7 kbp, 4.0 kbp and 1.8 kbp regions; by the DP1 isolate at 11.5 kbp, 8.0 kbp and 5.4 kbp regions, by the DP2 isolate at 14.4 kbp, 10.2 kbp, 7.1 kbp, 4.4 kbp and 1.4 kbp regions; by the DP3 isolate at 20.5 kbp, 12.8 kbp, 9.6 kbp, 6.6 kbp, 5.9 kbp, 4.0 kbp and 1.3 kbp regions and by the reference strain at 16.5 kbp, 13.8 kbp, 9.8 kbp, 6.1 kbp and 4.1 kbp regions, which were not present in any of the four field isolates.

4.9.2.3 REA using Hinf I

Hinf I digestion of the DNA samples of QP1, DP1, DP2, LKO and DP3 generated 15, 17, 1,7 and 12 fragments respectively and their molecular sizes are listed in Table 8 and represented diagrammatically in Fig.11 . The molecular sizes of the restriction fragments of the isolates ranged from 4.7 to 0.2 kbp (QP1 and DP1); 9.6 to 0.2 kbp (LKO) and 8.7 to 0.2 kbp (DP3).

The QP1, DP1 and DP3 isolates shared two bands each at the 0.7 kbp and 0.4 kbp molecular size positions. Sharing of DNA fragments were observed between the LKO strain and QP1, DP1 and DP3 isolates at 0.5 kbp, 0.3 kbp and 0.2 kbp regions; with QP1 alone at 0.6 kbp and with DP3 alone at 7.5 and 5.0 kbp regions.

The isolates QP1 and DP1 had six bands in common at 4.7 kbp, 1.6 kbp, 1.4 kbp, 1.1 kbp, 0.9 kbp and 0.8 kbp regions. The DP1 isolate shared two bands with DP3 at the 1.9 kbp and 1.3 kbp molecular size regions.

Unique restriction fragments were exhibited in the 3.7 kbp, 2.6 kbp and 2.1 kbp regions by the QP1 isolate; in the 4.2 kbp, 3.6 kbp, 2.0 kbp and 1.5 kbp regions by the DP1 isolate; in the 4.8 kbp region by DP2 isolate; in the 8.7 kbp, 6.7 kbp and 3.9 kbp regions by the DP3 isolate and in the 9.6 kbp region by the LKO strain.

4.9.2.4 REA using Hpa II

On digestion with *Hpa II*, the QP1, DP1, DP2, LKO and DP3 isolates yielded 8, 8, 7, 6 and 8 restriction fragments respectively and their molecular sizes are listed in the Table 9 and represented diagrammatically in Fig.12 The molecular sizes of the fragments of all the isolates ranged from 11.6 to 0.2 kbp (QP1); 12.6 to 0.3 kbp (DP1); 13.6 to 0.4 kbp (DP2); 7.5 to 0.2 kbp (LKO) and 14.7 to 0.3 kbp (DP3).

The LKO shared common fragments with QP1, DP1 and DP3 at 0.3 kbp; with DP2 and DP3 at 0.8 kbp; with QP1 alone at 0.5 and 0.2 kbp; with DP2 alone at 2.5 kbp and with DP3 alone at 7.5 kbp.

The QP1 isolate showed identical fragments with DP1 at 1.3 kbp and with DP3 at 11.6 and 1.0 kbp regions. The restriction fragment of 0.4 kbp was shared by DP2 and DP3 isolates.

The QP1 isolate had unique bands at 6.7 kbp and 0.6 kbp molecular size regions; the DP1 isolate at 12.6 kbp, 8.2 kbp. 6.2 kbp, 4.3 kbp, 3.0 kbp and 0.7 kbp regions; the DP2 isolate at 13.6 kbp, 6.1 kbp, 4.0 kbp and 1.2 kbp regions and the DP3 isolate at the 14.7 kbp and 4.9 kbp regions. The reference strain did not reveal any fragments specific for that strain alone.

Fig. 1 Mycoardial haemorrhage and enlarged liver with necrotic foci (DP 1 isolate)

Fig. 2 Haemorrhagic tracheitis (DP 1 isolate)

Fig. 3 Blood smear showing biopolar organisms (DP 1 isolate), Leishman's staining, 1000 X

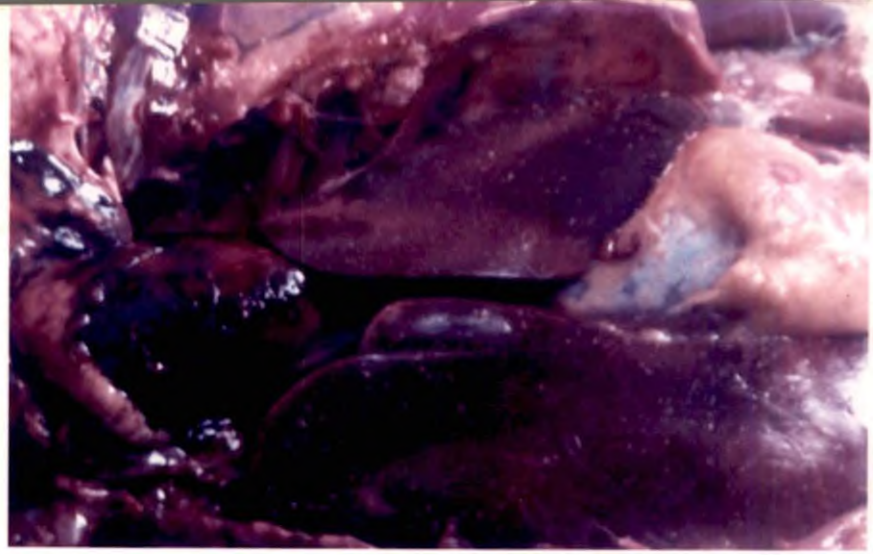


Fig- 1

Fig- 2

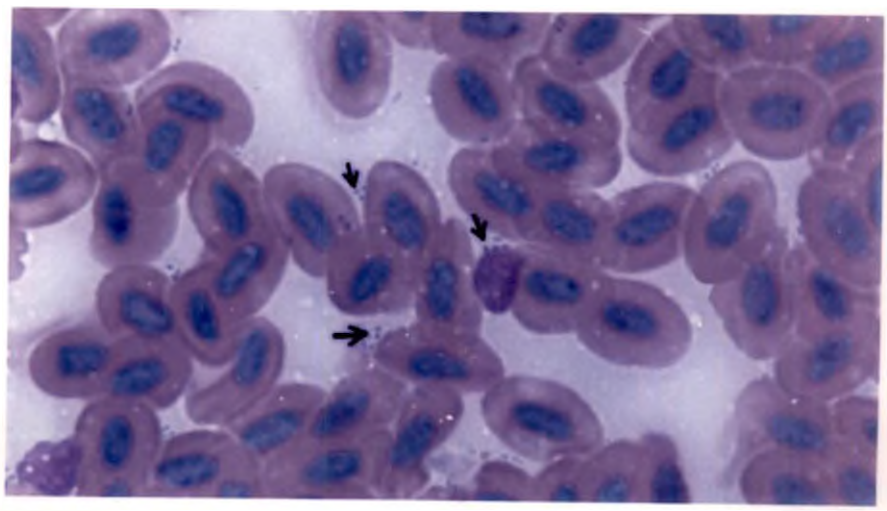


Fig- 3

Fig. 4 Gram's Staining showing gram negative coccobacilli (DP 1 isolate), 1000 X

Fig. 5 Liver impression smear of mice showing bipolars (DP 1 isolate), Leishman's staining, 1000 X

Fig. 6 Spleen impression smear of mice showing bipolars (DP 1 isolate), Leishman's staining, 1000 X

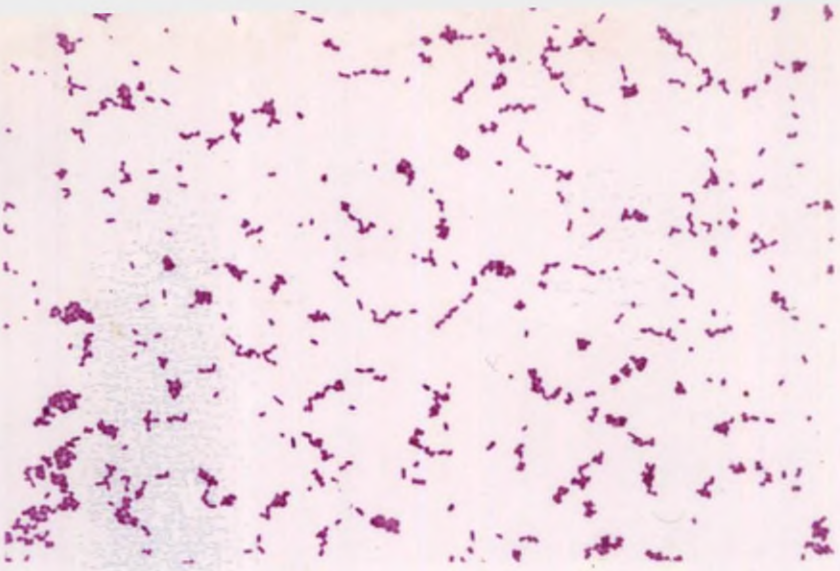


Fig- 4

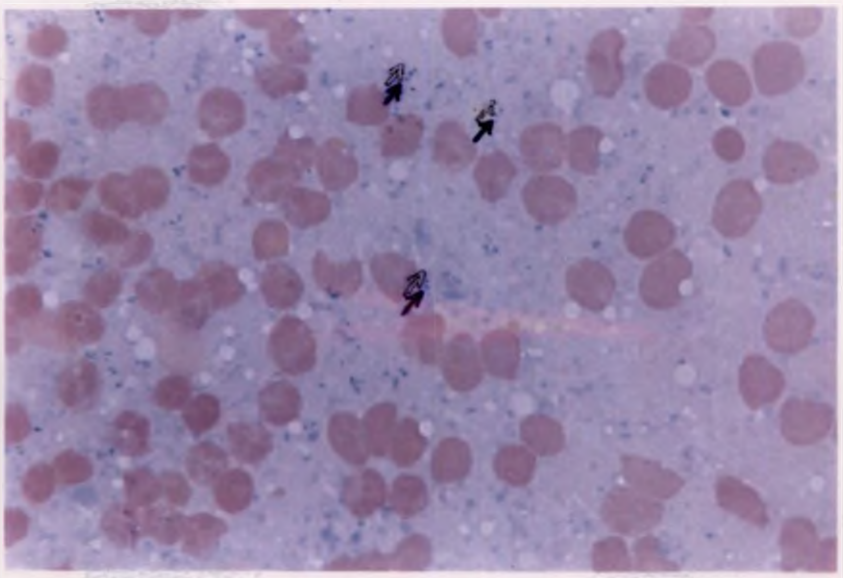


Fig- 5

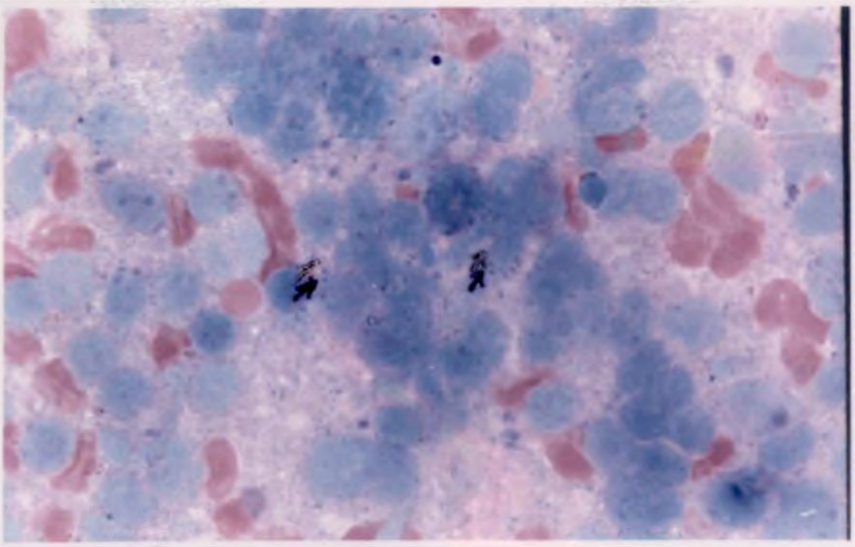


Fig- 6

Table 1. First stage of biochemical tests of isolates

Tests	Isolates				
	LKO	QP1	DP1	DP2	DP3
Gram's reaction	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Cocco-Bacilli	Cocco-Bacilli	Cocco-Bacilli	Cocco-Bacilli	Cocco-Bacilli
Presence of capsule	+	+	+	+	+
Motility	-	-	-	-	-
Growth in air	+	+	+	+	+
Growth anaerobically	+	+	+	+	+
Growth on MacConkey's agar	-	-	-	-	-
Haemolysis on blood agar	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Oxidation/ Fermentation of glucose (O/F)	F	F	F	F	F

Table 2. Second stage of biochemical tests of isolates

Tests	Isolates				
	LKO	QP1	DP1	DP2	DP3
Indole production	+	+	+	+	+
Methyl red test	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-
Urease	-	-	-	-	-
H_2S production	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Citrate utilization	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-
Beta-galactosidase activity	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	+	-	+	-	+
Sugar fermentation					
Galactose	+	+	+	+	+
Glucose	+	+	+	+	+
Inositol	-	-	-	-	-
Lactose	-	-	-	-	-
Maltose	-	-	-	-	-
Mannitol	-	+	+	-	-
Mannose	+	+	+	+	+
Melibiose	-	-	-	-	-
Raffinose	-	-	-	-	-
Salicin	-	-	-	-	-
Sucrose	+	+	+	+	+

Table 3. Biotyping of isolates

Sugars	Isolates				
	LKO	QP1	DP1	DP2	DP3
Arabinose	+	+	+	+	+
Dulcitol	-	-	-	-	-
Sorbitol	+	-	+	+	+
Trehalose	-	-	-	-	-
Xylose	+	+	+	+	+

Table 4. Antibiogram of isolates

Antibiotics/ Antibacterials	Isolates				
	LKO	QP1	DP1	DP2	DP3
Ampicillin	R	R	R	R	R
Chloramphenicol	S	S	S	S	S
Cloxacillin	R	R	R	R	S
Cotrimoxazole	S	S	S	S	S
Enrofloxacin	S	S	S	S	S
Erythromycin	S	R	S	S	S
Furazolidone	R	R	R	R	R
Gentamicin	S	S	S	R	R
Metronidazole	R	R	R	R	R
Nitrofurantoin	R	R	S	R	S
Penicillin G	R	R	R	R	S
Pefloxacin	S	S	S	S	S
Streptomycin	R	R	R	R	R
Tetracycline	S	S	S	S	S

S - Sensitive

R - Resistant

Table 5. SDS-PAGE profile of whole cell proteins

Protein fraction	Molecular weights (kDa) of the proteins of the isolates				
	QP1	DP1	DP2	DP3	LKO
I	-	197.4	-	-	-
II	-	-	94.9	-	-
III	87.8	-	-	87.8	87.8
IV	-	81.2	-	-	-
V	-	-	77.1	-	-
VI	-	-	-	71.3	71.3
VII	62.1	-	-	-	-
VIII	-	56.6	-	-	-
IX	-	-	53.3	-	-
X	-	-	-	-	51.7
XI	-	-	-	47.1	-
XII	42.5	-	-	-	-
XIII	-	-	42.0	-	-
XIV	38.2	-	-	-	-
XV	-	37.9	-	-	-
XVI	-	-	-	-	37.4
XVII	-	-	37.0	-	-
XVIII	-	-	-	36.7	-
XIX	31.8	-	-	-	-
XX	-	-	-	-	30.0

Protein fraction	Molecular weights (kDa) of the proteins of the isolates				
	QP1	DP1	DP2	DP3	LKO
XXI	-	-	29.7	-	-
XXII	-	-	-	29.3	-
XXIII	-	-	25.5	-	-
XXIV	22.0	-	-	-	-
XXV	-	20.9	-	-	-
XXVI	-	-	-	-	20.5
XXVII	-	-	19.8	-	-
XXVIII	-	-	-	19.5	-
XXIX	16.6	-	-	-	-
XXX	-	-	-	-	16.3
XXXI	-	-	15.6	-	-
XXXII	-	10.9	-	-	10.9
XXXIII	10.7	-	-	-	-
XXXIV	-	-	10.6	-	-
XXXV	-	-	-	10.4	-
XXXVI	-	-	4.6	-	-
XXXVII	-	2.6	-	-	-
XXXVIII	-	-	-	2.5	-
Total number of bands	8	7	11	8	8

Fig.7 SDS – PAGE profile of whole cell proteins

Lane 1 - Standard protein molecular weight marker

Lane 2 - QP1 isolate

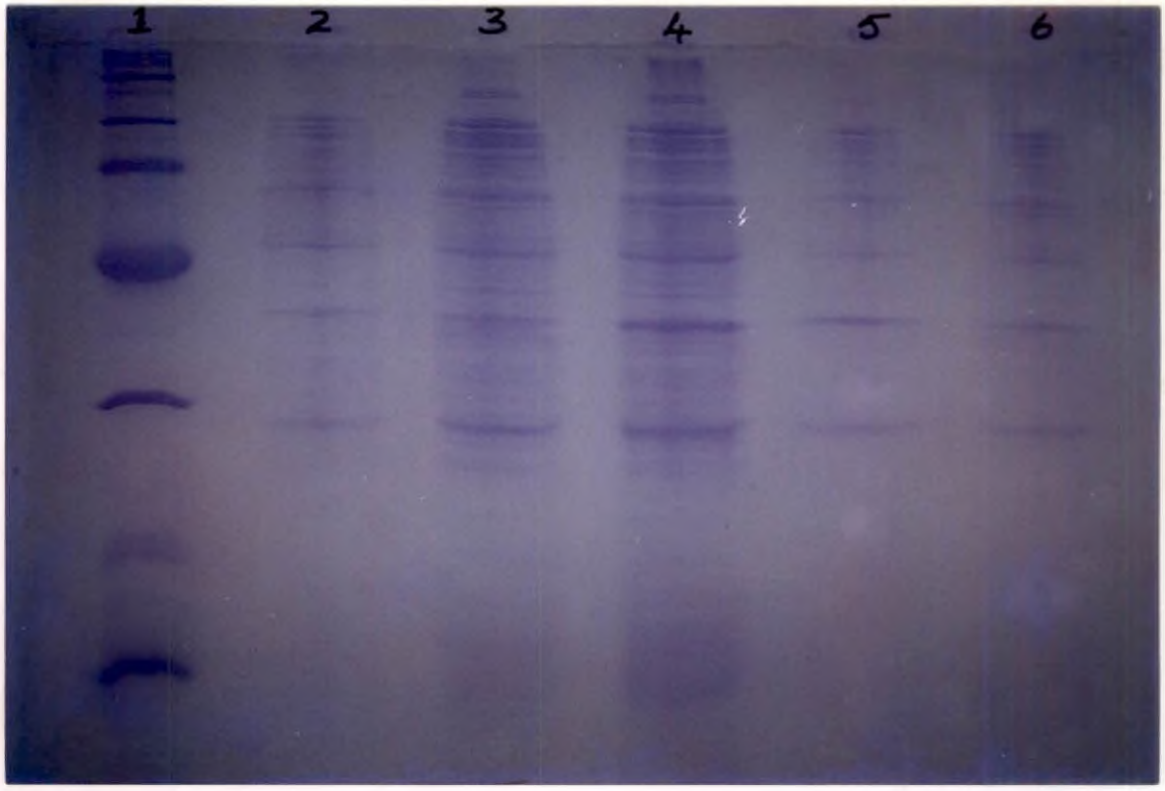
Lane 3 - DP1 isolate

Lane 4 - DP2 isolate

Lane 5 - DP3 isolate

Lane 6 - LKO strain

Fig.7a Diagrammatic representation of whole cell proteins profile


1
2
3
4
5
6

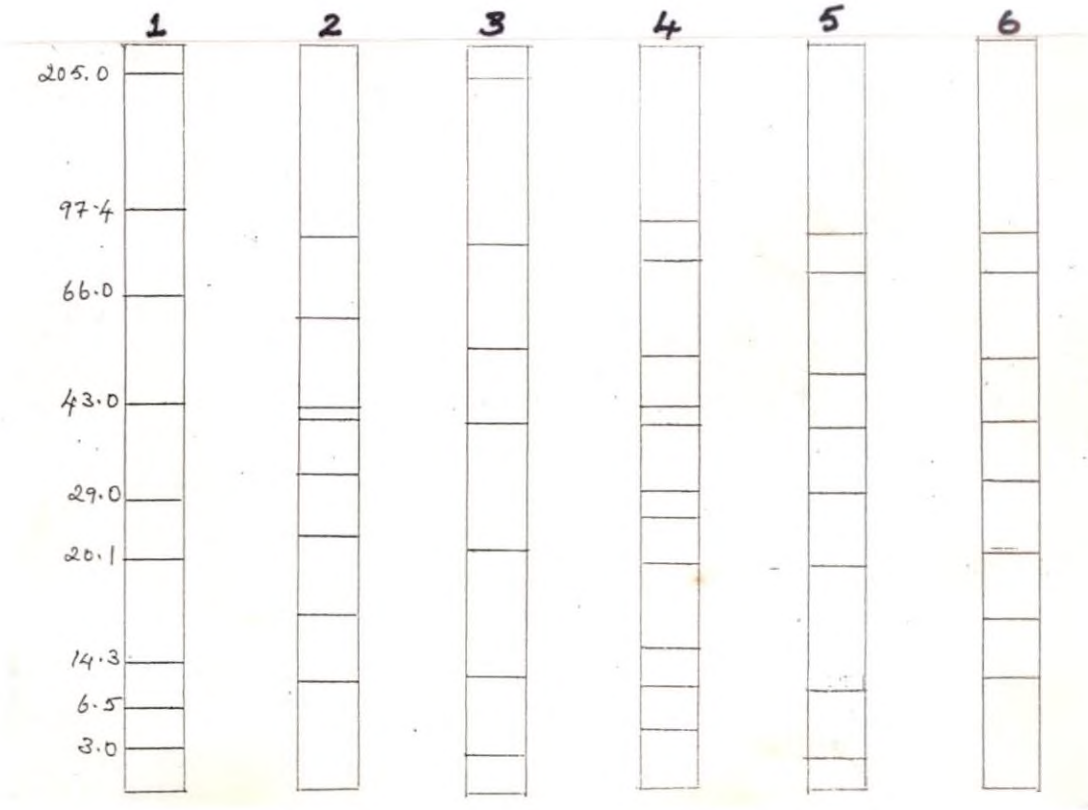

1
2
3
4
5
6
205.0
97.4
66.0
43.0
29.0
20.1
14.3
6.5
3.0

Table 6. SDS-PAGE profile of OMP

Protein fraction	Molecular weights (kDa) of the proteins of the isolates				
	QP1	DP1	DP2	DP3	LKO
I	93.1	-	93.1	-	-
II	-	91.3	-	-	91.9
III	87.2	87.8	87.1	87.8	87.2
IV	-	-	-	82.3	-
V	-	74.2	-	-	74.7
VI	-	-	70.0	-	70.0
VII	69.5	69.4	-	69.5	-
VIII	-	-	-	-	67.3
IX	66.4	66.9	66.9	66.9	-
X	49.6	-	-	49.2	49.2
XI	45.6	-	45.1	-	-
XII	-	44.0	-	-	44.0
XIII	-	-	-	43.0	-
XIV	42.3	-	-	-	-
XV	-	33.3	-	-	-
XVI	32.5	-	32.7	32.7	32.7
XVII	27.1	27.5	27.5	27.3	27.3

Protein fraction	Molecular weights (kDa) of the proteins of the isolates				
	QP1	DP1	DP2	DP3	LKO
XVIII	21.4	21.8	-	-	-
XIX	-	-	-	-	20.6
XX	-	17.4	-	-	-
XXI	-	-	-	-	16.7
XXII	11.9	-	11.9	-	-
XXIII	-	10.6	-	-	-
XXIV	-	-	-	-	9.9
XXV	-	-	-	6.5	6.2
XXVI	-	5.1	-	-	-
XXVII	-	-	4.9	-	-
XXVIII	-	3.1	-	-	-
Total number of bands	11	13	9	9	13

Fig.8 SDS – PAGE profile of OMP

Lane 1 - Standard protein molecular weight marker

Lane 2 - QP1 isolate

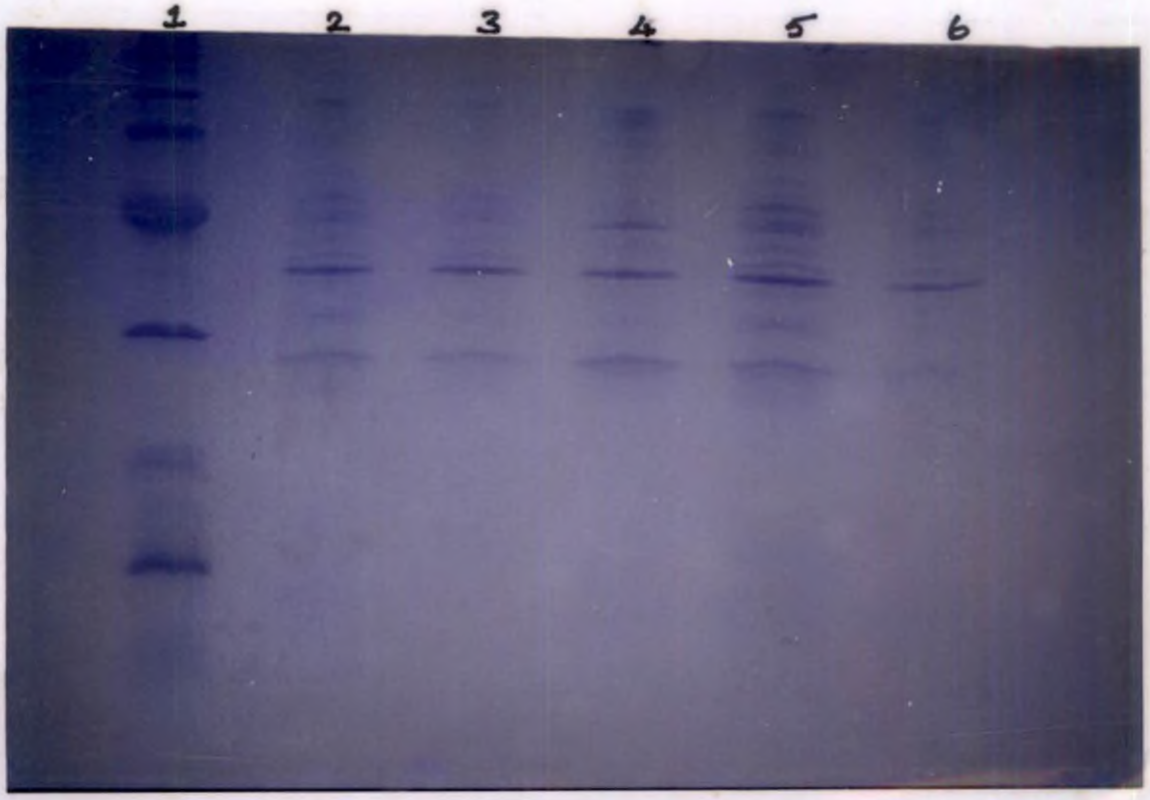
Lane 3 - DP1 isolate

Lane 4 - DP2 isolate

Lane 5 - DP3 isolate

Lane 6 - LKO strain

Fig.8a Diagrammatic representation of OMP profile


1
2
3
4
5
6

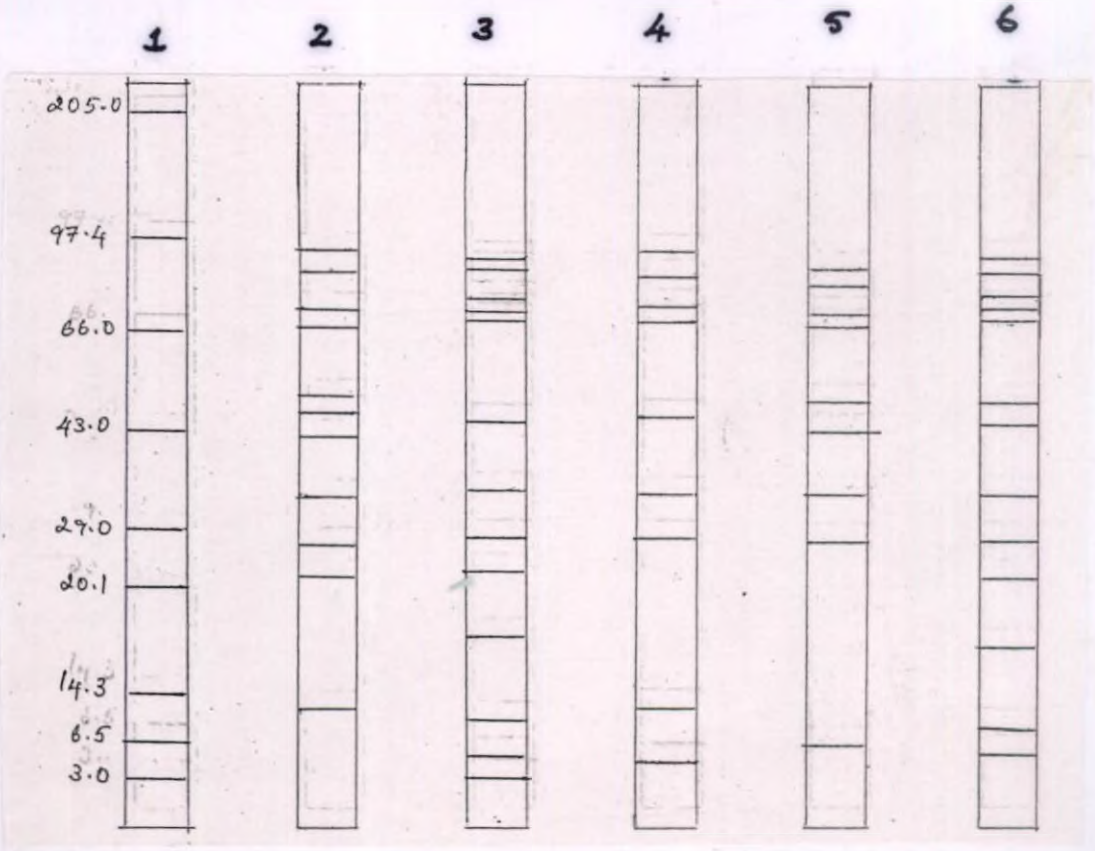

1
2
3
4
5
6
205.0
97.4
66.0
43.0
29.0
20.1
14.3
6.5
3.0

Fig.9 REA using *Not I*

Lane 1 - Lambda DNA *Hind III* digest molecular weight marker

Lane 2 - QP1 isolate

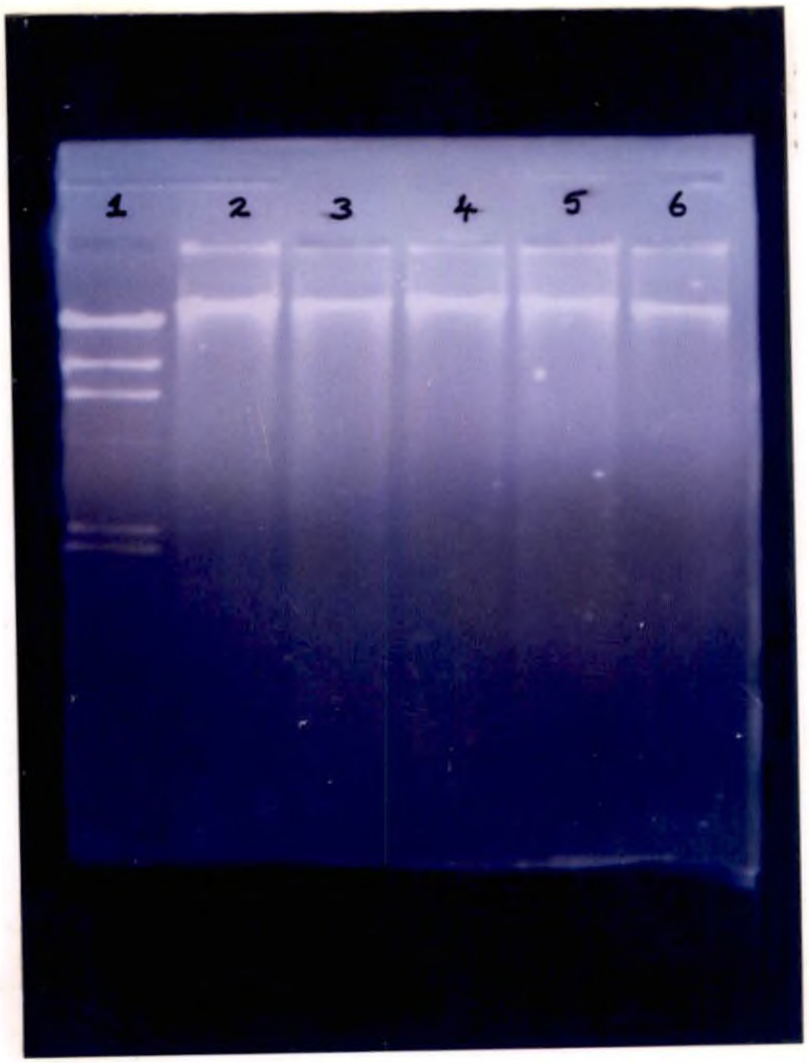
Lane 3 - DP1 isolate

Lane 4 - DP2 isolate

Lane 5 - LKO strain

Lane 6 - DP3 isolate

Fig.9a Diagrammatic representation of *Not I* profile


1
2
3
4
5
6

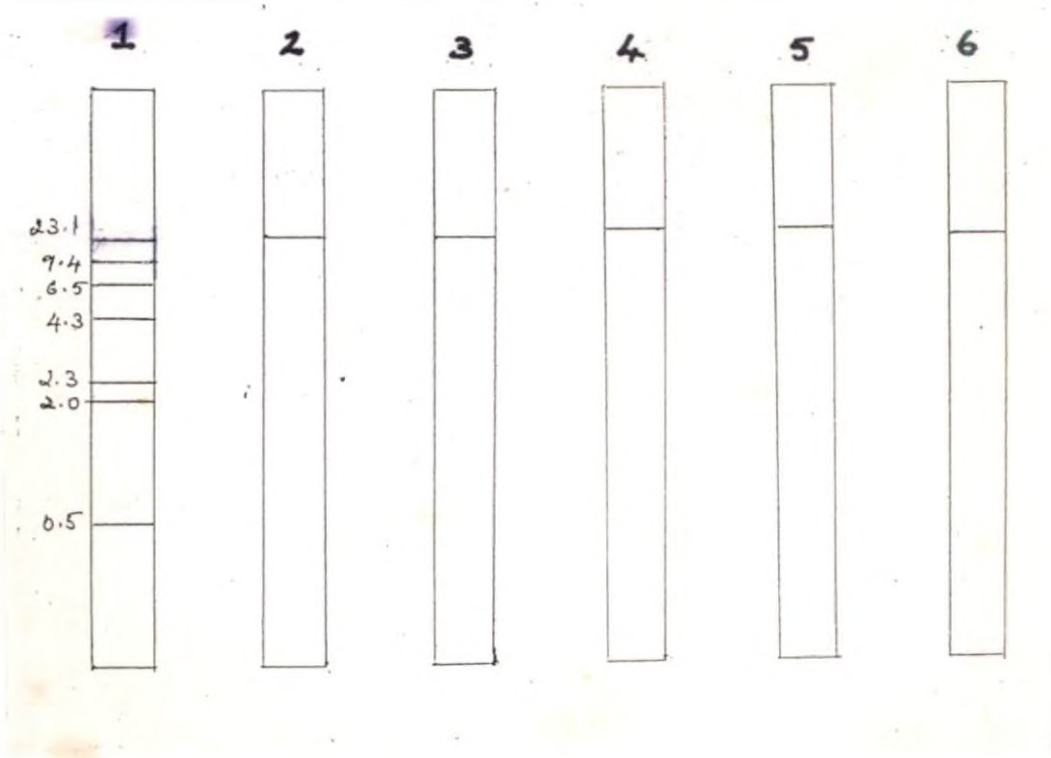

1
2
3
4
5
6
23.1
9.4
6.5
4.3
2.3
2.0
0.5

Table 7. REA using *Eco RI*

DNA fraction	Molecular sizes (kbp) of the restriction fragments of the isolates				
	QP1	DP1	DP2	LKO	DP3
I	-	-	-	-	20.5
II	-	-	19.7	19.7	-
III	19.5	-	-	-	-
IV	-	19.3	-	-	-
V	17.5	-	-	-	17.5
VI	-	-	-	16.5	-
VII	15.8	-	-	-	-
VIII	-	-	14.5	-	-
IX	14.0	-	-	-	14.0
X	-	-	-	13.8	-
XI	-	-	-	-	12.8
XII	12.2	-	-	-	12.2
XIII	-	11.5	-	-	-
XIV	-	-	-	11.1	11.1
XV	10.6	-	-	-	-
XVI	-	-	10.2	-	-
XVII	-	-	-	9.8	-
XVIII	-	-	-	-	9.6
XIX	-	8.0	-	-	-
XX	-	-	7.1	-	-
XXI	-	-	-	-	6.6

DNA fraction	Molecular sizes (kbp) of the restriction fragments of the isolates				
	QP1	DP1	DP2	LKO	DP3
XXII	-	-	-	6.1	-
XXIII	-	-	-	-	5.9
XXIV	5.7	-	-	-	-
XXV	-	5.4	-	-	-
XXVI	-	-	4.4	-	-
XXVII	-	-	-	4.1	-
XXVIII	4.0	-	-	-	-
XXIX	-	-	-	-	2.4
XXX	1.8	-	-	-	-
XXXI	-	-	1.4	-	-
XXXII	-	-	-	-	1.3
XXXIII	1.1	1.1	-	-	-
XXXIV	0.8	-	0.8	0.8	0.8
XXXV	0.7	-	-	-	0.7
XXXVI	0.6	0.6	0.6	0.6	-
XXXVII	0.5	-	-	0.5	0.5
XXXVIII	0.4	-	0.4	0.4	-
XXXIX	-	-	-	0.3	0.3
XL	-	0.2	0.2	0.2	0.2
Total number of fragments	15	7	10	13	16

Fig.10 Diagrammatic representation of *Eco RI* REA profile

Lane 1 - Lambda DNA *Hind III* digest molecular weight marker

Lane 2 - QP1 isolate

Lane 3 - DP1 isolate

Lane 4 - DP2 isolate

Lane 5 - LKO strain

Lane 6 - DP3 isolate

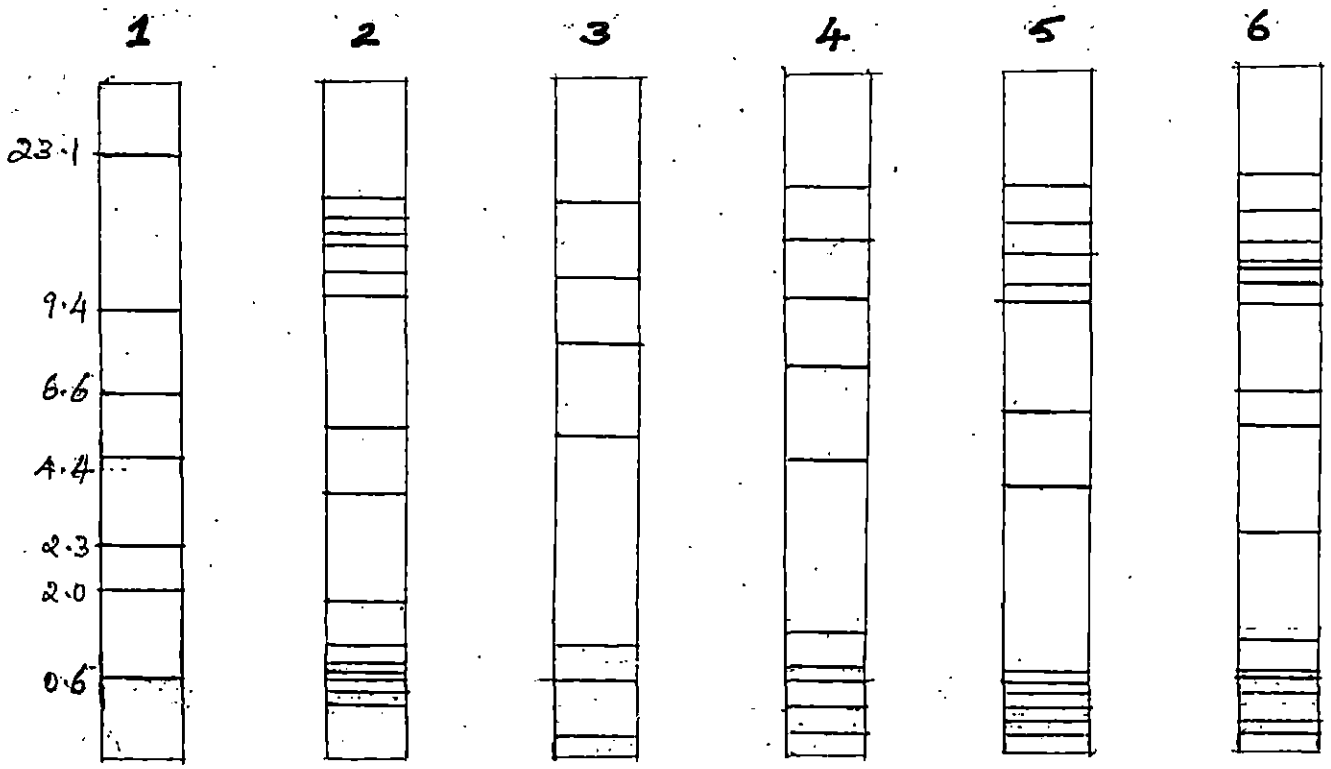

1
2
3
4
5
6
23·1
9·4
6·6
4·4
2·3
2·0
0·6

Table 8. REA using *Hinf I*

DNA fraction	Molecular sizes (kbp) of the restriction fragments of the isolates				
	QP1	DP1	DP2	LKO	DP3
I	-	-	-	9.6	-
II	-	-	-	-	8.7
III	-	-	-	7.5	7.5
IV	-	-	-	-	6.7
V	-	-	-	5.0	5.0
VI	-	-	4.8	-	-
VII	4.7	4.7	-	-	-
VIII	-	4.2	-	-	-
IX	-	-	-	-	3.9
X	3.7	-	-	-	-
XI	-	3.6	-	-	-
XII	2.6	-	-	-	-
XIII	2.1	-	-	-	-
XIV	-	2.0	-	-	-
XV	-	1.9	-	-	1.9
XVI	1.6	1.6	-	-	-
XVII	-	1.5	-	-	-

DNA fraction	Molecular sizes (kbp) of the restriction fragments of the isolates				
	QP1	DP1	DP2	LKO	DP3
XVIII	1.4	1.4	-	-	-
XIX	-	1.3	-	-	1.3
XX	1.1	1.1	-	-	-
XXI	0.9	0.9	-	-	-
XXII	0.8	0.8	-	-	-
XXIII	0.7	0.7	-	-	0.7
XXIV	0.6	-	-	0.6	-
XXV	0.5	0.5	-	0.5	0.5
XXVI	0.4	0.4	-	-	0.4
XXVII	0.3	0.3	-	0.3	0.3
XXVIII	0.2	0.2	-	0.2	0.2
Total number of fragments	15	17	1	7	12

Fig.11 Diagrammatic representation of *Hinf I* REA profile

Lane 1 - Lambda DNA *Hind III* digest molecular weight marker

Lane 2 - QP1 isolate

Lane 3 - DP1 isolate

Lane 4 - DP2 isolate

Lane 5 - LKO strain

Lane 6 - DP3 isolate

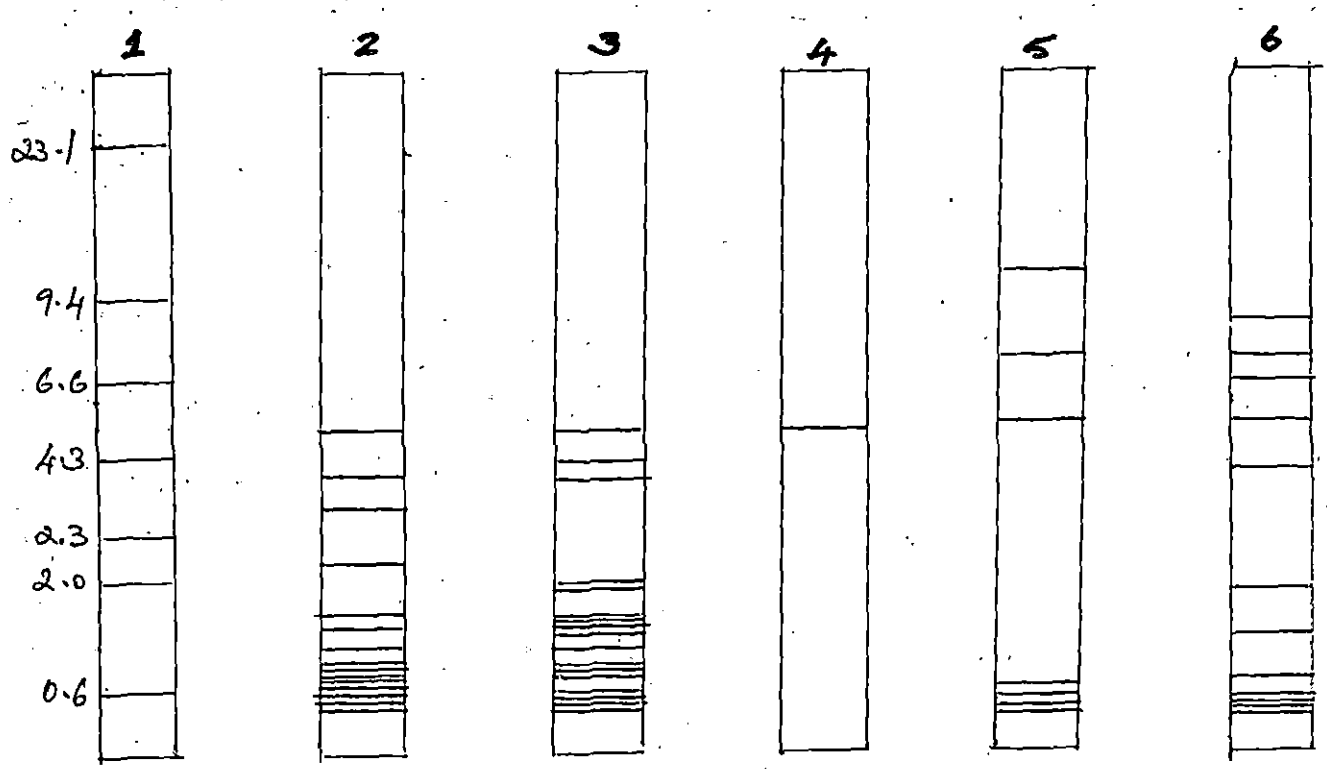

1
2
3
4
5
6
23.1
9.4
6.6
4.3
2.3
2.0
0.6

Table 9. REA using *Hpa II*

DNA Fraction	Molecular sizes(kbp) of the restriction fragments of the isolates				
	QP1	DP1	DP2	LKO	DP3
I	-	-	-	-	14.7
II	-	-	13.6	-	-
III	-	12.6	-	-	-
IV	11.6	-	-	-	11.6
V	-	8.2	-	-	-
VI	-	-	-	7.5	7.5
VII	6.7	-	-	-	-
VIII	-	6.2	-	-	-
IX	-	-	6.1	-	-
X	-	-	-	-	4.9
XI	-	4.3	-	-	-
XII	-	-	4.0	-	-
XIII	-	3.0	-	-	-
XIV	-	-	2.5	2.5	-
XV	1.3	1.3	-	-	-
XVI	-	-	1.2	-	-
XVII	1.0	-	-	-	1.0

DNA Fraction	Molecular sizes(kbp) of the restriction fragments of the isolates				
	QP1	DP1	DP2	LKO	DP3
XVIII	-	-	0.8	0.8	0.8
XIX	-	0.7	-	-	-
XX	0.6	-	-	-	-
XXI	0.5	-	-	0.5	-
XXII	-	-	0.4	-	0.4
XXIII	0.3	0.3	-	0.3	0.3
XXIV	0.2	-	-	0.2	-
Total number of fragments	8	8	7	6	8

Fig.12 Diagrammatic representation of *Hpa II* REA profile

Lane 1 - Lambda DNA *Hind III* digest molecular weight marker

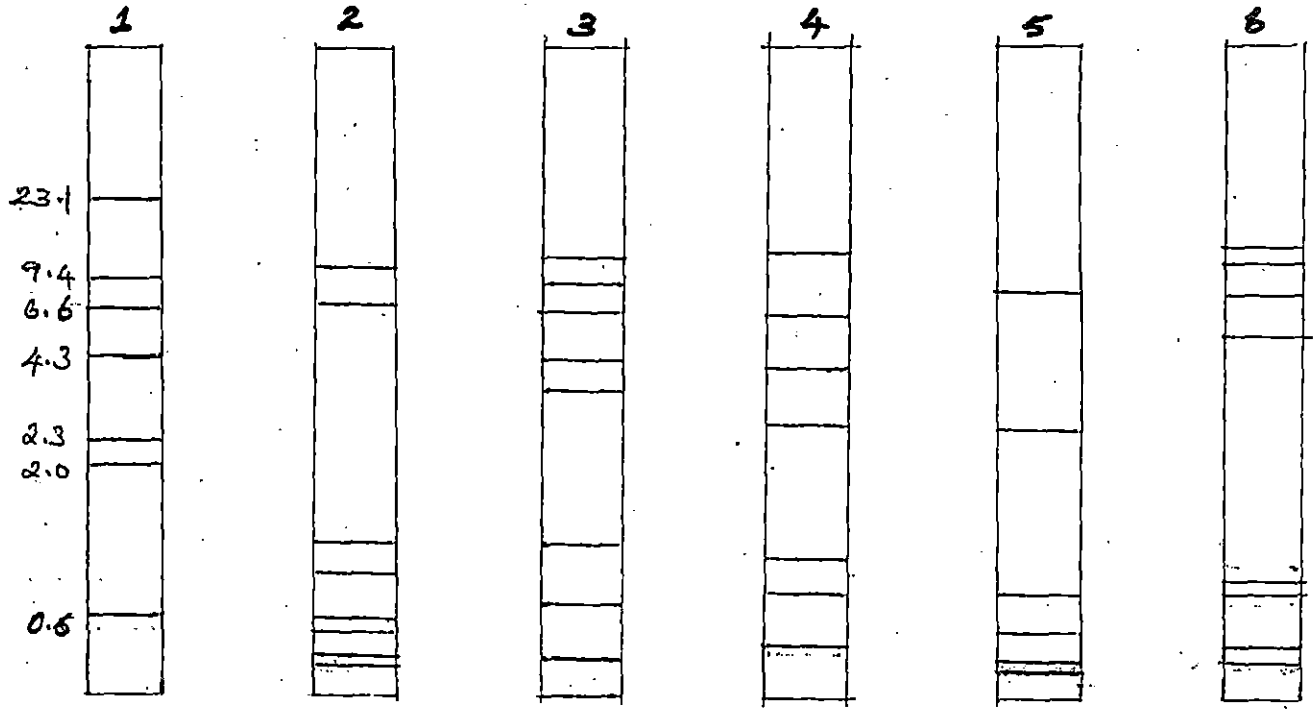
Lane 2 - QP1 isolate

Lane 3 - DP1 isolate

Lane 4 - DP2 isolate

Lane 5 - LKO strain

Lane 6 - DP3 isolate


1
2
3
4
5
6
23.1
9.4
6.6
4.3
2.3
2.0
0.6

Discussion

5.DISCUSSION

5.1 Isolation

The birds showing the gross lesions suggestive of pasteurellosis presented considerable number of bipolar organisms on blood smear examination except in quail. The lesser number of bacteria in the blood smear of quail could be attributed to the acute nature of the disease, while the ducks exhibited a per acute form of the disease.

The isolation rate of pasteurella from the biosamples of the four field biomaterials was quite high on sheep blood agar, which indicated the efficiency of sheep blood agar for primary isolation. This is in agreement with the findings of Carter (1981).

The incubation carried out in a candle jar with mild CO_2 tension at 37°C for 24 h was found to be optimum for the culture of pasteurella from the field biomaterials. Burrows and Gillet (1966) had suggested that the growth of *P. multocida* was more luxuriant at 37°C than at lesser temperature. Carter (1981) had reported that the organism grew well at 37°C in aerobic or anaerobic condition with five per cent CO_2 on bovine or sheep blood agar and that the carbonate ions favoured the growth of Pasteurella. The findings of the present study were in agreement with the observations made by the earlier workers.

P. multocida has been isolated from different species of animals and birds using different media incorporating various nutritive ingredients. Yeast Proteose Cystine (YPC) agar (Namioka and Murata, 1961), nutrient agar with five per cent sheep or bovine blood, tryptic soy blood agar (Carter, 1967), Casein sucrose-yeast (CSY) agar (Wijewardana *et al.*, 1986), *P.multocida* sensitive agar (PMSA) (Moore *et al.*, 1994) have all been reported to be useful for the primary isolation of *P. multocida*.

5.2 Identification

The colonies produced by all the isolates on blood agar plates were mucoid and non-haemolytic. The coalescing nature of the individual colonies might have been due to the high content of capsular material. All the field isolates and LKO strain produced mucoid colonies and exhibited the presence of capsule on Nigrosine staining. These observations were in accordance with the findings of Carter (1981).

The gram – negative, non-motile, coccobacillary forms of all the isolates were catalase and oxidase positive, grew anaerobically, fermented glucose, and neither grew on Mac Conkey's agar nor produced haemolysis on blood agar. Similar findings were reported by Cowan (1974) for the identification of *P. multocida*.

The second stage biochemical tests used to characterize *P. multocida* as described by Cowan (1974) were also identical for all the five isolates studied except for ornithine decarboxylase reaction. The two duck isolates (DP1 and DP3) and the reference strain showed a positive reaction while the quail isolate (QP1) and DP2 isolate were ornithine decarboxylase negative.

The importance of ornithine decarboxylase in the subspeciation, and the relatedness of ornithine negative strains of *P. multocida* by genomic studies, had been emphasised by Bisgaard *et al.* (1991), Mohan *et al.* (1994) and Fegan *et al.* (1995).

All the isolates uniformly fermented sugars such as galactose, glucose, mannose and sucrose without gas production. None of the isolates fermented inositol, lactose, maltose, melibiose, raffinose and salicin. These results were in agreement with the findings of Rimler and Rhoades (1989).

Variations in the fermentation of mannitol were noted between isolates: QP1 and DP1 isolates fermenting mannitol, while DP2, DP3 and LKO strains did not ferment. Similar observations with respect to fermentation of mannitol have also been reported by Mohan *et al.* (1994). However, Rajini *et al.* (1995) had reported fermentation of mannitol by only 63.3 per cent of *P. multocida* isolates used in their study while several workers

(Chandran *et al.*, 1995 and Fegan *et al.*, 1995) have reported fermentation of mannitol by all their *P. multocida* isolates.

Thus, based on the growth on culture media, morphological and biochemical characteristics and sugar fermentation reactions, all the four field isolates and the reference strain were identified as *P. multocida*.

5.3 Biotyping

The classification of *P. multocida* isolates into subgroups or biotypes was done based on the reaction patterns observed with acid production from arabinose, dulcitol, sorbitol, trehalose and xylose (Rimler and Rhoades, 1989).

In this study, two biotypes were observed among the five isolates. All the isolates fermented arabinose and xylose but dulcitol and trehalose were not utilized by any of them. The isolates DP1, DP2, DP3 and LKO strain fermented sorbitol. Mutters *et al.* (1985) had suggested the use of fermentation pattern of sorbitol and dulcitol in the differentiation of *P. multocida* into the various subspecies; the subsp. *gallicida* was sorbitol and dulcitol positive; sorbitol and dulcitol negative was subsp. *septica* and sorbitol positive but dulcitol negative constituted subsp. *multocida*.

None of the isolates in the present study fermented dulcitol and so none could be classified under the subsp. *gallicida*.

This was in accordance with the observations of Mohan *et al.* (1994).

The quail isolate (QP1) was negative for sorbitol and dulcitol and hence classified as *P. multocida* subsp. *septica*, while the three duck isolates (DP1, DP2 and DP3) and LKO strain were positive for sorbitol but negative for dulcitol, and hence are classified as *P. multocida* subsp. *multocida*.

Rajini *et al.* (1995) reported that the difference in the fermentation of carbohydrates was attributed for the geographical variations of the isolates and the use of chemotherapeutic agents, as these factors influenced the enzyme profiles of the microbes.

5.4 Antibiogram

In vitro sensitivity testing of antibiotics/ antibacterials is recommended for an effective chemotherapy owing to the variation in the responsiveness of *P. multocida* to the various antibiotics.

All the isolates were sensitive to chloramphenicol, cotrimoxazole, enrofloxacin, pefloxacin and teteracycline.

Waltman and Horne (1993) opined that the *P. multocida* isolates were highly susceptible to all antibiotics/ antimicrobicals tested, except for the sulphonamides. Balakrishnan (1998) reported on the sensitivity of *P. multocida*

isolates to oxytetracycline, pefloxacin and streptomycin and Devi *et al.* (2000) found a high degree of sensitivity of duck *P. multocida* isolates to pefloxacin and gentamicin.

The variations in the antibiograms of the isolates in the present study could be attributed to the indiscriminate use of antibiotics in treatment of the infection, or their increased use as feed additives which might have resulted in acquired drug resistance.

The antibiotic resistance in pathogenic pasteurellae may also be due to the occurrence of transferrable plasmids or chromosomal changes as opined by Willson (1990).

In the present study, no attempt was made to separate plasmids from the isolates.

5.5 Whole cell protein profile

The whole cell proteins of each of the four field isolates and the reference strain of *P. multocida* were analysed on SDS-PAGE. The number of polypeptide bands in QP1, DP1, DP2, DP3 and LKO strains, varied from 7 to 11, consisting of 8, 7, 11, 8 and 8 protein fractions respectively. The protein concentration of each isolate was found to be directly related to the number of protein bands resolved on SDS-PAGE.

Of these, two bands of molecular weight around 36 to 38 kDa and 10 to 11 kDa were common in all the five isolates.

The major difference between *P. multocida* isolates of porcine origin had been resolved in the 34 to 38 kDa region by Lugtenberg *et al.* (1984) who named the major protein in that region as the heavy ('H') protein. The immunogenicity of the protein-H was also identified (Lugtenberg *et al.*, 1986).

All the isolates in the study exhibited a similar major protein band in the molecular weight range of 36 to 38 kDa region with slight variations in the size, amongst them. Similar results have been reported by Ireland *et al.* (1991) who identified differences between avian isolates of *P. multocida* in the same molecular weight range.

The isolates QP1 and DP2 had two polypeptide bands of almost similar molecular mass of around 42 kDa, which were not observed in the other three isolates. The protein band of 42 kDa in addition to the 23 kDa and 10 kDa bands, were reported to be unique to *P. multocida* isolates from ducks (Sulochana, 1998). Although the protein band of 23 kDa was absent, bands in the molecular weight range of 19.5 to 22 kDa were present in all the isolates.

All the four field isolates and reference strain had a polypeptide band of molecular weight of around 10 kDa, which had been reported by Sulochana, (1998), to be one of the protein bands specific for *P. multocida*.

The high molecular weight protein polypetides in the range of 120 to 60 kDa obtained form sonicated cells of *P. multocida* as described by Srivastava (1998) were not clearly visible in this study. This could be attributed to the fact that these proteins were not precipitable at 45 per cent ammonium sulphate saturation. But proteins in the molecular weight range of 36 to 56 kDa were precipitated at this concentration.

The other low molecular weight proteins unique to each isolate could correspond to the minor polypetides of the isolates.

5.6 Outer membrane protein profile

The Outer Membrane Protein (OMP) and associated proteins of the various field isolates and the reference strain were analysed on SDS-PAGE. The number of polypeptide bands (QP1 DP1, DP2, DP3 and LKO) varied from 9 to 13, consisting of 11, 13, 9, 9 and 13 protein fractions respectively.

The major OMPs resolved from all the five isolates were around 27 kDa, 32 to 33 kDa, 42 to 45 kDa molecular weight

range. In addition, several other minor associated proteins were present in each of the isolates.

Of the major bands, the 27kDa protein band and bands in 42 to 45 kDa range were intensely – staining and common in all the isolates. The bands in the 32 to 33 kDa protein of all the isolates were faintly staining on the gel.

Brogden and Rimler (1983) had resolved four bands of 14.3 kDa, 28.6 kDa, 42.9 kDa and 57.2 kDa in turkey-grown cells, but were absent in the broth – grown cells. In the present study, no such bands were seen in any of the isolates except for the DP3 isolate, which exhibited a protein band of 43kDa.

Two major OMPs of around 27 and 32 to 33 kDa molecular weights were resolved in all the five isolates. Similar findings had been reported by Choi *et al.* (1989), with the major OMPs identified in the 34.5 kDa and 29 kDa.

Four of the isolates (QP1, DP1, DP2 and DP3) expressed an associated protein of molecular weight of around 66 kDa, absent in the reference strain. Lee *et al.* (1990) had described an isolate which had expressed bands in the 66 kDa region, more intensely staining than the vaccine strain.

Choi-Kim *et al.* (1991) had identified the major OMPs with molecular masses of 29kDa, 34.5 kDa and 45 kDa which were expressed by *P. multocida* grown in iron replete media which

included Brain Heart Infusion (BHI) broth, but failed to express 76 kDa, 85 kDa, and 94 kDa proteins. The results of the present study were in accordance with the above findings.

Chevalier *et al.* (1993) had demonstrated a protein band of around 34 kDa molecular mass from *P. multocida* isolates, probably representing the denatured monomers of protein-H. The present study resolved a protein band of around 32 to 33 kDa in all the isolates, which may be the protein-H.

All the four field isolates and reference strain expressed protein bands of around 87 kDa molecular mass which were weakly staining. The 87 kDa protein was one among the OMPs from avian isolates of *P. multocida* whose antibodies cross reacted with the OMPs from porcine strains (Zhao *et al.*, 1995).

The major and associated polypetides obtained from the OMP of the different isolates were in the range of 27 to 93 kDa molecular weight. Pati *et al.* (1996) had reported that presence of the major polypetides in the 25 to 88 kDa molecular weight range.

Higher and intermediate molecular weight bands (93 to 27 kDa), more or less revealed homogeneity among the isolates, while the differences were to some extent with the low molecular weight proteins (less than 20 kDa).

5.7 Restriction endonuclease analysis of chromosomal DNA

The DNA samples of all the field isolates and the reference strain had the OD260/OD280 ratio of more than 1.8, which indicated the purity of the DNA. The DNA concentrations of all the five isolates were found to be approximately similar.

5.7.1 Not I profile

Treatment of the isolates with *Not I* restriction enzyme did not reveal any fragments, thereby indicating that no digestion had taken place.

Townsend *et al.* (1993) had reported that digestion of *P.multocida* of bovine and porcine origin with *Not I* enzyme, which resulted in the production of three to four restriction fragments in all of the isolates except one, which appeared to have no recognition site for the enzyme. They also observed that the *Not I* enzyme recognised sequences composed of high G+C content and that since the G+C content of *P. multocida* was low, this enzyme would not cut the DNA as frequently as it would a G+C rich bacterial genome.

5.7.2 Eco RI profile

Eco RI digestion of all the five isolates used in the study yielded DNA fragments ranging from 20.5 kbp to 0.2 kbp molecular size.

Snipes *et al.* (1990) used *Eco RI* to initially group isolates of *P. multocida* from turkeys, and the results were confirmed by ribotyping.

Harel *et al.* (1990) observed that the analysis of bacterial genomic digests by *Eco RI* was a practical method for differentiating previously serotyped *P. multocida* isolates. Carpenter *et al.* (1991) could resolve distinct ribotypes of *P. multocida* isolates using *Eco RI* and *Pst I* - digested DNA.

In the present study, REA with *Eco RI* only was done. The banding patterns were complex and not easily distinguishable on the gel. However, 7 to 16 fragments were produced for the various isolates. The presence of unique fragments in each isolate indicated the differences between the five isolates.

Diallo *et al.* (1995) had reported a similar finding of complex patterns in the band profiles on digesting DNA with *Eco RI*, and they opined that it was not useful in designating the differences between isolates.

5.7.3 Hinf I profile

There were no published reports on the restriction pattern of *P. multocida* isolates with *Hinf I*.

Rimler and Nordholm (1998) had reported the use of *Hinf I* in the digestion of DNA extracts from *Rimerella anatipestifer* isolates. They resolved the presence of well-separated and distinguishable bands in the 2.3 to 6.6 kbp range, while no bands could be detected above and below the range.

In the present study, the three field isolates (QP1, DP1 and DP3) of *P. multocida* and the reference stain generated restriction fragments in the range of 9.6 kbp to 0.2 kbp, but one isolate (DP2) exhibited only one fragment.

The DNA fragments in the similar range were exhibited by *P. multocida* isolates used in this study also. In addition, bands above 6.6 kbp and below 2.3 kbp were also present.

5.7.4 Hpa II profile

Hpa II digestion of the field isolates and the reference strain generated six to eight restriction fragments in the molecular size range of 14.7 kbp to 0.2 kbp detected by auto-query. But the bands were poorly stained on the gel and indistinguishable, although clear patterns of the bands using the same enzyme had been reported by other workers (Zhao *et al.*, 1992; Wilson *et al.*, 1995a and b; Christensen *et al.*, 1998).

Unique bands were detected for all the isolates except LKO strain, the different fractions of which, showed similarity

with one or more of the isolates. This was indicative of the differences existing between the isolates and the reference strain.

5.8 Conclusion

The ornithine negative strains of *P. multocida* (QP1 and DP2) exhibited variations in their fermentation of mannitol. Bisgaard *et al.* (1991) had also reported the variations in the fermentation of mannitol in ornithine – negative strains of *P. multocida.*

With regard to the other three isolates (DP1, DP3 and LKO), they were ornithine positive and were biotyped as *P. multocida* subsp. *multocida,* based on the fermentation of dulcitol and sorbitol. The isolate DP2 in the present study, hence appears to be a ornithine negative variant of *P. multocida* subsp. *multocida.* The QP1 isolate did not ferment both sorbitol and dulcitol and hence designated as *P. multocida* subsp. *septica.* This observation is in accordance with the findings of various workers who also described ornithine negative variants of the species, emphasising the need of hybridisation studies in resolving the isolates (Mohan *et al.*, 1994; Fegan *et al.*, 1995; Blackall *et al.*, 1997).

The antibiograms were distinct for each of the field isolates and reference strain.

By biotyping, the three duck isolates were found to belong to the same biotype as that of the reference strain. Though the whole cell and outer membrane protein profiles of these isolates and the reference strain shared common bands, the presence of unique bands in each, indicated that the duck isolates could be variant forms of the reference strain. The QP1 isolate shared an even lesser degree of homology by protein profile, probably because of its different subspecies level.

The differences in the unique protein bands produced by each of the isolates obtained from different geographical areas, indicated that the isolates could be variants of *P. multocida.*

Restriction endonuclease analysis resolved similarities in between the duck isolates, as well as with the reference strain. On comparing the QP1 isolate with DP1, a higher degree of homology was observed unlike the protein profiles. Even though QP1 and DP1 belonged to two subspecies of *P. multocida*, this observation is indicative of a basic genetic relatedness among the *P. multocida* isolates from the two species; namely, quails and ducks.

The differences or similarities between the isolates could still be better resolved by serotyping, ribotyping and DNA hybridisation studies. Further research is required for the identification of the immunogenic proteins unique to the different isolates for attempting preparation of an effective vaccine.

Summary

6. SUMMARY

Four field isolates viz., one from quail (QP1) and three from ducks (DP1, DP2, DP3) suggestive of *Pasteurella sp.* were characterised by morphological, cultural and biochemical tests, and compared with the avian reference strain (LKO) of *Pasteurella multocida*.

Biochemically, all characters were similar for all the isolates except for ornithine decarboxylation reactions. The DP1, DP3 and LKO strain showed a positive reaction, while QP1 and DP2 isolates were negative for ornithine decarboxylase. Variations in the fermentation pattern of mannitol by the QP1 and DP1 isolates were also observed.

Two biotypes were observed among the four isolates. The sorbitol and dulcitol negative, QP1 isolate was classified as *P. multocida* subsp. *septica*, while the three duck isolates which were sorbitol positive, dulcitol negative were classified as *P. multocida* subsp. *multocida*.

Five different antibiogram patterns were observed among the four field isolates and reference strain. All the isolates were sensitive to chloramphenicol, cotrimoxazole, enrofloxacin, pefloxacin and tetracycline. Resistance to ampicillin, furazolidone, metronidazole and streptomycin was common to all the four field isolates and reference strain.

Analysis of the four field isolates and reference strain of *P. multocida* using the protein profiles revealed their diverse nature, with respect to the number and molecular weights of the polypeptides. The whole cell protein bands varied from 7 to 11, with molecular weights ranging from 197.4 kDa to 2.5 kDa among the four field isolates and reference strain. Intensely-staining major bands in the molecular weight range of 36 to 38 kDa, 29 to 32 kDa and approximately similar polypeptide bands around the 10 to 11 kDa region were present in all the five isolates.

The outer membrane protein (OMP) bands varied from 9 to 13, with molecular weights ranging from 93.1 kDa to 3.1 kDa among the four field isolates and the reference strain. The major OMPs were located in 32 to 33 kDa and 27 kDa regions in all the four field isolates and reference strain.

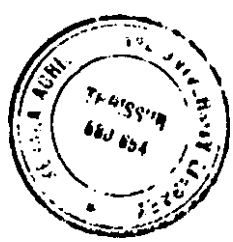
Yet, the differences in the isolate - specific bands indicated the variant nature of the basically similar organism.

Restriction Enzyme Analysis (REA) of the chromosomal DNA of all the isolates with *Not I* enzyme revealed no digestion, probably due to lack of recognition site in this bacterial genome for the enzyme.

Restriction enzyme digestion with *Eco RI*, *Hinf I* and *Hpa II* yielded indistinguishable band patterns on the gel, but the automated record of molecular sizes revealed 7 to 16, 7 to 15 and 6

to 8 restriction fragments respectively. Thus, REA was indicative of a basic genetic relatedness among the different isolates.

Phenotypic characterisation, antibiogram and protein profiles could be used as more effective tools in differentiating the avian field isolates of *P. multocida* than restriction enzyme analysis of chromosomal DNA.



References

REFERENCES

Anon, (1998). Farm guide, ed. K.K. Gangadharan, Farm information bureau, Kerala books and publications society, Kochi. pp. 64-69.

*Arif, M. and Champlin, F.R. (1998). Adaptive acquisition of novobiocin resistance in *Pasteurella multocida* strains of avian origin. *Vet. Res. Commun.* 22(**7**): 445-455.

Balakrishanan, G. (1998). Plasmid profile of avian strains of *Pasteurella multocida.* M.V.Sc. thesis submitted to Kerala Agricultural University, Thrissur.

Balasubramanian, (1960). Cited by Panda *et al.* (1981).

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(**5**): 668-676.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardised single disk method. *Am. J. Clin. Pathol.* 45(**4**): 493-496.

*Bermudez, A.J., Munger, L.L. and Ley, D.H. (1991). Pasteurellosis in bob white quails. *Avian Dis.* 35: 618-620.

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

Blackall, P.J., Pahoff, J.L. and Bowles, R. (1997). Phenotypic characterisation 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(**4**): 135-138.

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(**3**): 428-432.

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.

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(**8**): 1345-1349.

Carter, G.R. (1967). Pasteurellosis. *Pasteurella multocida* and *Pasteurella haemolytica*. In "Advances in Veterinary Science", Vol.2. (Brandly, C.A. and Cornelius, C. Eds.) Academic Press, New York. pp. 321-367.

Carter, G.R. and Rundell, S.W. (1975). Identification of type A strains of *Pasteurella multocida* using staphylococcal hyaluronidase. *Vet. Rec.* 96: 343.

Carter, G.R. (1981). In "Prokaryotes. A handbook on habitats, isolation and identification of bacteria" (Starr, M.P. Stolp, H., Truper, H.G., Balows, A. and Selegal, H.G. Eds.) Springer-Verlag, New York. pp. 1383-1391.

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.

Chawla, R. (1998). Poultry has major role in India's economy. *World Poultry*. 14: 12-17.

Chevalier, G., Duclohier, H., Thomas, D., Shechter, E. and Wroblewski, H. (1993). Purification and characterisation of protein H, the major porin of *Pasteurella multocida*. *J. Bacteriol.* 175 (**1**): 266-276.

Choi, K.H., Maheswaran, S.K. and Felice, L.J. (1989). Characterisation of outer membrane protein-enriched extracts of *Pasteurella multocida* isolated from turkeys. *Am. J. Vet. Res.* 50(**5**): 676-683.

Choi-Kim, 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(**2**): 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.

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.

Cowan, S.T. (1974). In Cowan and Steel's " Manual for the identification of medical bacteria". 2nd edition. Cambridge University Press, Cambridge. pp. 93-97.

Curtis, P.E. (1979). Observations on avian pasteurellosis in Britian. *Vet. Rec.* 104: 471-474.

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.

Das, U., Biswas, G., Bhattacharya, H.M., Mohanta, S.K. and Mukherjee, M. (1991). Outbreak of duck cholera in West Bengal. *Indian J. Poult. Sci.* 26: 60-61.

De Alwis, M.C.L. (1973). Cited by De Alwis (1999).

De Alwis, M.C.L. (1999). Haemorrhagic septicaemia. Australian Centre for International Agricultural Research (ACIAR) monograph No.57, Canberra, Australia. pp. 91-93.

*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.

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 biochemic study of avian *Pasteurella multocida* strains. *Avian Dis.* 7: 386-392.

Fegan, N., Blackall, P.J. and Pahoff, J.L. (1995). Phenotypic characterisation of *Pasteurella multocida* isolates from Australian poultry. *Vet. Microbiol.* 47: 281-286.

*Fujihara, Y., Onai, M., Koizumi, S., Satoli, N. and Sawada, T. (1986). An outbreak of fowl cholera in wild ducks (*Rosyibilled pochard*) in Japan. *Jpn. J. Vet. Sci.* 48: 35-43.

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.

Halder, B.R. (1972). Cited by Rammanath, K.R. and Gopal, T. (1993).

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.

Holmes, B. (1998). Actinobacillus, Pasteurella and Eikenella "In Topley and Wilson's Microbiology and Microbial infections". Vol.3. Bacterial infections. 9th edn., (Balows, A. and Duerdern, B.I. Eds.) Oxford University press Inc., New York. pp. 1198-1209.

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 fowlcholera. *Vet. Microbiol.* 27: 175-185.

Ireland, L.A., Milner, A.R. and Smart, I.J. (1989). Serotyping of isolates of *Pasteurella multocida* from chickens. *Aust. Vet. J.* 66(4): 119- 120.

Jayakumar, P.S. (1998). Comparative efficacy of different vaccines against pasteurellosis in ducks, M.V.Sc. thesis submitted to Kerala Agricultural University, Thrissur.

*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(1): 34-42.

Karim, (1987). Cited by Pillai *et al.* (1993).

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(5): 915-921.

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. Pasteurellosis in production animals. International workshop sponsored by ACIAR held at Bali, Indonesia, 10-13 August, 1992. 1993 edn. pp.144-148. ACIAR proceedings No. 43.

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(**2**): 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.

Knights, J.M., Adlam, C. and Owen, P. (1990). Characterisation of envelope proteins from *Pasteurella hemolytica* and *Pasteurella multocida*. *J. Gen. Microbiol.* 136: 495-505.

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(**4**): 237-238.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.

Lee *et al.* (1991). Cited by Woolcock, (1993).

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

Lee, M.D., Wooley, R.E., Glisson, J.R. and Brown, J. (1988). Comparison of *Pasteurella multocida* serotype 3,4 isolates from turkeys with fowl cholera. *Avian Dis.* 32: 501-508.

Lee, M.D., Burch, F.T., Maurer, J.J., Henk, A. and Thayer, S. (2000). DNA fingerprinting of plasmid-containing serotype A: 3,4 *Pasteurella multocida* isolated from cases of fowl cholera in chickens and turkeys. *Avian Dis.* 44:201-204.

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(**6**): 1532-1537.

Lu, Y.S., Lai, W.C., Pakes, S.P. and Nie, L.C. (1991) A monoclonal antibody against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against pasteurellosis. *Infect. Immun.* 59(**1**): 172-180.

Lugtenberg, B., Boxtel, R.V., and Jong, M.D. (1984). Cited by Lugtenberg *et al.* (1986).

Lugtenberg, B., Boxtel, R.V., Evenberg, D., Jong, M.D., Storm, P. and Frik, J. (1986). Biochemical and immunological characterisation of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect. Immun.* 52(**4**): 175-182.

Maillet (1836). Cited by Rhoades, K.R. and Rimler, R.B. (1991) in "Diseases of Poultry".

Mannheim, W. (1984). Pasteurellaceae, "In Bergey's Manual of Systematic bacteriology" Vol.1 (Krieg, N.R. and Holt, J.G. Eds.), Williams and Wilkins, Co., Baltimore, London, 1st edn. pp. 559-569.

Mannheim, W. and Carter, G.R. (1984). Cited "In Bergey's Manual of Systematic bacteriology" Vol.1 (Krieg, 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. (1996). Characterisation of an outer membrane protein of *Pasteurella multocida* belonging to the Omp A family. *Vet. Microbiol.* 53: 303-314.

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

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

Mohetada, S.N. and Bhadury, S.K. (1947). Cited by Rammanath, K.R. and Gopal, T. (1993).

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.

Morishita, T.Y., Lowenstine, L.J., Hirsh, D.C. and Brooks, D.L. (1996a). *Pasteurella multocida* in psittacines: prevalence, pathology and characterization. *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., Mtambo, M.M.A., Christensen, J.P. and Bisgaard, M. (2001). 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, N.B. and Paranjape, V.L. (1972). Some observations on pasteurellosis in ducks. *Indian Vet. J.* 49: 544-546.

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(**3 & 4**): 109-111.

Mutters *et al.* (1985). Cited by Bisgaard *et al.* (1991).

*Nakamine, M., Ohshiro, M. Amaku, Y., Ohshiro, K., Keruma, T., Sawada, T. and Ezaki, T. (1992). The first outbreak of fowl cholera in Muscovy ducks (*Cairina moschata*) in Japan. *J. Vet. Med. Sci.* 54(**6**): 1225-1227.

Namioka and Murata, (1961). Cited by De Alwis (1999).

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.

*Panigrahy, B. and Glass, S.E. (1982). Outbreaks of fowl cholera in quail. *Avian Dis.* 26: 200-203.

Pasteur, L. (1880). Cited by Carter, G.R. (1967) in "Advances in Veterinary Science".

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.

Perroncito (1878). Cited by Carter, G.R. (1967) in "Advances in Veterinary Science".

Pillai, R.M., James, P.C., Punnoose, K.T., Sulochana, S., Jayaprakasan, V. and Nair, G.K. (1993). Outbreak of pasteurellosis among duck population in Kerala. *Kerala J. Vet. and Anim. Sci.* 24: 34-39.

*Raemdonck, D.L., Tanner, A.C., Tolling, S.T. and Michener, S.L. (1992). *In vitro* susceptibility of avian *Escherichia coli* and *Pasteurella multocida* to danofloxacin and five other antimicrobials. *Avian Dis.* 36(**4**): 964-967.

Ratafia, M. (1988). Cited by Rhoades, K.R. and Rimler, R.B. (1989) in "Diseases of Poultry".

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.

Rammanath, K.R. and Gopal, T. (1993). A note on the isolation and characterisation of *Pasteurella multocida* from ducks. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 14 (**1&2**): 32-33.

Rao, G. (1964). Cited by Rammanath, K.R. and Gopal, T. (1993).

Rhoades, K.R. and Rimler, R.B. (1991). Pasteurellosis. In "Diseases of Poultry". 9th edn. (Calnek, B.W., Barnes, H.J., Beard, C.W., Reid, W.M. and Yoder, H.W. Eds.), Wolfe Publishing Ltd., England. pp. 145-162.

*Rimler, R.B. (2000). Restriction endonuclease analysis using *Hha I* and *Hpa II* to discriminate among group B *Pasteurella multocida* associated with haemorrhagic septicaemia. *J. Med. Microbiol.* 49(**1**): 81-87.

Rimler, R. B. and Rhoades, R. (1989). Fowl cholera. "In Pasteurella and Pasteurellosis". (Adlam, C. and Rutter, J.M. Eds.), Academic Press. pp. 52.

Rimler, R.B. and Nordholm, G.E. (1998). DNA fingerprinting of *Rimerella anatipestifer*. *Avian Dis*. 42: 101-105.

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

Sambrook, J., Fritsch, E.T. and Maniatis, T. (1989). Molecular cloning. A Laboratory manual 2nd edn. (Irwin, N., Ford, N., Nolan, C. and Ferguson, M. Eds.), Cold Spring Harbor Laboratory, New York.

Sander, J.E. and Glisson, J.R. (1989). Fowl cholera in broilers. *Avian Dis*. 33(**4**): 816-819.

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(**9**): 1059-1060.

Sankaranarayan and Banerjee (1944). Cited by Panda *et al*., (1981).

Schneider, L. (1948). Cited by Rimler and Rhoades (1989) in "Pasteurella and Pasteurellosis".

Smith, G.K. and Phillips, J.E. (1990). Pasteurella, In "Topley and Wilson's Principles of Bacteriology, Virology and Immunity", Vol.2. (Parkar, M.T. and Duerden, B.I. Eds.), 8th edn. pp. 335-351.

Snipes, K.P., Hansen, L.M. and Hirsh, D.C. (1988). Plasma and iron regulated expression of high molecular weight outer membrane proteins of *Pasteurella multocida*. *Am. J. Vet. Res.* 49(**8**): 1336-1338.

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 characterisation. *Avian Dis.* 34: 419-424.

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(**2**): 130- 135.

Srivastava, S.K. (1998). Characteristics of whole cell proteins of *Pasteurella multocida* serotype B:6. *Indian Vet. J.* 75:395-398.

Srivastava, S.K. (1999). Immunogenecity of protein fractions of *Pasteurella multocida* in animals. *Indian J. Anim. Sci.* 69(**9**): 653-656.

Srivastava, S.K. (2000). Efficacy of vaccines prepared from *Pasteurella multocida* cells grown in chemically defined media. *Indian J. Anim. Sci.* 70(**2**): 114-117.

Sulochana, (1998). Final report of the 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. pp. 28-30.

Topley, W.W.C and Wilson, G.S. (1936). Cited by Carter, G.R. (1967) in "Advances in Veterinary Science".

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. Pasteurellosis in production animals. International workshop sponsored by ACIAR held at Bali, Indonesia, 10-13 August, 1992. 1993 edn. pp.60-63. ACIAR proceedings No. 43.

Truscott, W.M. and Hirsh, D.C. (1988). Demonstration of an outer membrane protein with antiphagocytic activity from *Pasteurella multocida* of avian origin. *Infect. Immun.* 56(**6**): 1538- 1544.

Walser, M.M. and Davis, R.B. (1975). *In vitro* characterization of field isolants of *Pasteurella multocida* from Georgia turkeys. *Avian Dis.* 19(**3**): 525-532.

*Waltman, W.D. and Horne, A.M. (1993). Characteristics of fowl cholera diagnosed in Georgia, 1989-91. *Avian Dis.* 37: 616-621.

Warner, (1996). Cited by De Alwis (1999).

Wijewardana, T.G., De Alwis, M.C.L. and Bastiansz, H.L.G. (1986a). Cited by De Alwis (1999).

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

Wilson, M.A. Duncan, R.M., Nordholm, G.E. and Berlowski, B.M. (1995a). Serotypes and DNA fingerprint profiles of *Pasteurella multocida* isolated from raptors. *Avian Dis.* 39: 94-99.

Wilson, M.A. Duncan, R.M., Nordholm, G.E. and Berlowski, B.M. (1995b). *Pasteurella multocida* isolated from wild birds of North America: a serotype and DNA fingerprint study of isolates from 1978 to 1993. *Avian Dis.* 39: 587-593.

Wilson, M.A., Duncan, R.M., Roffe, T.J., Nordholm, G.E. and Berlowski, B.M. (1995c). Pasteurellosis in elk (*Cervus elaphus*): DNA fingerprinting of isolates. *Vet. Rec.*: 195-196.

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(**2**): 255-259.

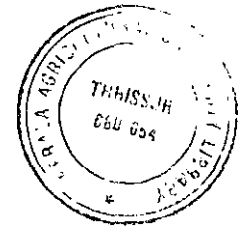
Wilson, M.A., Rimler, R.B. and Hoffman, L.J. (1992). Comparison of DNA fingerprints and somatic serotypes of serogroup B and E *Pasteurella multocida* isolates. *J. Clin. Microbiol.* 30(**6**): 1518-1524.

Woolcock, J.B. (1993). The biology of *Pasteurella multocida* and *Pasteurella haemolytica.* Pasteurellosis in production animals. International workshop sponsored by ACIAR held at Bali, Indonesia, 10-13 August, 1992. 1993 edn. pp.27-28. ACIAR proceedings No. 43.

Zhao, G., Pijoan, C., Choi, K., Maheswaran, S.K. and Trigo, E. (1995). Expression of iron-regulated outer membrane proteins by porcine strains of *Pasteurella multocida*. *Can. J. Vet. Res.* 59: 46-50.

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. and Immun.* 60(**4**): 1401-1405.

* Originals not consulted



NUCLEIC ACID AND PROTEIN PROFILE OF *Pasteurella multocida* OF AVIAN ORIGIN

By

S. RAJALAKSHMI @ RADABAI

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences

Kerala Agricultural University

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR - 680651

KERALA

2001

ABSTRACT

Four avian field isolates (one from quail and three from ducks) suggestive of *Pasteurella sp.* were compared with the avian *Pasteurella multocida* reference strain (LKO) for their biochemical properties, drug resistance patterns, whole cell and outer membrane protein profiles and restriction enzyme digestion pattern of chromosomal DNA.

Biochemical characterisation revealed two biotypes among the four field isolates; namely *P. multocida* subsp. *septica* (quail isolate) and *P. multocida* subsp. *multocida* (duck isolates).

The study of antibiogram using 14 chemotherapeutic agents revealed the presence of multiple drug resistance in all the four field isolates and reference strain.

Whole cell protein profiles of the four field isolates and reference strain revealed 7 to 11 bands of molecular weights ranging from 197.4 kDa to 2.5 kDa. Polypeptide bands in the molecular weight range of 36 to 38 kDa (which were attributable to the protein-H), 29 to 32 kDa and 10 to 11 kDa were common to all the isolates of *P. multocida*.

The major outer membrane proteins (OMPs) in the molecular weight range of 32 to 33 kDa and 27 kDa region were common to all the four field isolates and reference strain.

Unique protein bands in each of the isolate were indicative of variant forms of the same organism.

Restriction enzyme analysis of chromosomal DNA yielded different restriction fragments in each of the isolates which were not easily distinguishable.

Hence, of the tests used, phenotypic characters, biotype, antibiogram and protein profile were found to be more effective in differentiating the isolates rather than restriction enzyme digestion patterns of genomic DNA.