PLASMID PROFILE OF AVIAN STRAINS OF *Pasteurella multocida*

By
G. BALAKRISHNAN

THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Veterinary Science

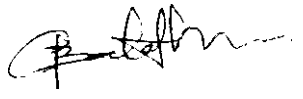
Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

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

1998

DECLARATION

I hereby declare that the thesis entitled "**PLASMID PROFILE OF AVIAN STRAINS OF PASTEURELLA MULTOCIDA**" 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.



Mannuthy
28.9.1993

G. BALAKRISHNAN

CERTIFICATE

Certified that the thesis entitled "**PLASMID PROFILE OF AVIAN STRAINS OF PASTEURELLA MULTOCIDA**" is a record of research work done independently by **Sri. G. Balakrishnan**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

Mini

Dr. M. Mini, Ph.D.
(Chairperson, Advisory Committee)
Assistant Professor
Department of Microbiology
College of Veterinary & Animal Sciences
Mannuthy

Mannuthy
28-9-1998

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Sri. G. Balakrishnan**, a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled "**PLASMID PROFILE OF AVIAN STRAINS OF PASTEURELLA MULTOCIDA**" may be submitted by Sri. G. Balakrishnan, in partial fulfilment of the requirement for the degree.

mini

Dr. M. Mini, Ph.D.
(Chairperson, Advisory Committee)
Assistant Professor
Department of Microbiology
Mannuthy

28/9

Dr. S. Sulochana, Ph.D.
Professor & Head
Department of Microbiology
(Member)

Dr. K.T. Punnoose, Ph.D.
Professor
Department of Microbiology
(Member)

Dr. Sisilamma George, Ph.D.
Assistant Professor
Department of Physiology and
Biochemistry
(Member)

28/V/98

External Examiner

R. Madhusoodanan Pillai

ACKNOWLEDGEMENT

There are no words to pay my regard and deep sense of gratitude to ***Dr. M. Mini****, Chairperson of the Advisory Committee, Department of Microbiology. I cannot accord my own effort a prime place but the timely guidance, motivation, urge, zeal, feeling in me by my major advisor, lending an air of self assurance and confidence in each phase of my thesis work, stands out with clear distinction. It is a matter of great pleasure, pride and a privilege to get an opportunity to work under the dynamic guidance of an able and affectionate academician of her calibre.*

With deep reverence shall I express my extreme indebtedness and obligation to ***Dr. S. Sulochana****, Professor and Head, Department of Microbiology for her meticulous help, fore bearance, affectionate advice and sustained interest given throughout my course. In spite of her busy schedule, she had been a support during each step of the way and I owe a deep sense of gratitude for that.*

With profound gratitude, I wish to place on record my indebtedness to ***Dr. K.T. Punnoose****, Professor, Department of Microbiology, for his expert advice, scrupulous navigation, constructive suggestions and valuable discussions during the execution of this work.*

I am cordially obliged to ***Dr. Sisilamma George****, Assistant Professor, Department of Physiology for her valuable suggestions, fruitful discussions and incessant help rendered throughout the tenure of the study.*

I am at a loss of words to express my heartfelt gratitude to ***Dr. V. Jayaprakasan****, Associate Professor, Department of Microbiology for his timely help which destined my future when the going was tough. His willingness to help could never be forgotten.*

I sincerely acknowledge the helping hands extended by ***Dr. G. Krishnan Nair****, Associate Professor and* ***Dr. Koshy John****, Assistant Professor, Department of Microbiology for their fruitful discussions, ideas, and for having words of inspiration.*

I am thankful to Dr. Raghunathan Nair, Professor, Centre of Advanced Studies in Poultry Sciences, ***Dr. K.V. Reghunandanan****, Associate Professor, Centre of Advanced Studies in Animal Breeding and Genetics, Dr. Sreekumaran, Professor, Centre of Excellence, Department of Pathology,* ***Dr. Chandrasekharan Nair****, Associate Professor, Department of Pharmacology,* ***Dr. Kumanan****, Associate Professor, Department of Biotechnology, M.V.C.,* ***Dr. Ramnath****, Assistant Professor, Department of Physiology and* ***Dr. Thirupathy****, Assistnat Professor, Department of Genetics, for their valuable help and support for the completion of my research work.*

I am thankful to the Dean, College of Veterinary and Animal Sciences, Mannuthy for the facilities provided for this research work.

I acknowledge my deep thanks to ***Dr. Padalkar, Dr. Pradeep, Dr. Tressa and Dr. Mohan****. The help rendered by Research Associates* ***Mr. Ranjit, Mrs. Sreekalakumari****, Department of Microbiology and Mr. Sanjay, Department of Pathology are acknowledgeable.*

With great fondness, I express my heartful thanks to my friends ***Dr. Mal, Dr. Bindu, Dr. Rini, Dr. Senthil, Dr. Biju, Dr. Krishnakumar, Dr. Mahadevappa, Dr. Vinu, Dr. Reghu, Dr. Nagaraj, Dr. Vathsala and Mr. Jeeva Mathewson*** *for their help and co-operation in the completion of this work.*

I cordially obliged to ***Dr. Prabhakaran*** *who paved the way to me enter in this veterinary fied.*

I acknowledge ***my parents*** *for their unstinted love which have been a perennial source of inspiration to me.*

I am thankful to ***ICAR*** *for awarding me the fellowship for post-graduate study.*

Lastly, my heartful thanks to ***M/s Peagles****, Mannuthy for its well timed assistance in the preparation of this thesis.*

G. BALAKRISHNAN

To

Ross

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

INTRODUCTION

Poultry industry is a major sector of employment generation and is expected to generate three million additional jobs by the year 2005. India's poultry sector contributes at least Rs.8,082 crores in terms of value of annual agri-based produce (Chawla, 1998).

The 1996 livestock census indicated that the poultry population in Kerala state is 269.45 lakhs. This includes 256.46 lakhs of domestic fowl, 11.87 lakhs of ducks and 1.12 lakhs of other avian species.

Presently, the poultry industry in Kerala is facing a major setback due to the repeated outbreaks of certain bacterial and viral diseases. Pasteurellosis is a major disease problem among birds especially chicken and ducks. Fowl cholera due to *Pasteurella multocida* is a highly contagious septicaemic disease with high morbidity and mortality resulting in significant economic loss. World wide economic loss due to this disease is estimated to be 200 million US dollars for the year 1986 (Ratafia, 1988).

The most important factor which causes failure in the treatment of this disease is the emergence of multiple drug resistant strains as a result of indiscriminate use of antimicrobial agents.

The genes responsible for drug resistance may be located either on chromosome or plasmids. Antibiotic resistance plasmids have been isolated from *P. multocida* which causes fowl cholera (Hirsh *et al.*, 1985).

For characterisation of an isolate, several typing methods have been evolved in which serotyping formed the primary system. Phage typing was more useful as a secondary system. A third have been evolved to differentiate between similar strains, being the molecular characterisation of the genome (Pitt, 1994).

Various researchers (Purushothaman. 1990); David *et al.*, 1991 and Mini, 1996) have emphasised the use of different typing methods for differentiation of bacterial isolates from the epidemiological point of view.

The aim of the present study is to isolate *P. multocida* from dead and apparently healthy chicken and ducks and characterise the isolates by

1. Biotyping
2. Antibiogram
3. Pathogenicity studies in species such as mice, rabbits, chicken and ducks
4. Plasmid profile analysis

Review of Literature

REVIEW OF LITERATURE

2.1 History and nomenclature

It was Perroncito (1878) who first isolated and described an organism named bacillus of fowl cholera. A detailed description of fowl cholera and the organism was made by Pasteur in 1880. Subsequently, several others have reported the isolation of similar organisms from cattle (Bollinger, 1878), rabbit (Davaine, 1880; Gaffky, 1881) and pig (Loeffler and Schutz, 1882).

A comparative study of the organisms producing fowl cholera, rabbit septicaemia, swine septicaemia and septicaemia of cattle and wild animals was carried out by Kitt (1885). He found that they were similar in many respects and referred to as *Bacterium bipolare multocida*. These organisms were later redesignated with the generic name Pasteurella in recognition of the voluminous work carried out by Louis Pasteur on the causative agent of fowl cholera.

Trevisan (1887) assigned the name *Pasteurella* for the bipolar stained organism from septicaemia in animals.

Flugge (1896) named the Haemorrhagic septicaemia organisms of bovine and swine origin as *Bacillus boviseptica* and *Bacillus suiseptica* respectively. Since different

isolates from divergent species had common characters, Lignieres (1900) assigned the generic name *Pasteurella* to these organisms with an indication of species of animals from which they were isolated.

According to Topley and Wilson (1936), *Pasteurella septica* was the most suitable name to indicate bacteria of septicaemia.

The name *P. multocida*, proposed by Rosenbusch and Merchant (1939), has now been widely accepted and it is listed as the type species of the genus (Mannheim and Carter, 1984).

Rimler and Rhoades (1989) suggested that the most suitable name to represent this heterogeneous species is *P. multocida*.

2.2 Media for isolation

Namioka and Murata (1961) described a solid medium called yeast proteose cystic (YPC) agar to demonstrate colony morphology of *P. multocida*. This medium consisted of yeast extract, proteose-peptone, L-cystine, glucose, sucrose, sodium sulphite, agar, and potassium diphosphate.

Heddleston *et al.* (1964) reported that dextrose starch agar can be used as a solid medium to study the colony morphology of *P. multocida*.

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

Pasteurella grew well on blood agar (Carter, 1967) and this medium was routinely used for the isolation of *P. multocida* from clinical specimens. But blood agar was not satisfactory for the identification of colonial variants. Carter (1967) found Brucella agar containing two per cent haemolysed rabbit serum was satisfactory. Other media used were tryptic soy blood agar and tryptose blood agar containing five per cent sheep or bovine blood. To avoid contamination with proteus, a high concentration of agar was used (Carter, 1967).

Targowski and Fargowski (1979) isolated a *Pasteurella* strain with an unusual growth requirement. This grew copiously only on media supplemented with serum and incubated in the candle jar.

It was suggested by Neter and Dryja (1981) that the antibiotic nafcillin might be useful in the selective media for *P. multocida*.

Carter (1981) reported that *P. multocida* being a facultative anaerobe grew best at temperatures of 35 to 37°C in presence of air or air plus five per cent Co_2 on bovine or bovine and sheep blood agar.

Knight *et al.* (1983) proposed a selective medium (CGT medium) containing clindamycin, gentamicin, potassium tellurite and amphotericin-B in five per cent horse blood agar for the isolation of *P. multocida*.

A selective medium for the isolation of *P. multocida* and *Bordetella bronchiseptica* has been described by De Jong and Borst (1985) which contained Tryptose Soy Agar, five per cent defibrinated sheep blood, gentamicin sulphate, potassium tellurite, amphotercin-B and bacitracin. This medium was found to be more efficient in the isolation of toxigenic strains of *P. multocida* from nasal swabs as compared to mice inoculation and modified Mac Conkey's media.

According to Smith and Phillips (1990) *P. multocida* grew well at an optimum temperature of 37°C.

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

2.3 Identification of *P. multocida*

Identification of *P. multocida* was done primarily by testing for biochemical reactions. Several methods were available for the performance of biochemical tests. The classical biochemical tests should be considered because they formed the basis for taxonomy and identification.

Cowan (1974) reported that the primary biochemical tests for the identification of *P. multocida* are the tests for catalase, oxidase, production of acid from glucose, and oxidative/fermentative utilisation of glucose. The second stage tests include the growth on KCN medium, acid production from various sugars, ornithine decarboxylase activity, indole production, and production of H_2S.

The primary biochemical tests for identification of *P. multocida* included growth on the Mac Conkey's Agar, haemolysis on blood agar, production of indole, hydrogen sulphide and the urease activity (Buxton and Fraser, 1977).

According to Mannheim (1984) *P. multocida* was identified by various tests like haemolysis on blood agar, growth on Mac Conkey's agar, indole production, urease activity, gas production from carbohydrates and acid production from lactose and mannitol.

Serological identification of *P. multocida* is a very valuable tool in the identification of the agent. Strains of capsular serogroup A produced the most severe form of the disease. Most of the 16 somatic serotypes with capsular group A strains of *P. multocida* were isolated from avian species (Rhodes and Rimler, 1987)

Identification of *P. multocida* was carried out by morphological and biochemical tests as per the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

2.4 Isolation of *P. multocida* from avian species

In 1600, Androvandus reported a deadly epizootic disease of fowls in Italy, which is presumed to be the earliest published report on the occurrence of fowl cholera. The disease occurred in Italy and France from 1770 to 1800, and was first studied by Chabert in France in 1780. Maillet named it as fowl cholera in 1835-36 (Rhoades and Rimler, 1989).

In 1880, Pasteur (1880) isolated the causative organism and grew pure cultures in chicken broth.

Acute fowl cholera causing high mortality was reported in quails and causative organism, *P. multocida* serotype 3 was identified (Panigrahy and Glass, 1982).

An outbreak of avian cholera in Mississippi valley was reported for the first time in Canada geese (*Branta canadensis*) (Windingstad *et al.*, 1983).

Fowl cholera occurred in a wild duck (*Rosyibilled pochard*) in Japan, and the organism was isolated in pure culture from heart blood, lung, liver, kidney and spleen. This is the first report on the occurrence of fowl cholera in wild waterfowl in Japan (Fujihara *et al.*, 1986).

Williams *et al.* (1987) reported an outbreak of avian cholera in gyrfalcon (*Falco rusticolus*) and isolated *P. multocida* serotype 1 from tissues of the falcon.

P. multocida was isolated from the samples of lung and liver of infected Japanese quail (*Coturnix coturnix japonica*) in Georgia (Glisson *et al.*, 1989).

Bermudez *et al.* (1991) isolated *P. multocida* from a case of peracute pasteurellosis in Bobwhite quails.

Blackall *et al.* (1995) isolated *P. multocida* from fowl cholera outbreaks on turkey farms in Australia.

Morishita *et al.* (1996b) reported the prevalence of fowl cholera in raptors and isolated *P. multocida* from respective birds. The prevalence of fowl cholera in psittacine birds was also reported by him.

2.5 Incidence of fowl cholera in India

Avian pasteurellosis (Fowl Cholera) is a serious problem in poultry. Sankaranarayan and Banerjee (1944) recorded outbreaks of fowl cholera in ducks and poultry from different parts of India.

In India, the incidence of fowl cholera in ducks has been reported as early as 1947 by Mohetada and Bhadury from Anthra Pradesh and Bengal respectively.

Rao (1964), recorded the incidence of duck pasteurellosis in Anthra Pradesh.

Madhusudan (1967) reported the occurrence of fowl cholera in Tamil Nadu.

Several outbreaks of duck pasteurellosis in government farms were reported by Halder (1972) from Assam.

Mulbagal *et al*. (1972) reported pasteurellosis in muscovy ducks in Bombay and isolated *P. multocida* from the heart blood of the affected ducks.

An acute outbreak of fowl cholera in one month old chickens was investigated. *Pasteurella gallinarum* was isolated in pure culture from the heart blood of two moribund chicks. One of the isolants on experimental inoculation was found to

be non-pathogenic for rabbits, mice and chickens. It did not provide protection in rabbit against a virulent strain of *P. multocida*. This seems to be the first record of the isolation of *P. gallinarum* in India (Yadav *et al.*, 1977).

An outbreak of fowl cholera occurred in the central poultry breeding farm in Orissa. *P. multocida* was isolated from the oedema fluid of wattles of the affected birds but not from the heart blood (Panda *et al.*, 1981).

Clinical pasteurellosis in ducks was reported by Karim (1987) in Assam.

Kulkarni *et al.* (1988) reported the occurrence of fowl cholera in six day old chicks in an organised poultry farm in Parbhani city. *P. multocida* was isolated from heart blood, liver, lung and spleen.

Fowl cholera in ducks was reported from a duck farm in Srinagar (Sambyal *et al.*, 1988), West Bengal (Das *et al.*, 1991), Bangalore (Rammanath and Gopal, 1993) and in Tripura (Murugkar and Ghosh, 1995).

An outbreak of pasteurellosis in a commercial traders outlet of Japanese quails at Madras was reported by Chandran *et al.* (1995).

Isolation and identification of *P. multocida* was carried out by Rajini *et al.* (1995) from outbreaks of avian pasteurellosis in Anthra Pradesh.

P. multocida was isolated from cases of pasteurellosis in ducks from Kerala (Jayakumar, 1998).

2.6 Biotyping

Separation of *P. multocida* into subgroups based on variation in biochemical characters has been reported (Schneider, 1948). This subgrouping has been based mostly upon acid production from xylose, arabinose, maltose, trehalose, sorbitol, mannitol and dulcitol.

Dorsey (1963) reported that 81.5 per cent of the 432 isolates from fowl cholera were dulcitol positive.

Donahue and Olson (1971) carried out biotyping of turkey isolates of *P. multocida* with 16 carbohydrates and reported that all the 121 isolates fermented dextrose, sucrose, mannose, mannitol, galactose and levulose. The isolates varied in their ability to ferment arabinose, dulcitol, lactose and xylose.

Pasteurella gallinarum, isolated from chicks showed a low rate of fermentation of dextrose, maltose and sucrose and

was unable to ferment lactose or mannitol (Yadav *et al.*, 1977).

Sharma *et al.* (1979) observed that strains of *P. multocida* isolated from pneumonic lungs of domestic animals fermented dextrose, lactose, raffinose and xylose.

Mutters *et al.* (1985) reported that fermentation of dulcitol and sorbitol was of taxonomic significance in dividing the taxon *P. multocida* into following three subsp., the sorbitol and dulcitol positive variety became subsp. *gallicida*; negative for both constituted subsp. *septica* and positive for sorbitol but negative for dulcitol constituted subsp. *multocida*.

P. multocida isolated from green pheasants did not ferment xylose, arabinose, trehalose, sorbitol, inositol and raffinose (Sakurai *et al.*, 1986).

Fujihara *et al.* (1986) studied the biotyping of *P. multocida* from wild ducks (Rosyibilled pochard). All the isolates fermented arabinose, sorbitol, xylose, sucrose, glucose, galactose and maltose but not lactose, salicin and trehalose.

A vaccine strain of *P. multocida* and those isolated from turkeys were found to ferment ribose, galactose, D-glucose,

D-fructose, D-mannose, mannitol, sorbitol, N-acetyl glucosamine, esculin and saccharose (Lee *et al.*, 1988).

Dwivedi and Sodhi (1989) observed fermentation of glucose, galactose, fructose, sucrose and xylose by the strains of *P. multocida*. Majority of them fermented mannose, mannitol and sorbitol. None of the strains utilised lactose and dulcitol.

Ikeda and Hirsh (1990) performed biotyping of *P. multocida* from turkeys and reported that the subsp. multocida fermented trehalose.

Hirsh *et al.* (1990) characterised *P. multocida* isolated from water fowl and associated avian species into three subsp. based on biotyping.

Das *et al.* (1991) biotyped *P. multocida* isolates of duck cholera. They observed acid production in glucose, galactose and mannitol but not in maltose and lactose. There was no gas production in any of the sugars.

Droual *et al.* (1992) isolated a Fresno Strain of *P. gallinarum* which gave a positive reaction in ONPG broth and fermented xylose. The ATCC strain was negative for ONPG test and did not ferment xylose.

P. multocida isolates showed variability in the ornithine decarboxylase, glucose fermentation and oxidase tests (Waltman and Horne, 1993).

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

Rajini *et al.* (1995) studied the biotypes of *P. multocida* from poultry. All the isolates fermented glucose, sucrose, fructose, galactose and maltose and 90 per cent of the isolates fermented sorbitol. Sixty three per cent, 66.6 per cent and 53.3 per cent of the isolates fermented mannitol, dulcitol and lactose respectively. It was concluded that these differences in the fermentation reactions of carbohydrates might be due to geographical variation of the isolates and the use of chemotherapeutic agents, as these factors may influence the enzyme profiles of microbes.

Chandran *et al.* (1995) reported fermentation of glucose, sucrose and mannitol without gas production by *P. multocida* of quail origin.

Ten different biochemical biovars were recognised amongst the *P. multocida* isolates from Australian poultry, on the

basis of fermentation of sorbitol, trehalose and xylose and distinguished the isolates into various subspecies (Fegan *et al.*, 1995).

P. multocida of turkey origin were grouped into four groups (A, B, C, D) based on the biochemical profile, three (A, B, C) were variants of *P. multocida* subsp. *multocida* and the fourth (D) were *P. multocida* subsp. *septica*. The biovar A fermented sorbitol and xylose similar to reference strain of *P. multocida* subsp. *multocida*. The biovar B fermented sorbitol, the biovar C fermented sorbitol, trehalose and xylose. The biovar D fermented maltose, trehalose and xylose but not sorbitol (Blackall *et al.*, 1995).

Bhaumik and Dutta (1995) studied the fermentation pattern of various sugars by *P. multocida*. Acid was produced by fermentation of glucose, sucrose and fructose without gas production. There was no reaction with lactose, maltose and raffinose.

2.7 Antibiogram

The drug resistance of bacteria was reported in Japan with *Shigella* species showing resistance to four common antibiotics such as sulphonamides, streptomycin, chloramphenicol and tetracycline (Ochiai *et al.*, 1959).

P. multocida isolates of turkey origin were, in general, sensitive to chlortetracycline, chloromycetin, erythromycin, novobiocin, penicillin, polymixin-B, terramycin, tetracycline, furazine, furoxone, kanamycin and neomycin and resistant to bacitracin and sulfadiazine. Many of the isolates resistant to streptomycin were slightly sensitive to kanamycin and neomycin. These three antibiotics are aminoglycosides and of similar chemical structure, indicating the occurrence of cross-resistance (Donahue and Olson, 1972).

Sharma *et al.* (1979) reported that Pasteurellae isolated from calves, sheep, pigs, rabbits, guinea pigs and mice were sensitive to furaxon and resistant to ampicillin, dihydrostreptomycin, terramycin and tetracycline.

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

Pasteurella multocida isolated from avian species were subjected to *in vitro* drug sensitivity test and found to be highly sensitive to tetracycline (Panda *et al.*, 1981).

The long acting oxytetracycline product was highly efficient in preventing disease caused by experimental

inoculation with the P-1059 strain of *P. multocida* (Skeeles *et al.*, 1983).

Pasteurella multocida isolates from ducks were highly sensitive to chloramphenicol, nitrofurantoin and tetracycline, moderately to gentamicin and resistant to neomycin, ampicillin, trimoxazole, erythromycin and streptomycin (Sambyal *et al.*, 1988).

Antibiotic sensitivity pattern of *P. multocida* isolated from Japanese quail showed all isolates to be sensitive to sulfonamides and tetracycline (Glisson *et al.*, 1989).

Pasteurella multocida isolated from clinical cases of fowl cholera were resistant to sulfisoxazole, rifampin and bacitracin (Lee *et al.*, 1991).

The antibiogram of *P. multocida* isolates from ducks indicated that the organisms were sensitive to chloramphenicol, chlortetracycline, oxytetracycline, cotrimoxazole, nalidixic acid, gentamicin, nitrofurantoin, streptomycin, kanamycin and neomycin. They were sensitive to a lesser extent to polymixin-B, penicillin-G, amoxycillin, cloxacillin, lincomycin and vancomycin (Rammanath and Gopal, 1993).

Pasteurella multocida isolated from chicken, turkey and game birds in Georgia showed resistance to sulphonamides (Waltman and Horne, 1993).

In vitro drug sensitivity pattern of *P. multocida* chicken isolates in Anthra Pradesh showed high sensitivity to chloramphenicol followed by doxycycline hydrochloride, chlortetracycline, nitrofurantoin, penicillin-G and cotrimaxazole and complete resistance to colistin (Rajini *et al.*, 1995).

Diallo *et al.* (1995) reported that avian strains of *P. multocida* were resistant to streptomycin, trimethoprim and lincomycin.

Bhaumik and Dutta (1995) stated that *P. multocida* isolated from Khaki Campbell ducklings showed maximum sensitivity to gentamicin, erythromycin, kanamycin, moderate to cotrimoxazole and furazolidone. Resistance was shown to tetracycline, oxytetracycline, ampicillin and cloxacillin.

All the isolates of *P. multocida* of Psittacine origin were susceptible to penicillin-G, sulfisoxazole, gentamicin, erythromycin, tetracycline and trimethoprim and sulfamethoxazole but resistant to streptomycin (Morishita *et al.*, 1996a).

All the isolates of *P. multocida* from Raptors were susceptible to penicillin-G, sulfisoxazole, tetracycline, trimethoprim and sulfamethoxazole (Morishita *et al.*, 1996b).

2.8 Pathogenicity studies

Pasteurella multocida is found in a wide variety of animals and birds and its main habitat is the respiratory tract (Smith, 1955).

Murata *et al.* (1964) tested the pathogenicity of serotypes 1:A and 5:A for chicken and mice and reported that serotype 1:A was not pathogenic for chicken while serotype 5:A was pathogenic. Both serotypes were found to be pathogenic to mice.

Sharma *et al.* (1974) inoculated 1 ml of 1:30 dilution of 18 h broth culture of *P. multocida* into Rhode Island Red birds of 8 to 12 weeks of age. They observed acute, subacute and chronic forms of the disease in different birds. The acute form was evidenced by the death of four birds within 18 to 24 h. Three birds passed on to the chronic phase of the disease, whereas the remaining birds which were destroyed between 30 to 196 h manifested lesions of subacute form.

It has been reported that in many cases *P. multocida* isolated from avian hosts exhibited little virulence for avian

hosts (Heddleston *et al.*, 1975; Brogden and Rebers, 1978; Curtis *et al.*, 1980).

Sharma *et al.* (1979) observed the death of swiss mice in 24 to 48 h after receiving 0.5 ml of 6 h old broth cultures of *pasteurella* intraperitoneally.

Okerman *et al.* (1979) determined the virulence of *P. multocida* strains isolated from rabbits in mice and correlated with the colony type. All the smooth strains were highly virulent to mice and blue colonies were of low virulence and found that 1 to 10 bacterial cells were sufficient to cause death of mice.

Panda *et al.* (1981) inoculated 0.5 ml of 18 h old nutrient broth culture of *P. multocida* subcutaneously into pigeons, rabbits and poultry birds. The pigeons and rabbits remained healthy for 10 days but the fowls died four days after inoculation *P. multocida* was isolated in pure culture from the heart blood of the dead birds indicating selective pathogenicity.

Heart blood from goose infected with *P. multocida* was injected I/P into swiss mice and they died within 24 h. The organism was isolated from the liver of the dead mice (Windingstad *et al.*, 1983).

Sambyal *et al.* (1988) reported that the isolates of *P. multocida* of duck origin were highly virulent for chicken and mice, killing them within 24 h of inoculation showing typical signs of fowl cholera in chicken.

Glisson *et al.* (1989) indicated that chickens, turkeys and Japanese quails were susceptible to experimental infection with *P. multocida*.

Pasteurella multocida caused peracute mortality in bob-white quails within 24 h of inoculation (Bermudez *et al.*, 1991).

Intraperitoneal inoculation of *P. multocida* into swiss mice resulted in death of the swiss mice within 18 to 24 h and smears taken from heart blood, lung, liver and spleen revealed characteristic bipolar stained organisms (Das *et al.*, 1991).

The chicken inoculated intramuscularly with 10 cfu of the Fresno strain of *Pasteurella gallinarum* developed severe myositis at the inoculation site, pericarditis, perihepatitis, air sacculitis and synovitis. The same organism could be reisolated from the infected chicken (Droual *et al.*, 1992).

Sheela (1992) studied the pathogenicity of *P. multocida* in rabbits and observed lesions such as typical haemorrhagic

tracheitis, congestion of the lung, liver and spleen and haemorrhagic enteritis.

Matsumoto and Strain (1993) demonstrated the pathogenicity of *P. multocida* after *in vivo* passages. To increase virulence, the organism was passaged intravenously in turkeys. After five passages, the encapsulated organism caused 67 per cent mortality with a 10^2 cfu and 50 per cent of the in contact control birds also died. After four intraperitoneal passages increased virulence of the non-encapsulated variant was observed suggesting that both encapsulated and non encapsulated form of *P. multocida* can increase their pathogenicity by bird to bird transmission in a short period of time.

Rammanath and Gopal (1993) studied the pathogenicity of *P. multocida* in mice, rabbits and chicken by S/C route. The experimental animals and birds died within 18 to 24 h post infection. The organisms were recovered from heart blood and liver of these animals and birds.

Murugkar and Ghosh (1995) injected 0.5 ml of 6 h broth culture of *P. multocida* I/P into pigeons and ducks and S/C into rabbits and mice (0.1 ml was injected in mice) and observed the death of all the animals and birds within 48 h (Pigeon and rabbits in 48 h, ducks in 36 h and mice in 12 h).

Avian strains of *P. multocida* culture containing 100 cfu in 0.1 ml of medium was inoculated in mice I/P resulted in death of the mice within 24 h of inoculation (Diallo *et al.*, 1995).

In mouse bioassay study, all the isolates of *P. multocida* of avian origin were found to be lethal to mice, although they varied in their virulence as determined by mean death time of inoculated mice (Rajini *et al.*, 1995).

The inoculation of rabbits by skin scarification with heart blood of dead quails infected with *P. multocida* resulted in death of the rabbits overnight (Chandran *et al.*, 1995).

Jayakumar (1998) studied the pathogenicity of duck isolates of *P. multocida* in mice and ducks. The inoculated mice died in 12 to 48 h and ducks within 24 to 72 h post inoculation.

2.9 Plasmids

2.9.1 Bacterial plasmids and their classification

Plasmids are extrachromosomal autonomous DNA molecules (replicons) found in many bacterial cells. The term plasmid was proposed by Lederberg (1952) for all extrachromosomal genetic structures that can replicate autonomously. They have

attracted a disproportionate degree of research interest because they carry genes which determine many interesting features displayed by bacteria like resistance to antibiotics and heavy metals, virulence, symbiosis, nitrogen fixation, etc. (Stanisich, 1988).

Plasmids range in size from approximately 1 kb to greater than 400 kb. Small plasmids are relatively abundant and may confer no obvious phenotypic relationship on their host. Such plasmids are said to be cryptic.

Identification and classification should be based on genetic traits that are universally present and are constant. These criteria are best met by traits concerned with plasmid maintenance, especially replication control (Couturier *et al.*, 1988).

Low copy number plasmids (one to four copies of the plasmid per bacterial chromosome) have been described as being under stringent replication control. Some other plasmids maintain themselves at a relatively high plasmid/chromosome ratio. Plasmids of this type are said to be subjected to relaxed replication control. Plasmids with same replication control are incompatible and plasmids with different replication control are compatible (Bennet and Howe, 1990).

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

2.9.2 Bacterial plasmid DNA isolation

Several techniques are available for extraction and separation of plasmid DNA. Many strains of bacteria contained in addition to a single large unit of chromosomal DNA, one or more copies of second DNA molecule which are smaller in size than those of chromosome. These plasmids have been found to exist as covalently closed circles (CCC).

For isolation of plasmid DNA, one usually uses any one of the following properties such as (1) difference in base composition from chromosomal DNA, (2) small size relative to the chromosome, (3) transferability and (4) circularity. Some methods are more or less appropriate to a particular system and depend on the amount of interval that one wishes to recover and whether it is necessary to obtain native circular DNA (Freifelder, 1970).

Many methods have been developed to prepare pure plasmid DNA from bacteria. These methods invariably involve three

basic steps (Sambrook *et al.*, 1989). (1) growth of the bacteria, culture, (2) harvesting and lysis of the bacteria and (3) purification of plasmid DNA.

2.9.2.1 Alkaline extraction method

Most of the techniques employed for the isolation of DNA are based on their supercoiled, covalently closed circular (CCC) configuration within the bacterial cell. Thus, ethidium bromide density gradient centrifugation, nitrocellulose absorption and alkaline sucrose sedimentation are all dependent upon certain characteristics unique to CCC DNA. The Briji lysis technique, or cleared lysate method (Clewell and Helinski, 1969) has been widely used.

Guerry *et al.* (1973) isolated plasmid DNA ranging from 5.4×10^6 to 65×10^6 dalton from the chromosomal DNA by the preferential precipitation of the higher molecular weight chromosomal DNA in the presence of sodium lauryl sulphate and a high concentration of sodium chloride.

A simple procedure for the rapid assay to detect the of plasmid was described by Barnes (1977).

Birnboim and Doly (1979) described a procedure for extracting plasmid DNA from bacterial cells, wherein plasmid DNA of high purity could be obtained. The principle of this

method is selective alkaline denaturation of high molecular weight chromosomal DNA, while covalently closed circular DNA remains double stranded. Adequate pH control is accomplished without using a pH meter. Upon neutralisation, chromosomal DNA renatures to form an insoluble clot, leaving plasmid DNA in the supernatant following centrifugation. Large and small plasmid DNAs have been extracted by this method.

Kado and Liu (1981) described a procedure for the detection and isolation of plasmids of sizes varying from 2.6 to 350 MDa that are harboured in various species of bacteria. This method utilised the molecular characteristics of CCC DNA that is released from cells under conditions that denatures chromosomal DNA by using alkaline sodium dodecyl sulphate (pH 12.6), at elevated temperatures. Proteins and cell debris were removed by extraction with phenol-chloroform.

Birnboim (1983) reported that alkaline extraction method for the isolation of CCC DNA from bacterial cell was useful.

Sambrook *et al.* (1989) described a protocol by modification of the methods of Birnboim and Doly, 1979). In this procedure, the use of lysozyme at the first step of tissues has been avoided. Phenol chloroform step was taken as an optional step.

Azad *et al.* (1992) used an improved method for large scale preparation and purification of CCC plasmid DNA molecules of size ranging from 4.3 to 73 kb. This protocol involved an alkaline lysis procedure followed by acid-phenol extraction.

Sikka (1992) described a method of in gel lysis analysis of plasmid profile of bacteria. The principle is that the cells grown with selection are mixed with lytic mix and immediately loaded into the wells of SDS agarose gel.

The method of Basha and Palanivelu (1994) is a modified alkaline lysis procedure for isolation of plasmid DNA from *E. coli*. This method utilised lower concentration of NaOH and SDS and the time used for precipitation with potassium acetate was reduced to 15 min. This procedure was consistent, reproducible and gave better yields.

2.9.2.2 Boiling method

The boiling method described by Holmes and Quigley (1981) is an efficient procedure for removing bulk of chromosomal DNA.

MacNeil (1986) developed a rapid and small scale procedure for the isolation of plasmid DNA from streptomyces cells which were washed with an EDTA solution, subjected to an

extended lysozyme treatment and the supernatent after boiling and centrifugation was extracted with acid phenol.

Gomez-marquez *et al.* (1987) reported a simple, rapid and reliable procedure for large scale purification of plasmid DNA from non amplified bacterial cultures. It was a modified method of Holmes and Quigley (1981).

The boiling method is not recommended while making small scale preparations of plasmid DNA from strains of *E. coli* that express endonuclease A (Sambrook *et al.*, 1989).

2.9.3 Purification and precipitation of plasmid DNA

Most of the techniques devised in recent years for purification of plasmid DNA have depended on the covalently closed circular nature of plasmid DNA (Helinski and Clewell, 1971) and its resistance to denaturation.

Centrifugation of lysates of plasmid-bearing strains on alkaline sucrose gradients has also been used to purify plasmid DNA (Freifelder, 1968).

Guerry *et al.* (1973) described a method for preparing cleared lysates in which high molecular weight chromosomal DNA was selectively precipitated from crude lysates of plasmid bearing strain in the presence of sodium dodecyl sulphate and a high concentration of sodium chloride.

Kado and Liu (1981) reported that proteins and cell debris were removed by extraction with phenol chloroform. Under these conditions, chromosomal DNA concentrations were reduced or eliminated.

Gomez-Marquez *et al.* (1987) described a simple, rapid and reliable procedure for large scale purification of plasmid DNA from non-amplified bacterial cultures. This involves extraction of plasmid DNA with phenol-choloroform, then with chloroform-isoamyl alcohol followed by ethanol precipitation. The final step is gel filtration chromatography employing S-sephacryl S-1000.

Sambrook *et al.* (1989) described the use of ion-exchange gel filtration chromatography or differential precipitation to separate plasmid and host DNAs. Differential precipitation with polyethylene glycol yielded plasmid DNA of extremely high purity.

Sullivan and Klaenhammer (1993) suggested a rapid mini preparation for isolation of high quality plasmid DNA from Lactococcus and Lactobacillus species. After alkaline extraction the pellet was resuspended in 7.5 M ammonium acetate containing ethidium bromide and further purified by phenol-chloroform treatment followed by precipitation with ethanol.

2.9.4 Agarose Gel Electrophoresis of Plasmid DNA

The application of agarose gel electrophoresis in the study of plasmid DNA molecules has been well established (Meyers *et al.*, 1976; Willshaw *et al.*, 1979; Maniatis *et al.*, 1982; Sambrook *et al.*, 1989).

2.9.5 Purity of plasmid DNA

DNA fraction were deemed to be acceptable when the ratios of absorbance at 260 nm and 280 nm (A 260/A 280) were greater than or equal to 1.8 and the ratios of absorbance at 260 nm and 230 nm (A 260/A 230) were greater than or equal to 1.5 (Orgam *et al.*, 1987).

The shape of the UV absorption spectra obtained were compared with published data and the purity of the preparation, was commented. The extinction values at 260 nm and 280 nm can be used to detect protein. The E-260/E-280 ratio should be between 1.8 and 1.9 for pure DNA and a value less than 1.75 indicate significant protein contamination (Plummer, 1988).

2.9.6 Molecular size estimation of plasmid DNA

Plasmid DNA molecular size may be estimated conveniently by agarose gel electrophoresis using either purified plasmid DNA or plasmid containing cell lysates that are devoid of most

chromosomal DNA. Such estimations are made possible by inclusion of plasmid molecules of known molecular size in the same electrophoretic run.

Meyers *et al.* (1976) have shown that there was a linear relationship between the logarithms of the relative migration of covalently closed circular (CCC) DNA molecules and the logarithm of the plasmid molecular size.

The electron microscopy of plasmid DNA was performed to find contour length of DNA (Meyers *et al.*, 1976). Molecular weights were calculated from the contour lengths by using the conversion factor of 1 μm = 2.07×10^6.

Macrina *et al.* (1978) described a strain of *E. coli* that contains eight plasmid species ranging in size from 1.36×10^6 to 35.8×10^6 daltons, this strain can be employed as a single source of covalently closed circular deoxyribonucleic acid molecules of different sizes for use as reference in agarose gel electrophoretic analysis.

Several regression methods were tested for estimating the sizes of a wide range of plasmids (1.37 to 31.2 MDa) and restriction fragments (2.2 to 14.2 MDa) by agarose gel electrophoresis. The accurate and least variable method was the multiple regression of $\log_{10}$ molecular size against $\log_{10}$

relative mobility and the reciprocal square root of the relative mobility (Rochelle *et al.*, 1985).

Genthner *et al.* (1985) developed relatively rapid methods for the determination of relative genome molecular mass and estimation of plasmid copy number.

David *et al.* (1993) estimated the molecular size of plasmid DNA of two *E. coli* isolates and one salmonella isolate by the smooth curve method and the least square technique of fitting the regression line using covalently closed circular *E. coli* V517 as a standard marker and concluded that either of the methods could be used for precise molecular weight determination of bacterial plasmid DNA.

2.10 Plasmid profile of *P. multocida*

Antibiotic resistance has been associated with *P. multocida* that caused fowl cholera. Two non transmissible R-plasmids of molecular weight 4.4 and 3.44 MDa were isolated by Berman and Hirsh (1978). They coded resistance to tetracycline, streptomycin and sulphonamides.

A strain of *P. multocida* isolated from turkeys during an outbreak of septicaemic disease (fowl cholera) was shown to possess the ability to transfer streptomycin and sulphadiazine resistance. The genes necessary for the transfer of

resistance and resistence were associated with a 28.5 MDa plasmid and 7.2 MDa plasmid respectively (Hirsh *et al.*, 1981).

In a survey for the detection of plasmids in *P. multocida*, Hirsh *et al.* (1985) reported that 41 of 58 isolates contained plasmids with molecular weights ranging from 2 to 10 MDa. Seven of them encoded resistance to tetracycline, streptomycin and sulphonamides.

According to Snipes *et al.* (1989) three isolates were found to contain plasmid DNA, all of which were isolated from dead turkeys. The plasmids in these isolates were of the same size (approximately 2 MDa) and were cryptic.

Plasmid profile of selected strains of *P. multocida* from pneumonic lesions of slaughter weight swine was studied by Hoie *et al.* (1990). The toxigenic strain contained three plasmids with approximate sizes of 2.8, 3.0 and 10 MDa, the other non-toxigenic strain contained one plasmid of about 30 MDa. The results indicated that plasmid characterisation was not a suitable tool for studying the pathogenicity of *P. multocida*.

The characteristics of *P. multocida* isolated from water fowl and associated avian species were studied by Hirsh *et al.* (1990) and reported that none of the *P. multocida* isolates from avian species contained plasmid DNA.

A 5.2 kb plasmid with single restriction site for Eco RI and Hind III was isolated from dermonecrotic, atrophic rhinitis strains of *P. multocida* type D clones from which the plasmid was removed with acridine orange. This plasmid was associated with resistance to streptomycin, lincomycin polymixin-B and amikacin. The same plasmid also encoded for dermonecrotoxin (Mendosa-Elvira *et al.*, 1990).

Yamamoto *et al.* (1990) isolated R-plasmids from *P. multocida* swine origin. Sulfamethoxine and streptomycin resistance was encoded on a 3.6 MDa plasmid and 5.5 MDa plasmid encoded resistance to sulfadimethoxine streptomycin, kanamycin and chloramphenicol.

Ikeda and Hirsh (1990) reported that possession of an identical non-conjugative R-plasmid by different isolates of *P. multocida* does not imply clonality.

Thirteen out of 25 of *P. multocida* strains contained two plasmids of 2.6 kb and 48 kb (Lee *et al.*, 1991).

Cote *et al.* (1991) observed that streptomycin and sulphonamide resistance were associated with 5.6 kb and 5.9 kb plasmids respectively in *P. multocida*.

Gunther *et al.* (1991) assayed 28 isolates of rabbit *P. multocida* for plasmids to correlate their presence with

clinical or pathologic findings, serotype, toxin production, possession of pili and biochemical characteristics. Fourteen isolates contained a single 1.6 MDa plasmid. An additional isolate had two plasmids which migrated as a closely spaced doublet, centred around 1.6 MDa.

According to Droual *et al.* (1992) the ATCC strain of *P. gallinarum* had two plasmids of approximately 2.4 and 4.5 kilo dalton and no plasmids were detected in Fresno strain of *P. gallinarum*.

Twelve small cryptic plasmids were isolated by Price *et al.* (1993) from Asian strains of *P. multocida*.

A collection of 45 strains of *P. multocida* were examined for plasmids and attempts were made to correlate their presence with resistance to antimicrobial substances and to pathogenicity for mice. Twenty strains contained no detectable plasmids but showed pathogenicity for mice. Seven strains contained a single plasmid of 1.3 kilobase pair (kbp) and 18 strains contained two plasmids of 2.4 and 7.5 kbp and were not virulent for mice. There was no obvious correlation between the presence of plasmids and antimicrobial resistance (Diallo *et al.*, 1995).

According to Pande and Singh (1997), there was no correlation between virulence and the presence of plasmids in *P. multocida*.

Materials and Methods

MATERIALS AND METHODS

3.1 Materials for isolation of pasteurella organisms

3.1.1 Biosamples for isolation of pasteurella organisms

Biosamples for isolation of pasteurella were collected from ailing/dead chicken and ducks brought by the farmers to the Department of Microbiology and Department of Pathology, College of Veterinary and Animal Sciences, Mannuthy for diagnosis of disease.

Throat swabs were also collected from healthy chicken maintained in the Kerala Agricultural University Farm, Mannuthy and ducks reared by the duck farmers in and round Thrissur.

3.1.2 Sheep blood for blood agar

The sheep blood for the preparation of blood agar was collected from the animals maintained in the University Goat and Sheep Farm, Mannuthy. Blood collection was carried out under sterile conditions in round bottom flasks containing glass beads. The defibrinated blood was stored at 4°C in 10 ml aliquotes until use.

3.1.3 Blood agar

The dehydrated blood agar base (Hi-Media) was prepared as per the manufacturer's instructions, to which sterile blood was added at five per cent level to make blood agar.

3.1.4 Pasteurella multocida sensitive agar (PMSA) (Moore *et al.*, 1994)

Basal medium of Brain Heart Infusion Agar dehydrated was prepared as per the manufacturer's instructions. Sterilised by autoclaving and added the following substances.

Gentamicin	-	0.75 µg/ml
Potassium tellurite	-	2.5 µg/ml
Amphotericin B	-	5.0 µg/ml
Defibrinated blood	-	5 per cent (5 ml/100 ml)

pH adjusted to 10 ± 0.1 with sterile IN NaOH

3.1.5 Dextrose Starch Agar (DSA) dehydrated (Hi-media) was reconstituted as per the manufacturer's instructions.

3.1.6 Nutrient broth

Dehydrated (Hi-Media) was used as per manufacturer's instructions.

3.1.7 Glycerol broth

Rehydrated nutrient broth	-	70 ml
Glycerol	-	30 ml

3.2 Methods

3.2.1 Isolation of pasteurella organisms

Aseptic precautions were taken in procedures wherever necessary. Samples of heart blood, lung, liver and spleen collected from chicken and ducks died of suspected pasteurellosis were streaked onto sheep blood agar plates and incubated at 37°C in a candle jar (under increased carbondioxide tension). The incubated plates were examined after 24 h. In negative cases the plates were again incubated under the same conditions for another 24 h.

The throat swabs collected from healthy chicken and ducks were also used for the isolation of pasteurella organisms on PMSA following the above procedures.

After isolation the colony characters of the organisms were studied using Dextrose Starch Agar (DSA).

Colonies suggestive of pasteurella (round, slight convex colonies, mucoid in consistency and sticky in nature) were

stained with gram's staining technique to confirm the morphological features.

3.2.2 Identification

The bacterial isolates were identified based on morphology, colony characters and various biochemical tests described by Cowan (1974).

3.2.3 Bacterial cultures

Four isolates of *P. multocida* were employed in this study. The isolate DC2 was the one isolated from a duck and identified during the period of this study. The isolate DC1 was a culture maintained in the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, which was a duck isolate. Two isolates designated as FC1 and FC2 were from throat swabs of healthy chicken.

The pure cultures of all the four isolates were maintained in the laboratory by preserving in glycerol broth, and kept at -20°C.

3.3 Biotyping

3.3.1 Materials

Various sugars such as arabinose, dulcitol, sorbitol, trehalose and xylose were prepared at one per cent

concentration in peptone water. Phenol red (0.2 per cent) was used as an indicator to determine the acid production.

3.3.2 Methods

Stock solutions (10 per cent) of each carbohydrate was prepared in sterile distilled water and sterilised by filteration. The stock solutions were further diluted to one per cent solutions in peptone water. Transferred 3 ml of each solution (1 per cent) into 5 ml tubes and inoculated with *P. multocida*. The tubes were incubated at 37°C for a minimum of seven days and examined daily for any acid production as indicated by a colour change in the phenol red indicator.

3.4 Antibiogram

3.4.1 Materials

The following antibiotic discs with known concentration of antibiotics noted against each (Hi-Media Laboratories Pvt. Ltd., Bombay, India) were used to study the antibiotic sensitivity pattern of the isolates.

i Ampicillin (A) - 10 μg

ii Chloramphenicol (C) - 30 μg

iii Cloxacillin (Cx) - 5 μg

iv Erythromycin (E) - 15 μg

v	Furazolidone (Fr)	-	100 μg
vi	Gentamicin (G)	-	30 μg
vii	Kanamycin (K)	-	30 μg
viii	Metronidazole (Mt)	-	5 μg
ix	Nalidixic acid (Na)	-	30 μg
x	Nitrofurantoin (Nf)	-	300 μg
xi	Oxytetracycline (O)	-	30 μg
xii	Penicillin-G (P)	-	10 units
xiii	Pefloxacin (Pf)	-	5 μg
xiv	Streptomycin (S)	-	10 μg
xv	Trimethoprim (Tr)	-	5 μg
xvi	Triple sulphas (S3)	-	300 μg

3.4.2 Method

Antibiotic sensitivity test was done based on the disc diffusion method of Bauer *et al.* (1966).

Five colonies of each pure culture were picked up with sterile platinum loop and was used as the inoculum in 4 ml of trypticase soy broth. The inoculated cultures were incubated at 37°C for 5 h. The bacterial suspension was absorbed on to a sterile cotton swab and uniformly inoculated on blood agar plates of 15 cm diameter. The plates were dried at room temperature for 5 min.

Six antibiotic discs were placed at equidistant hexagonally and one disc in the middle and three such plates were used for each test isolate.

After incubation at 37°C for 18 to 24 h, the diameter of clear zone (including the diameter of the disc) was measured, recorded and interpreted according to the zone size interpretation chart of Brown and Blowers (1978).

3.5 Pathogenicity studies

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.

3.5.1.2 Rabbits

Pasteurella multocida free rabbits aged two to three months were employed. The animals were received from SABS, Mannuthy.

3.5.1.3 Chicken

Thirty, day old male chicks were procured from University Poultry Farm, Mannuthy and were reared. All the birds were

fed with chick mash. Feed and water were given *ad libitum*. After two weeks the chicks were divided into five groups and reared in separate cages. Four groups were inoculated with *P. multocida* and maintained for further seven days.

3.5.1.4 Ducks

Ten ducks of two months age were procured from the local market and were fed with concentrate duck feed purchased from Meat Products of India, Koothattukulam. Feed and water were given *ad libitum*.

3.5.2 Method

3.5.2.1 Pathogenicity in mice

The method described by Kawamoto *et al*. (1990) was followed. Four mice per isolate were used for this purpose. Each animal was injected intraperitoneally (I/P) with 0.1 ml of inoculum containing $3x10^8$ organisms/ml of sterile saline. The control mice were injected with 0.1 ml of sterile saline. All the animals were observed for clinical symptoms of infection/death. Blood smears were prepared from dead ones and stained with Wright's stain. The reisolation of *P. multocida* from dead mice was carried out from liver, spleen, lung and heart blood.

3.5.2.2 Pathogenicity in rabbits

Two rabbits were used for each isolate. Each animal received 0.2 ml of the inoculum containing $3x10^8$ organisms/ml in sterile saline by nasal route (I/N) and two rabbits were injected with sterile saline only. They were observed for a period of 21 days for clinical symptoms/death. All the dead animals were subjected to post mortem examination and reisolation was carried out from heart blood, trachea, lung, liver and spleen.

3.5.2.3 Pathogenicity in chicken

Six chicks were used for each isolate. The birds received 0.1 ml of the bacterial culture containing $3x10^8$ organisms/ml of sterile saline by oral route. All the birds were observed for a period of seven days for clinical symptoms/death. Six control chicks received 0.1 ml of sterile saline. Reisolation of the organism was carried out from heart blood, liver, lung and spleen of dead birds.

3.5.2.4 Pathogenicity in ducks

Each isolate was inoculated into two ducks. The birds were injected s/c with 0.1 ml of the culture containing $3x10^8$ organisms/ml of sterile saline. All the injected birds were observed for a period of 14 days for clinical symptoms/death.

Two birds remained as control and they received 0.1 ml of sterile saline by S/C route. The dead birds were subjected to post mortem examination and the reisolation of the organisms was carried out from heart blood and appropriate visceral organs.

3.6 Plasmid profile analysis

3.6.1 Materials for plasmid profile analysis

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

3.6.1.1 Luria Bertani Medium (Hi-Media)

Bacto-tryptone	-	10 g
Bacto-yeast extract	-	5 g
Sodium chloride	-	10 g
Distilled water to	-	1 L

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

3.6.1.2 TEG buffer (pH 8.0)

Glucose	-	50 mM
Tris-Cl (pH 8.0)	-	25 mM
EDTA (pH 8.0)	-	10 mM
Distilled water	-	100 ml

TEG buffer was prepared in 100 ml quantity, autoclaved at 10 lbs pressure for 20 min and stored at 4°C.

3.6.1.3 Lysozyme (Sigma)

3.6.1.4 SDS-NaOH solution

Sodium hydroxide	-	0.2 N
Sodium dodecyl sulphate	-	1.0 per cent
Distilled water	-	100 ml

SDS-NaOH solution was made up from stock solutions of 10 per cent SDS and 1N NaOH.

3.6.1.5 Sodium acetate solution

3 M sodium acetate	-	40.81 g
Distilled water	-	100 ml

pH adjusted to 4.8 with glacial acetic acid. Autoclaved at 121°C for 15 min at 15 lbs pressure.

3.6.1.6 Distilled ethanol

3.6.1.7 Tris-EDTA (TE) buffer pH 8.0

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

Autoclaved at 121°C for 15 min at 15 lbs pressure.

3.6.1.8 RNAase that is free from DNAase

Pancreatic RNAase (Sigma), was dissolved at a concentration of 10 mg/ml in TE buffer (previously boiled for 15 min and allowed to cool at room temperature slowly). Dispersed in small quantities and stored at -20°C.

3.6.1.9 Tris Borate Electrophoresis buffer (TBE), pH 8.0

Stock solution (5x)		
Trizma base	-	54.0 g
Boric acid	-	27.5 g
EDTA, 0.5 M	-	20.0 ml
Distilled water	-	980 ml

Autoclaved. The stock solution (5x) was diluted to 1x before use.

3.6.1.10 Gel loading buffer

Bromophenol blue	-	0.25 per cent
Xylene cyanol	-	0.25 per cent
Glycerol	-	30 per cent (w/v)

3.6.1.11 Agarose (Sigma)

3.6.1.12 Ethidium bromide (Sigma)

Ethidium bromide 0.5 μg/ml of sterile distilled water.

3.6.2 Plasmid profile analysis of *P. multocida*

The plasmid DNA was prepared from avian strains of *P. multocida* by alkaline lysis technique with the modification of Sambrook *et al.* (1989).

The three basic steps involved in the plasmid DNA preparations are:

1. Growth of the bacteria
2. Harvesting and lysis of bacteria
3. Isolation and precipitation of plasmid DNA

3.6.2.1 Growth of the bacteria

A colony from pure culture of *P. multocida* from sheep blood agar plate was inoculated into 2 ml of LB medium and incubated overnight at 37°C with frequent shaking.

3.6.2.2 Harvesting and lysis of bacteria

Bacterial culture of 1.5 ml was taken in an eppendorf tube and the cells were harvested by centrifuging at 6000 rpm

for 10 min. The supernatent was discarded and the pellet was taken for lysis.

The bacterial pellet was resuspended in 100 µl of ice cold TEG solution containing lysozyme at the rate of 2 mg/ml to get a uniform suspension and was vortexed gently. It was kept on ice for 10 min and then shifted to room temperature.

Added 200 µl of SDS-NaOH solution and the contents were mixed by inverting the tube five times and placed on ice for 15 min.

After the addition of 150 µl of 3 M sodium acetate solution, the contents of the tube were mixed by gentle inversion and kept ice for 15 min.

Centrifuged at 8000 rpm for 10 min in a microfuge.

The supernatent was transferred to a fresh eppendorf tube and 450 µl of cold ethanol was added and kept at -20°C overnight.

3.6.2.3 Precipitation with ethanol

The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 20 min. The pellet was rinsed in 1 ml of 70% ethanol. The supernatent was discarded and the pellet was allowed to dry. The DNA was dissolved in 50 µl of TE buffer.

3.6.2.4 RNAase treatment

To the DNA solution, added 5 µl of RNAase and incubated at 37°C for 30 min.

3.6.2.5 Spectrophotometric determination of DNA

The purity and concentration of plasmid DNA were assessed by spectrophotometry. The UV spectrophotometer reading after standardization with TE buffer as blank, was used for testing the purity of plasmid DNA. The OD readings of the preapred plasmid DNA was measured at 260 nm and 280 nm. The concentration was calculated using the formula, OD 1 at 260 nm = 50 µg/ml (for double stranded DNA).

3.6.2.6 Agarose gel electrophoresis

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

Agarose (120 mg) was dissolved in 15 ml of TBE buffer and boiled to dissolve. Allowed to cool to 60°C. Ethidium bromide was added to a final concentration of 0.5 µg/ml. The clean, dry glass plate edges were sealed with adhesive tape and the comb was placed in position and a gap of 0.5 to 1.0 mm has been provided between the glass plate and the comb tip, so as to form a seal of agarose within the well beneath the tip. The melted agarose was poured and 30 min time lapse was given for

the agarose to form a gel. The comb and tape were removed and the agarose gel plate was placed within the electrophoresis tank filled with TBE (1x) buffer. More TBE buffer was added so that the buffer stood 0.5-0.8 cm above the gel surface.

To 20 µl of plasmid DNA sample containing 25 µg/ml of DNA, about 3 µl of loading buffer was added and was loaded into each slot of the gel. Electrophoresis was carried out at 50 V for 1 h or till the loading dye reached 3/4th of the gel. The gel was examined in Alpha Imager (Alpha Innotech Corporation, USA).

3.6.2.7 Photography of plasmid DNA

The gel was placed under the camera of Alpha Imager and viewed to obtain the real image of the sample to capture. The image the gel was illuminated with UV for 6 sec. The depth of grey scale was adjusted to Black: 0, White: 255, Gamma: 0.50. The images and data were printed at 1x magnification to a thermal (graphic) printer and presented as such.

3.6.2.8 Estimation of molecular size of plasmid DNAs

The molecular size of plasmid DNA was estimated by drawing a linear graph in comparison with the molecular size of the plasmids of *E. coli* V517 (Macrina *et al.*, 1978).

A standard curve was drawn by plotting the log 10kb values of the plasmid DNAs of the marker strain V517 on Y axis and distance migrated on X axis. The values of the distance migrated by the unknown plasmids were interpolated with the standard curve to arrive at the molecular weight of plasmid DNA.

Results

RESULTS

4.1 Isolation

4.1.1 Sick birds

Fifteen chicken and 10 ducks which were showing listlessness, ocular and nasal discharge, greenish diarrhoea and paralysis of the legs and wings, were subjected to detailed post mortem examination. Gross lesions in these birds varied much and were not suggestive of a particular disease entity. But in some birds, haemorrhages were observed in internal organs like proventriculus, lungs, intestine and pericardium. Pericardial fluid accumulation was noticed along with the enlargement of liver and spleen.

Heart blood smear and impression smear from liver, lungs and spleen of all the necropsied birds with symptoms and lesions suggestive of pasteurellosis were stained with Wright's stain. None of the smears from chicken revealed bipolar organisms. On examination of blood smears from ducks, four exhibited stray bipolar organisms and one was teaming with bipolar organisms. Such organisms were also seen in impression smears of various organs taken from the particular duck.

Although bacterial isolation was tried from biomaterials collected from birds, only one blood smear from a bird, yielded bacterial colonies suggestive of pasteurella, on blood agar after 24 h incubation at 37°C under increased CO2 tension.

4.1.2 Healthy birds

Throat swabs were collected from apparently normal birds, 50 chicken and 20 ducks. These swabs were inoculated onto PMSA. General bacterial contaminant growth was minimum in all the plates even after 48 h of incubation. Throat swabs from two chicken revealed colonies suggestive of pasteurella.

4.2 Identification

The colonies on blood agar were mucoid, convex and non-haemolytic and the colonies on PMSA were small, convex and smooth. Both colonies ie. from the blood agar and from PMSA were subcultured on DSA to describe the colony morphology. On DSA the colonies from blood agar were medium sized, mucoid and grey, while that from PMSA were small, smooth and grey.

Four isolates (DC1, DC2, FC1 and FC2) of *P. multocida* were employed in this study. The isolate DC1 was a culture maintained in the Department of Microbiology. The isolate DC2 was the one isolated from the duck suspected for

pasteurellosis and the other two isolates (FC1 and FC2) were isolated from apparently healthy chicken.

Identification of bacterial isolates of the first phase was based on the following tests, Gram's staining reaction, morphology, motility, growth under aerobic and anaerobic condition catalase activity, oxidase activity, carbohydrate break down, growth on Mac Conkey's agar and haemolysis on blood agar (Table 2; Fig.1 and 2).

All the isolates were gram negative, non motile coccobacilli. They were catalase and oxidase positive, grew aerobically and anaerobically, fermented glucose, did not grow on Mac Conkey's agar and showed no haemolysis on blood agar.

Further, the isolates (DC1, DC2, FC1 and FC2) were subjected to second stage biochemical tests such as indole production, nitrate reduction, methyl red, voges proskauer, H_2S production, urease, citrate utilisation, gelatin liquefaction, growth in KCN medium and sugar fermentation tests (Table 3).

4.3 Biotyping

Biotyping of *P. multocida* isolates was carried out by observing their ability to ferment sugars such as arabinose, dulcitol, sorbitol, trehalose, and xylose. Of the four

isolates two biotypes were observed. The isolates DC2, FC1 and FC2 came under one biotype fermenting xylose and trehalose, while DC1 came under another biotype fermenting xylose and sorbitol None of the isolates fermented arabinose and dulcitol.

4.4 Antibiogram

An antibiogram of *P. multocida* isolates was formulated based on its susceptability and resistance to various antibiotics/antibacterial agents is presented in Table 5.

All the isolates were resistant to furazolidone, metronidazole and nalidixic acid. All strains of *P. multocida* were sensitive to oxytetracycline, pefloxacin and streptomycin. The isolates DC1 and DC2 have shown resistance to eleven antibiotics/antibacterials. The isolate FC1 was resistant to six antibiotics/antibacterials and FC2 was resistant to eight antibiotics/antibacterials.

4.5 Pathogenicity studies

4.5.1 Pathogenicity in mice

Four isolates of *P. multocida* viz., DC1, DC2, FC1 and FC2 were tested for their pathogenicity in mice. Each culture was injected into a group of four mice by intraperitoneal route.

The DC1 and DC2 isolates killed all the inoculated groups of mice within 24 h of injection. All the mice inoculated with FC1 and FC2 died within 36 to 48 h of injection (Table 6).

The gross lesions observed in the internal organs of the dead mice were petechial haemorrhages in the liver, pericardium and congestion of lungs and spleen. Blood smear and tissue impression smears, collected from dead mice, stained with Wright's stain revealed the presence of bipolar organisms suggestive of *P. multocida*.

Bacteria could be isolated from heart blood, lungs, liver and spleen.

4.5.2 Pathogenicity in rabbits

The rabbits were inoculated intranasally with 0.2 ml of culture containing *P. multocida* isolates. The rabbits inoculated with DC1 and DC2 developed symptoms like general weakness, lethargy, inability to stand, laboured breathing, convulsions and torticollis. DC1 and DC2 killed all the rabbits on the fourth and fifth day of inoculation respectively (Table 6).

The lesions observed were characteristic haemorrhagic tracheitis, ecchymotic haemorrhages in the lung, liver, congestion of the spleen and haemorrhagic enteritis. Bacteria

could be reisolated from heart blood, liver, spleen and trachea.

Rabbits inoculated with isolates FC1 and FC2 did not develop any clinical symptom during the observation period of 21 days. These isolates were found to be non-pathogenic to rabbits.

4.5.3 Pathogenicity in chicken

The isolates were tested for their pathogenicity in chicks. The chicks received 0.1 ml of saline containing *P. multocida* isolates orally. All the chicks inoculated with DC1 and DC2 died within 48 h (Table 6). The birds developed symptoms like depression, ruffled feathers, anorexia, mucous discharge from the mouth, increased respiratory rate and diarrhoea.

Post mortem examination of the dead birds revealed petechial haemorrhages throughout the internal organs including myocardium and haemorrhagic enteritis. *P. multocida* were demonstrated in heart blood smear and were isolated from liver, lung and spleen.

Chicks inoculated with FC1 and FC2 did not develop any clinical symptom throughout the observation period of seven days and remained apparently normal.

4.5.4 Pathogenicity in ducks

Each culture of *P. multocida* was injected into a group of two ducks by subcutaneous route. All the ducks injected with DC1 and DC2 developed symptoms like nasolacrymal discharges, swollen and sticky eyelids, paralysis of the legs and wings, greenish diarrhoea and typical posture of breast touching on the ground and were dead within 24 h and 48 h of injection respectively (Table 6).

The gross lesions observed were petechial haemorrhages on the pericardium, liver, lungs and congestion of the spleen. Bacteria could be reisolated from the heart blood, liver, lung and spleen.

The FC1 and FC2 injected ducks did not develop any clinical signs upto 14 days of injection and remained apparently healthy.

4.6 Plasmid profile of *P. multocida* isolates

The plasmid profiles of four *P. multocida* isolates of avian origin have been analysed (Fig.3). The number of plasmids present in these strains varied from zero to five plasmids and the size of the plasmids ranged from 2.69 kb to 7.07 kb (Table 7).

The 4.99 kb plasmid is common to the duck isolates (DC1 and DC2) and 5.76 kb plasmid is common to the isolates DC1 and FC2.

4.7 Correlation between biotyping and plasmid profile of *P. multocida*

The biotypes obtained by the fermentation of certain sugars were compared with plasmid profile of the *P. multocida* isolates and the data are presented in Table 8.

By combination of these parameters all the isolates could be distinguished. *P. multocida* isolates DC2, FC1 and FC2 belonged to same biotype but could not be differentiated by biotyping alone. But they varied in their plasmid profile. DC2 and FC2 contained four and five plasmids respectively and FC1 did not possess any plasmid.

4.8 Correlation between antibiogram and plasmid profile of *P. multocida*

Using 16 antibiotics and anti-bacterials, four different resistance patterns were obtained. Correlation of this method with plasmid profile is presented in Table 9.

4.9 Correlation among biotyping, antibiogram and plasmid profile of *P. multocida* isolates

Data on biotyping and antibiogram were compared with those of plasmid profile analysis of *P. multocida* isolates of avian origin.

All the isolates could be differentiated using a combination of parameters such as biotyping, antibiogram studies and plasmid profile (Table 10). Eventhough the three isolates (FC1, DC2, FC2) come under the same biotype, they exhibited variation in the antibiotic resistance and plasmid profiles.

Table 1. Isolation of *P. multocida* from chicken and ducks

Type of specimens	Species of birds screened			
	Chicken		Ducks	
	No. of birds	No. of positive isolation	No. of birds	No. of positive isolation
Tissues from ailing/dead birds	15	Nil	10	One
Throat swabs from apparently healthy birds	50	Two	20	Nil

Table 2. Primary tests of *P. multocida* isolates

Tests	Isolates			
	DC1	DC2	FC1	FC2
Gram's reaction	-	-	-	-
Shape	cocco bacilli	cocco bacilli	cocco bacilli	cocco bacilli
Presence of capsule	+	+	-	-
Motility	-	-	-	-
Growth in air	+	+	+	+
Growth anaerobically	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
O/F	F	F	F	F
Growth in MacConkey's agar	-	-	-	-
Haemolysis on blood agar	-	-	-	-

Table 3. Second stage biochemical tests of *P. multocida*

Tests	Isolates			
	DC1	DC2	FC1	FC2
Indole	+	+	+	+
Nitrate reduction	+	+	+	+
Methyl red test	-	-	-	-
Voges-proskauer test	-	-	-	-
H_2S production	-	-	-	-
Urease	-	-	-	-
Citrate utilisation	-	-	-	-
Gelatin liquefaction	-	-	-	-
Growth in KCN medium	+	+	+	+
Sugar fermentations				
Glucose (Dextrose)	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Mannose	+	+	+	+
Mannitol	+	+	+	+
Sucrose	+	+	+	+
Lactose	-	-	-	-
Maltose	-	-	-	-
Raffinose	-	-	-	-

Table 4. Biotyping of *P. multocida* using sugars

	Sugars	Isolates			
		DC1	DC2	FC1	FC2
1.	Xylose	+	+	+	+
2.	Arabinose	-	-	-	-
3.	Dulcitol	-	-	-	-
4.	Sorbitol	+	-	-	-
5.	Trehalose	-	+	+	+

Table 5. Antibiogram pattern of *P. multocida* isolates

P. multocida isolates	Antibiotics and antibacterials															
	A	C	Cx	E	Fr	G	K	Mt	Na	Nf	O	P	Pf	S	S_3	Tr
DC1	R	R	R	S	R	S	S	R	R	S	S	S	S	S	S	S
DC2	R	S	R	R	R	R	R	R	R	R	S	S	S	S	R	R
FC1	S	S	S	S	R	S	S	R	R	R	S	S	S	S	R	R
FC2	S	S	S	R	R	S	S	R	R	R	S	R	S	S	R	R

A - Ampicillin
C - Chloramphenicol
Cx - Cloxacillin
E - Erythromycin
F - Furazolidone
G - Gentamicin
K - Kanamycin
Mt - Metronidazole
Na - Nalidixic acid
Nf - Nitrofurantoin
O - Oxytetracycline
P - Penicillin-G
Pf - Pefloxacin
S - Streptomycin
S_3 - Triple sulpha
Tr - Trimethoprim

Table 6. Pathogenicity studies of *P. multocida* isolates in experimental animals/birds

P. multocida isolates	Experimental animals/ birds used	No. of animals/ birds used	Quantity given	Route of inoculation	Time of death	No. of animals/ birds died
DC1	Mice	four	0.1 ml	I/P	24 h	four
	Rabbits	two	0.2 ml	I/N	96 h	two
	Chicks	six	0.1 ml	Orally	48 h	six
	Ducks	two	0.1 ml	S/C	24 h	two
DC2	Mice	four	0.1 ml	I/P	24 h	four
	Rabbits	two	0.2 ml	I/N	120 h	two
	Chicks	six	0.1 ml	Orally	48 h	six
	Ducks	two	0.1 ml	S/C	48 h	two
FC1	Mice	four	0.1 ml	I/P	36-48 h	four
	Rabbits	two	0.2 ml	I/N	-	-
	Chicks	six	0.1 ml	Orally	-	-
	Ducks	two	0.1 ml	S/C	-	-
FC2	Mice	four	0.1 ml	I/P	36-48 h	four
	Rabbits	two	0.2 ml	I/N	-	-
	Chicks	six	0.1 ml	Orally	-	-
	Ducks	two	0.1 ml	S/C	-	-

Table 7. Plasmid profile of avian strains of *P. multocida* isolates

Isolates	Number of plasmids	Molecular size of plasmid DNA (kb)*
DC1	3	6.73 5.76 4.99
DC2	4	6.93 5.81 4.99 3.30
FC1	Nil	
FC2	5	7.07 5.76 5.38 2.82 2.69

* Kilobase pair

Table 8. Correlation between biotypes and plasmid profile of avian strains of *P. multocida* isolates

P. multocida isolates	Biotypes	No. of plasmids	Molecular size of plasmid DNA (kb)*
DC1	Xyl, Sor	3	6.73 5.76 4.99
DC2	Xyl, Tre	4	6.93 5.81 4.99 3.30
FC1	Xyl, Tre	Nil	-
FC2	Xyl, Tre	5	7.07 5.76 5.38 2.82 2.69

Xyl - Xylose; Sor - Sorbitol
Tre - Trehalose

Table 9. Correlation between antibiogram pattern and plasmid profile of avian strains of *P. multocida* isolates

P. multocida isolates	Antibiogram	No. of antibiotics resistant	Plasmid profile	Molecular size of plasmids (kb)
DC1	A,C,Cx,Fr,Mt,Na	6	3	6.73 5.76 4.99
DC2	A,Cx,E,Fr,G,K,Mt, Na,Nf,S3,Tr	11	4	6.93 5.81 4.99 3.30
FC1	Fr,Mt,Na,Nf,S3, Tr	6	Nil	-
FC2	E,Fr,Mt,Na,Nf,P, S3, Tr	8	5	7.07 5.76 5.38 2.82 2.69

Table 10. Correlation among biotype antibiogram and plasmid profile of avian strains of *P. multocida* isolates

P. multocida isolates	Biotype	Antibiogram	Plasmid profile	
			No. of plasmid	Molecular size of plasmids (kb)
DC1	Xyl, Sor	A,C,Cx,Fr,Mt,Na	3	6.73 5.76 4.99
DC2	Xyl, Tre	A,Cx,E,Fr,G,K,Mt, Na,Nf,Tr,S3	4	6.93 5.81 4.99 3.30
FC1	Xyl, Tre	Fr,Mt,Na,Nf,S3,Tr,	Nil	-
FC2	Xyl, Tre	E,Fr,Mt,Na,Nf,P, S3,Tr	5	7.07 5.76 5.38 2.82 2.69

Fig.1. Gram staining showing gram negative coccobacilli

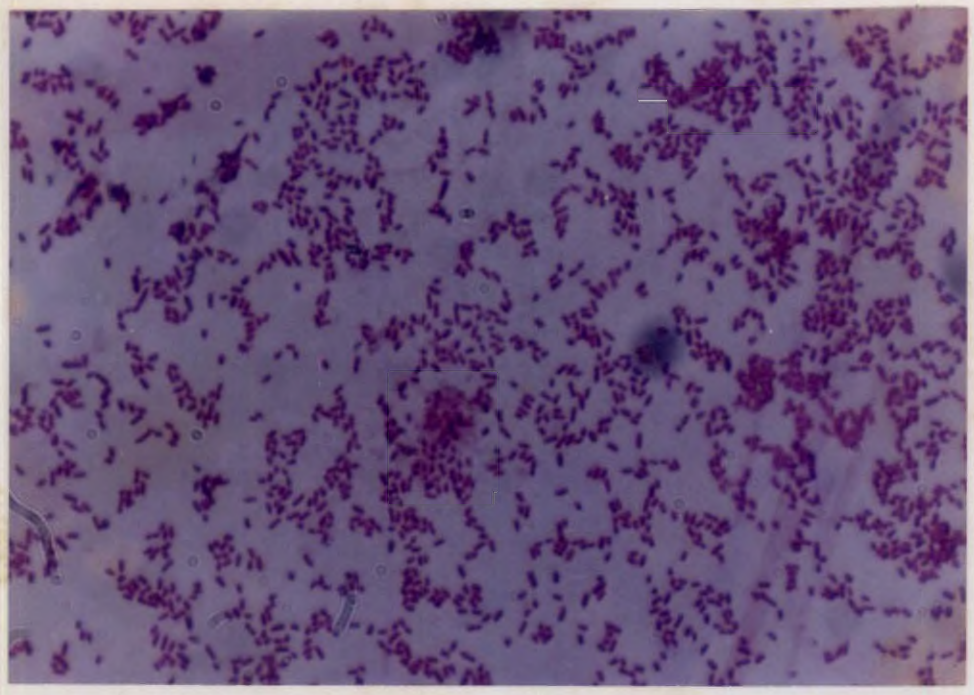


Fig.2. **Nigrosin staining method revealing capsulated *Pasteurella multocida* (DC2)**

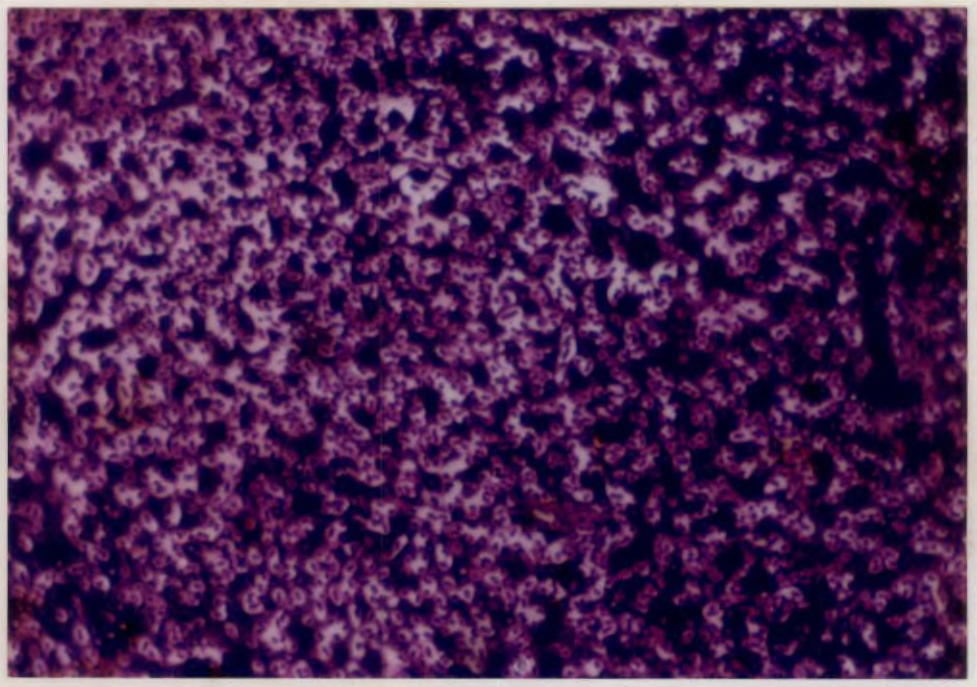


Fig.3. Plasmid profile of avian strains of *Pasteurella multocida* isolates

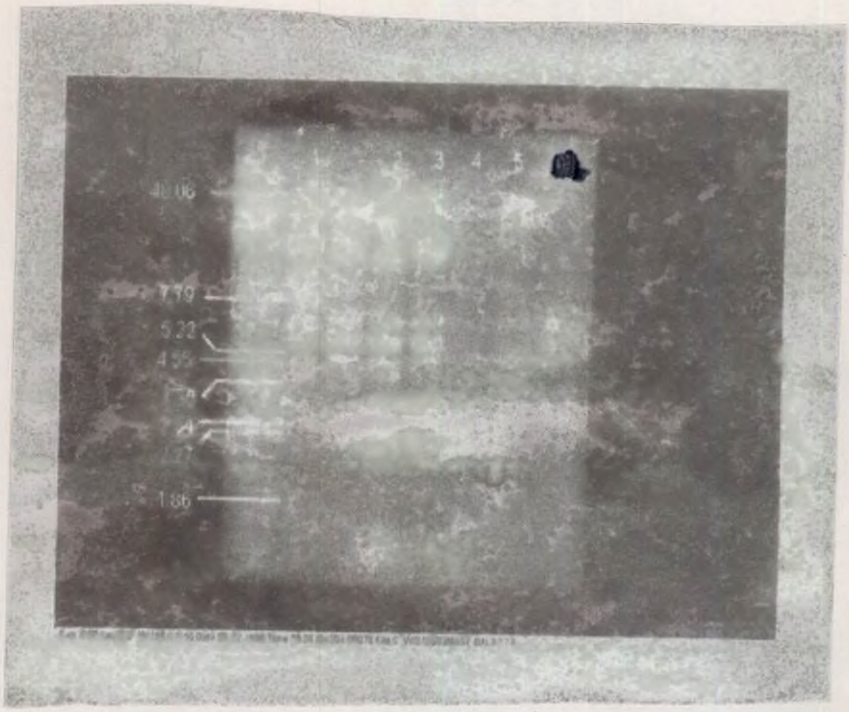
Lane 1 - ***E. coli*** **V517 (Molecular size marker) with 8 plasmids of standard size 48.06 kb, 7.79 kb, 5.22 kb, 4.55 kb, 3.36 kb, 2.54 kb, 2.27 kb and 1.86 kb**

Lane 2 - DC1 with 6.73 kb, 5.76 kb and 4.99 kb

Lane 3 - DC2 with 6.93 kb, 5.81 kb, 4.99 kb and 3.30 kb

Lane 4 - FC1 with no plasmids

Lane 5 -6 FC2 with 7.07 kb, 5.76 kb, 5.38 kb, 2.82 kb and 2.69 kb


1.86

Discussion

DISCUSSION

5.1 Isolation

5.1.1 Sick birds

Though a good proportion of birds were showing the clinical symptoms and gross lesions suggestive of pasteurellosis, only a few birds yielded bacteria on biosmear examination. Even from birds with the bacteriemia isolation was of low rate. This results indicate the difficulties encountered in isolation and identification as a method of diagnosis of pasteurellosis in birds employing only one medium.

Several workers have isolated *P. multocida* organisms from different species of animals and birds using different kinds of artificial media. Yeast Protease Cystic agar (YPC) (Namioka and Murata, 1961), blood agar with five per cent bovine blood (Dorsey, 1963), Difco's dextrose starch agar (Heddleston *et al.*, 1964), trypticase soy blood agar, tryptose agar, tryptose blood agar (Carter, 1967) have been reported to be useful for the primary isolation of *P. multocida*.

Reduced number of isolates of *P. multocida* from biosamples may be due to the use of a single medium such as sheep blood agar for primary isolation.

5.1.2. Healthy birds

For the isolation of *P. multocida* from throat swabs of apparently healthy birds, blood agar and BHIA were not suitable, because of profuse growth of contaminants present in the nasopharyngeal regions. Hence, a new selective enrichment medium known as Pasteurella multocida sensitive agar (PMSA) (Moore *et al.*, 1994) was used to isolate the organism. The medium inhibited the growth of other organisms, because of its high pH (10 ± 0.1) and presence of antibiotics such as amphotericin-B, and gentamicin and potassium tellurite. The incubation was carried out at five per cent CO_2 tension at 37°C for 24 h. It is likely that when CO_2 is used for incubation, the very high pH of the selective medium is reduced by the acidifying influence of the carbonate ion, resulting in conditions which, overall, are most favourable to *P. multocida*.

5.2 Identification

Eventhough, blood agar containing five per cent sheep/bovine blood is one of the media recommended for the primary isolation of *Pasteurella multocida*, it is unsuitable for determining the colony morphology (Carter, 1967).

The colonies produced by the isolates on blood agar plates under aerobic condition were mucoid and non haemolytic. But under increased CO_2 tension (5%), the colonies showed a flowing

nature and this may be due to enhanced formation of capsular material.

Heddleston *et al.* (1964) reported that dextrose starch agar was a highly suitable medium for describing the colony morphology of *P. multocida* and demonstrated three morphologically different variants viz., smooth, mucoid and rough.

All the isolates of the present study also grew well on dextrose starch agar. The isolates FC1 and FC2 produced small, smooth and grey coloured colonies and the colonies of DC1 and DC2 were medium sized, mucoid and grey coloured.

The mucoid nature of the colony indicated the presence of capsule and the isolates DC1 and DC2 showed capsule on Nigrosin staining which is in agreement with the findings of Carter (1967). These isolates were obtained from the biomaterials of ducks died of suspected Pasteurellosis.

Cowan (1974) described certain primary tests for the identification of *P. multocida*, viz., catalase, oxidase, production of acid from glucose and oxidative/fermentative utilisation of glucose. The gram negative, non-motile, coccobacilli isolates obtained were catalase and oxidase positive. They grew anaerobically and fermented glucose. They were also found to be negative for growth on Mac Conkey's agar

and did not produce haemolysis on blood agar. These results were in accordance with the characters described to the species by Mannheim (1984).

The second stage biochemical tests used to characterize *P. multocida* by Cowan (1974) were also identical for all four isolates studied.

In sugar fermentation tests, all the isolates uniformly metabolised the sugars such as, dextrose, fructose, galactose, mannose, mannitol and sucrose without gas production. None of the isolates fermented lactose, maltose and raffinose. These results agree with the findings of Rimler and Rhoades (1989).

Thus, based on the screening studies such as growth on culture media, morphologic characteristics and sugar fermentation tests, all the four isolates were identified as *P. multocida*.

5.3 Biotyping

Separation of *P. multocida* isolates into subgroups or biotypes based on variations in biochemical characters has been reported (Frederikson, 1973; Oberhofer, 1981). This subgrouping is mostly based on reaction patterns observed with acid production from xylose, arabinose, maltose, trehalose, sorbitol, mannitol and dulcitol (Rimler and Rhoades, 1989).

Mutters *et al.* (1985) also suggested the use of fermentation pattern of sorbitol and dulcitol for differentiation of *P. multocida* into different subsp., the sorbitol and dulcitol positive variety became subsp. *gallicida*; negative for both constituted subsp. *septica* and positive for sorbitol but negative for dulcitol constituted subsp. *multocida*.

In this study, all the isolates fermented xylose. The isolate DC1 fermented sorbitol. Trehalose was utilised by three isolates (DC2, FC1 and FC2). Dulcitol was not fermented by the isolates. On this basis, the isolates were subtyped as *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*.

None of the strains in the present study metabolised dulcitol and none appeared to be *P. multocida* subsp. *gallicida*. This is in agreement with the observations of Dwivedi and Sodhi (1989) and Mohan *et al.* (1994).

However, Dorsey (1963) reported that most of the pasteurellae isolates from fowl cholera fermented dulcitol. Rajini *et al.* (1995) reported that the differences in the fermentation of carbohydrates might be due to geographical variation and the use of chemotherapeutic agents which will influence the enzyme profile of microbes.

5.4 Antibiogram

Chemotherapy is widely used for the treatment of fowl cholera. A variety of chemotherapeutic agents, are often effective. Because of the variation in the responsiveness of *P. multocida* to these agents, *in vitro* sensitivity testing is recommended (Rhoades and Rimler, 1989).

Antibiogram studies revealed that all the isolates were sensitive to oxytetracycline, pefloxacin, and streptomycin and resistant to furazolidone, metronidazole and nalidixic acid. Moderate sensitivity was observed to chloramphenicol, gentamicin, kanamycin and penicillin. Rammanath and Gopal (1993) also reported that *P. multocida* of avian origin were found to be sensitive to chloramphenicol, tetracycline, gentamicin and streptomycin. The sensitivity of these organisms to penicillin was also reported (Donahue and Olson, 1972). Waltman and Horne (1993) observed that *P. multocida* was highly susceptible to all antimicrobial agents tested except sulphonamides. The differences in the antibiogram pattern may be either due to their frequent use in treatment of bacterial infections either through feed and water or their use as feed additives which may result in acquired drug resistance.

5.5 Pathogenicity studies

Pathogenicity of the isolates DC1, DC2, FC1 and FC2 was tested separately in mice by I/P injection. DC1 and DC2 killed the mice within 24 h and FC1 and FC2 were lethal within 48 h.

Sambyal *et al.* (1988) and Jayakumar (1997) observed that *P. multocida* of duck origin killed the mice within 24 h and 12 h of injection respectively.

Congestion of spleen and liver was observed in the internal organs of mice after experimental injection. Bacteria could be recovered from the visceral organs and heart blood which indicated the cause of death. Collins and Woolcock (1976) observed that an overwhelming increase in the number of *P. multocida* in visceral organs associated with death in mice when experimentally injected.

All the isolates were also tested for their pathogenicity in rabbits. Each isolate was injected into two rabbits by I/N route. The symptoms exhibited by rabbits inoculated with DC1 and DC2 were general weakness, recumbency, conjunctivitis, torticollis, convulsion and respiratory distress. The gross lesions observed were typical haemorrhagic tracheitis, congestion of the lung, liver and spleen and haemorrhagic enteritis. These symptoms and lesions observed in the present

study is in conformity with the observation made by Sheela (1992).

Isolates FC1 and FC2 neither produced any clinical symptoms nor death in inoculated rabbits. DC1 and DC2 killed the rabbits on fourth and fifth day respectively. Murugkar and Ghosh (1995) observed that *P. multocida* isolates killed rabbits in 48 h. The difference in this observation may be due to the variation in virulence of the isolates.

The isolates were tested for their pathogenicity in chicks and ducks. The chicken isolates (FC1 and FC2) were nonpathogenic to chicks and ducks. But the duck isolates (DC1 and DC2) killed the chicks in 48 h of administration, indicating the acute pathogenicity in this species. The symptoms noticed were depression, ruffled feathers, mucous discharges from the nose and mouth and greenish watery diarrhoea.

Sharma *et al.* (1974) reported the prompt clinical symptoms as dullness, incoordination of movements, greenish yellow diarrhoea, laboured painful breathing and unusual sitting posture.

The gross lesions observed were petechial haemorrhages in the myocardium, liver, spleen and on serosal surface of the duodenum. Similar types of lesions were also observed by Rhoades and Rimler (1989) in natural cases of fowl cholera.

The isolates DC1 and DC2 were found to be pathogenic to ducks but the DC1 was found to be more virulent because it took less time to kill the bird as compared to DC2. The clinical symptoms were paralysis of the wings and legs, greenish diarrhoea and typical posture of breast touching on the ground. Post mortem examination of dead birds revealed petechial haemorrhages throughout the internal organs, whitish necrotic foci on the liver and spleen. These symptoms and lesions are simulating to those observed in natural cases of duck pasteurellosis and are in accordance with the findings of Karim (1987) and Das *et al.* (1991).

5.6 Plasmid profile of *P. multocida* isolates

The plasmid DNA content of each of the four isolates of *P. multocida* was analysed on agarose gel electrophoresis. The number of plasmids in three strains varied from three to five and one isolate did not show the presence of any plasmids. The size of the plasmids ranged from 2.69 kb to 7.07 kb.

Strains of *P. multocida* isolated from cases of fowl cholera have been found to harbour plasmids ranging in size from 3.03 kb to 106 kb. The plasmids were found to be resistant to antimicrobial agents. The occurrence of plasmidless strains were also reported (Hirsh *et al.*, 1981, 1985 and 1989).

It is also observed that isolates with multiple drug resistance harboured plasmids of low molecular size. However, Gunther *et al.* (1991) suggested that small plasmids found in strains of *P. multocida* were not regarded as resistance plasmids. Diallo *et al.* (1995) also observed plasmids (1.3 kb to 7.5 kb) in *P. multocida* strains.

The isolate FC1 contained no plasmids but at the same time, it was resistant to six of the 16 antimicrobial agents tested. So the correlation between presence of plasmids and antibiotic resistance could not be confirmed. Similarly Lee *et al.* (1991) reported that all the organisms tested exhibited some level of resistance to some antibiotics irrespective of the presence of plasmids and concluded that there was no correlation to the occurrence of plasmids and antibiotic resistnace.

All the isolates which were used for plasmid extraction were examined for their pathogenicity in experimental animals and birds. All were pathogenic to mice within 24 to 48 h of I/P injection. The isolate FC1 which did not possess plasmids killed all the mice within 48 h of injection.

From the present investigation, the role of plasmids in virulence could not be understood as there was no correlation between virulence and presence of plasmids and this is in accordance with the observation of Diallo *et al.* (1995) and Pande and Singh (1997).

5.7 Correlation between biotyping and plasmid profile of *P. multocida*

By biotyping, two different pattern could be obtained. *P. multocida* isolate (DC1) formed a distinct biotype. Isolates DC2, FC1 and FC2 shared a common biotype. But by a combination of biotyping and plasmid profiling, the isolates with same biotype could be differentiated. Similarly, David *et al.* (1991) reported that biotyping alone could differentiate *E. coli* strains to the tune of 50 per cent and by combining biotyping with plasmid profile, the differentiation could be achieved to the extend of 80 per cent.

5.8 Correlation between antibiogram and plasmid profile of *P. multocida*

By a combination of antibiogram and plasmid profile, all the isolates could be completely distinguished. David *et al.* (1991) and Mini (1996) have also recorded similar findings.

5.9 Correlation among biotyping, antibiogram and plasmid profile of avian strains of *P. multocida* isolates

Among the epidemiological markers employed, the antibiogram testing alone could differentiate the *P. multocida* isolates followed by plasmid profile and biotyping. But a combination of

biotyping, antibiogram and plasmid profile was able to distinguish the isolate to the extent of 100 per cent.

In the present study, it is concluded that antibiogram is found to be superior to other typing methods, since it helps to identify maximum number of types. This is in agreement with the observation of Purushothaman (1990).

Among the tests carried out in this study, biotyping and antibiogram do not require sophisticated equipments or much skill. Plasmid profile analysis is the latest technique which can be carried out without much difficulty. Hence in a situation, where there is a need to trace the source of infection or for the differentiation of strains, plasmid profile analysis in combination with biotyping and antibiogram will be helpful.

Summary

SUMMARY

The isolation of *P. multocida* was attempted from ailing/ dead chicken and ducks brought to the Department of Microbiology and Department of Pathology for diagnosis of diseases. The organisms were isolated from the biomaterials such as heart blood, lung, liver and spleen of dead/ailing birds and from throat swabs of apparently healthy birds. Gross pathological lesions observed in post mortem examinations were haemorrhages in most of the internal organs such as proventriculus, intestine, lungs and pericardium and congestion of the spleen.

The isolates obtained from the biomaterials, DC2, FC1 and FC2 were subjected to biochemical tests (first and second stage). The results were compared with those of the *P. multocida* isolate (DC1) maintained in the Department of Microbiolgy. All the isolates were gram negative coccobacilli, non motile and produced catalase and oxidase, grew aerobically and anaerobically, fermented carbohydrates and were negative for growth on Mac Conkey's agar. The isolates did not show any haemolysis on blood agar. Capsule was present in two of the isolates (DC1 and DC2) and was absent in the other two isolates (FC1 and FC2).

The results of the second stage biochemical tests of all the four isolates (DC1, DC2, FC1 and FC2) were identical and

confirmed the character described for the species. The isolates were differentiated by biotyping, antibiogram and plasmid profile analysis.

All the isolates fermented xylose uniformly and did not ferment arabinose and dulcitol. Based on their ability to ferment sorbitol and trehalose the isolates are biotyped into two subspecies namely *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*.

In antibiogram testing, four different patterns were obtained and this was found to be the best method for differentiating the strains. All the isolates were sensitive to oxytetracycline, pefloxacin and streptomycin but shown resistance to furazolidone, metronidazole and nalidixic acid.

The isolates were tested for their pathogenicity in mice, rabbits, chicks and ducks. Intraperitoneal injection of 0.1 ml of inoculum containing 3 x 10^8 organisms/ml of all the four isolates killed mice between 24 h to 48 h of injection. When rabbits were inoculated intranasally (0.2 ml of overnight culture containing 3 x 10^8 organisms/ml), the isolates DC1 and DC2 killed the animals on the fourth and fifth day respectively. The rabbits inoculated with isolates FC1 and FC2 did not develop any clinical symptoms or die during the observation period of 21 days. The organisms could be reisolated from the dead mice and rabbits.

Two of the isolates DC1 and DC2 killed all the chicks within 48 h of oral administration. DC1 and DC2 were also found to be lethal to S/C inoculated ducks within 24 h and 48 h of injection respectively and the organisms were reisolated from the tissues of dead birds. The chicks and ducks inoculated with FC1 and FC2 did not develop any clinical signs throughout the observation period of seven and fourteen days respectively.

The isolates were screened for the presence of plasmids. Three isolates, DC1, DC2 and FC2 contained plasmids varying in size from 2.69 kb to 7.07 kb. None of the plasmids were common to all the isolates. The isolate, FC2 contained maximum number of plasmids (Five).

Plasmids were seen in an isolate which was non pathogenic to rabbits, chicks and ducks. Another isolate which was resistant to six antibiotics did not contain any plasmids.

Biotyping alone could differentiate the isolates into two biogroups, while antibiogram could differentiate all the isolates. Plasmid profiling presented three different patterns. Hence, a combination of plasmid profiling and one of the typing methods either biotyping or antibiogram could make it possible to distinguish *P. multocida* isolates of avian origin.

From the present study, the following conclusions were made.

1. *Pasteurella multocida* inhabit in the respiratory tract of apparently healthy chicken.

2. Based on biotyping, the isolates could be subtyped into two viz. *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*.

3. Indiscriminate use of antibiotics should be avoided to prevent the emergence of drug resistant strains. Antibiogram is a pre-requisite for starting therapy. The drugs of choice for avian pasteurellosis are oxytetracycline, pefloxacin and streptomycin.

4. *Pasteurella multocida* of avian origin harboured zero to five plasmids and the molecular size of which ranged from 2.69 to 7.07 kb.

5. Plasmids obtained in this study need not necessarily be associated with either antibiotic resistance or virulence.

6. Plasmid profile can be used as an adjunct with other typing methods (biotyping and antibiogram) to differentiate the avian strains of *P. multocida* in epidemiological studies.

References

REFERENCES

Azad, A.K., Coote, J.G. and Darton, R. (1992). Distinct plasmid profiles of *Pasteurella haemolytica* serotypes and the characterisation and amplification in *Escherichia coli* of ampicillin resistance plasmids encoding ROB-I beta-lactamase. *J. Gen. Microbiol.* **138**: 1185-1196.

Basha, S.Y. and Palanivelu, P. (1994). A modified alkaline lysis procedure for isolation of plasmid from *Escherichia coli*. *Curr. Sci.* **66**: 335-336.

Barnes, W.M. (1977). Plasmid detection and sizing in single colony lysates. *Science* **195**: 393-394.

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

Bennet, P.M. and Howe, T.G.B. (1990). Cited "In Topley and Wilson's Principles of Bacteriology, Virology and Immunity". 8th Edn. Vol.I. Edited by Edward Arnold, London, Melbourne and Auckland. pp. 173-174.

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

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

Bhaumik, A. and Dutta, S. (1995). Studies on bacteriological parameters of *Pasteurella multocida* isolated from Khaki Campbell ducklings in Tripura. *Indian Vet. J.* 72: 1092-1093.

Birnboim, H.C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Meth Enzymol.* **100**: 243-255.

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

Blackall, P.J., Pahoff, J.L., Marks, D., Fegan, N. and Morrow, C.J. (1995). Characterisation of *Pasteurella multocida* isolated from fowl cholera outbreaks on turkey farms. *Aust. Vet. J.* 72: 135-138.

Bollinger, (1878). Cited by Carter, G.R. (1967).

Borkataki, P., Baxi, K.K., Sambyal, D.S. and Sodhi, S.S. (1987). *In vitro* sensitivity of *Pasteurella multocida* field strains to antimicrobial agents. *J. Res. PAU* Ludhiana **24**: 307-310.

Brogden, K.A. and Rebers, P.A. (1978). Serological examination of the West Phal type lipopolysaccharides of *Pasteurella multocida*. *Am. J. Vet. Res.* 39: 1680-1682.

Brown, T.A. (1990). Gene cloning. 2nd Edn. Library of Congress Catalogueing. p.16.

Brown, D. and Blowers, R. (1978). Disc methods of sensitivity testing and semiquantitative methods. "In Laboratory Methods in Antimicrobial Agents Chemotherapy". Edited by Reeves, D.S., Phillips, D.I., Williams, J.D. and Wise, N. (1978). Churechill Livingstone, London. pp.8-30.

Burrows, T.W. and Gillett, W.A. (1966). Cited by Smith, U.K. and Phillips, J.E. (1990).

Buxton, A. and Fraser, G. (1977). Animal Microbiology. Vol.1. Blackwell Scientific Publication, Oxford. pp. 121-130.

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

Carter, G.R. (1981). In the procaryotes, a handbook on habitats, isolation and identification of Bacteria. Edited by Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Sclegal, H.G., Springer-Verlag, New York. pp. 1383-1391.

Chandran, N.D.J., Prabhakar, 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.

Clewell, D.B. and Helinski, D.R. (1969). Supercoiled circular DNA-protein complexes in *Escherichia coli*: purification and induced conversion to open circular DNA form. *Proc. Natl. Acad. Sci. USA* **62**: 1159-1166.

Collins, F.M. and Woolcock, J.B. (1976). Immune responses to *Pasteurella multocida* in the mouse. *J. Reticulo endothelial Soc.* **19**: 311-321.

Cote, S., Harel, J., Higgins,R. and Jacques, M. (1991). Resistance to antimicrobial agents and prevalence of R plasmids in *Pasteurella multocida* from swine. *Am. J. Vet. Res.* **52**: 1653-1657.

Couturier, M., Bex, F., Bergquist, P.L. and Mass, W.K. (1988). Identification and classification of bacterial plasmids. *Microbiol. Rev.* **52**: 375-395.

Cowan, S.T. (1974). In Cowan and Steel's Manual for the Identification of Medical Bacteria. 2nd Ed. Cambridge University Press, Cambridge.

Curtis, P.E., Ollerhead, G.E. and Ellis, C.E. (1980). *Pasteurella multocida* infection of poultry farm rats. *Vet. Rec.* **107**: 326-327.

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.

Davaine (1880). Cited by Carter, G.R. (1967).

David, B.P., Purushothaman, V. and Venkatesan, R.A. (1991). Comparison of plasmid profile, antibiogram testing, resistotyping and biotyping in the identification of *Escherichia coli* isolates from poultry. *Vet. Rec.* **129**: 94-97.

David, B.P., Purushothaman, V. and Venkatesan, R.A. (1993). Comparison of molecular weight estimation techniques: bacterial plasmid DNA. *Indian J. Anim. Sci.* **63**: 1146-1151.

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.

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. (1971). Biochemic, serologic and immunologic properties of isolates of *Pasteurella multocida* obtained from turkeys. *Avian Dis* **15**: 905-909.

Donahue, J.M. and Olson, L.D. (1972). The *in vitro* sensitivity of *Pasteurella multocida* of turkey origin to various chemotherapeutic agents. *Avian Dis* **16**: 506-511.

Dorsey, T.A. (1963). Studies on fowl cholera. 1. A biochemic study of avian *Pasteurella multocida* strains. *Avian Dis* **7**: 386-392.

Droual, R., Walker, R.L.,Shivaprasad, H.L., Jeffrey, J.S., Meteyer, C.U., Chin, R.P. and Shapiro, D.P. (1992). An atypical strain of *Pasteurella gallinarum*: pathogenic, phenotypic and genotypic characteristics. *Avian Dis.* **36**: 693-399.

Dwivedi, P.N. and Sodhi, S.S. (1989). Morphological, cultural and biochemical characterisation of *Pasteurella multocida*. III. isolated from poultry. *Poult. Advisor* **21**: 69-71.

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

Flugge (1896). Cited by Carter, G.R. (1967).

Frederiksen, W. (1973). Cited by Rimler, R.B. and Rhoades, K.R. (1989).

Freifelder, D. (1968). Cited by Humphreys, G.O., Willshaw, G.A. and Anderson, E.S. (1975). A simple method for the preparation of large quantities of pure plasmid DNA. *Biochem. Biphys. Aeta.* **383**: 457-463.

Freifelder, D. (1970). Isolation of extrachromosomal DNA from bacteria. "In Methods in Enzymology" Vol.XXI. Edited by Grossman, L. and Moldare, K. Academic Press Inc., New York. pp. 153-163.

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

Gaffky, (1881). Cited by Carter, G.R. (1967).

Genthner, F.J., Hook, L.A. and Strohl, W.R. (1985). Determination of the molecular mass of bacterial genomic DNA and plasmid copy number by high pressure liquid chromotography. *Appl. Environ. Microbiol.* **50**: 1007-1013.

Glisson, J.R., Cheng, I.H.N., Rowland, G.N. and Stewart, R.G. (1989). *Pasteurella multocida* infection in Japanese quail (*Coturnix coturnix japonica*). *Avian Dis.* **32**: 820-822.

Gomez-Marquez, J., Freire, M. and Segade, F. (1987). A simple procedure for large scale purification of plasmid DNA. *Gene.* **54**: 255-259.

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

Gunther, R., Manning, P.J., Bouma, J.E., DeLong, D. and Cook, D.B. (1991). Partial characterisation of plasmids from rabbit isolates of *Pasteurella multocida*. *Lab. Anim. Sci.* **41**: 423-426.

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

Heddleston, K.L., Watko, L.P. and Rebers, P.A. (1964). Dissociation of fowl cholera strain of *Pasteurella multocida*. *Avian Dis.* **8**: 649-657.

Heddleston, K.L., Rebers, P.A. and Klessman, G. (1975). Fowl choleria: Immunogenic and serologic response in turkeys to live *Pasteurella multocida* vaccine administered in the drinking water. *Poult. Sci.* **54**: 217-221.

Helinski, D.R. and Clewell, D.B. (1971). Cited by Humphreys, G.O., Hillshaw, G.A. and Anderson, E.S. (1975). A simple method for the preparation of large quantities of pure plasmid DNA. *Biochem. biophys. Acta.* **383**: 457-463.

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

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

Hirsh, D.C., Hansen, L.M., Dorfman, L.C., Snipes, K.P., Carpenter, T.E., Hird, D.W. and McCapes, R.H. (1989). Resistance to antimicrobial agents and prevalence of R-plasmids in *Pasteurella multocida* from turkeys. *Antimicrob. Agents Chemother.* **20**: 415-417.

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

Hoie, S., Nordby, K. and Falk, K. (1990). Plasmid profiles of selected strains of *Pasteurella multocida* from pneumonic lesions of slaughter weight swine. Proceedings, International Pig Veterinary Society, 11th Congress, July 1-5, 1990. Lausanne, Switzerland. 1990, 104.

Holmes, D.S. and Quigley, M.A. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**: 193-197.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Stanley, J.F. and Williams, S.T. (1994). "*In* Bergey's Manual of Determinative Bacteriology", 9th Edn. Edited by Williams and Wilkins, Baltimore.

Ikeda, T.S. and Hirsh, D.C. (1990). Possession of identical non conjugative plasmids by different isolates of *Pasteurella multocida* does not imply clonality. *Vet. Microbiol.* **22**: 79-87.

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

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

Karim, R. (1987). An outbreak of clinical pasteurellosis in ducks and its control. *Indian Vet. J.* **64**: 723-724.

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

Kitt, (1885). Cited by Carter, G.R. (1967).

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.

Kulkarni, D.D., Karpe, A.G., Bannalikar, A.S. and Kulkarni, G.B. (1988). Report of fowl cholera in six days old chicks. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 9: 237-238.

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., Wooley, K.E., Brown, J. and Glisson, J.R. (1991). A survey of potential virulence markers from avian strains of *Pasteurella multocida*. *Vet. Microbiol.* 26: 213-225.

Lederberg, J. (1952). Cell genetics and hereditary symbiosis. *Physiol. Rev.* 32: 403-430.

Liqnieres, J. (1900). Cited by Hussaini, S.N. (1975). A review on nomenclature and taxonomy of *Pasteurella multocida*. *Vet. Bullet.* 45: 130-132.

Loeffler and Schutz (1882). Cited by Carter, G.R. (1967).

Mac Neil, D.J. (1986). A flexible boiling procedure for isolating plasmid DNA from gram positive organisms. *J. Microbiol. Meth.* 5: 115-123.

Macrina, F.L., Kopecko, D.J., Jones, K.R., Ayers, D.J. and Mc Cowan, S.M. (1978). A multiple plasmid containing *Escherichia coli* strain. Convenient source of size reference plasmid molecules. *Plasmid* 1: 417-420.

Madhusudan, A.R. (1967). Cited by Panda *et al.* (1981).

Maniatis, T., Fritsch, E.F, and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York.

Mannheim, W. (1984). Pasteurellaceae. "In Bergey's Manual of Systematic Bacteriology". Vol.1. Edited by Krieg, N.R. and Holt, J.G., Williams and Wilkins. 1st ed. pp. 559-569.

Mannheim, W. and Carter, G.R. (1984). Cited "In Bergey's Manual of Systematic Bacteriology". Vol.1. Edited by Krieg, N.R. and Holt, J.G. Williams and Wilkins Co., Baltimore London. pp.550-558.

Matsumoto, M. and Strain, J.G. (1993). Pathogenicity of *Pasteurella multocida* : Its variable nature demonstrated by *in vivo* passages. *Avian Dis.* 37: 781-785.

Mendosa-Elvira, S., Ciprian-Carrasco, A., ColmenaresViladomat, G., Alvarez-de-la-Cuadra, J., Camacho-Machin, J. and De-la-Cuadra, J.A. (1990). Dermonecrotoxin of *Pasteurella multocida* encoded in plasmid. *Veterinaria Mexico* 21: 265-268.

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.

Mini, M. (1996). Studies on *Escherichia coli* of bovine mastitis origin. Ph.D. thesis submitted to TANUVAS, Madras.

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

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

Moore, M.K., Cicnjak-Chubbs, L. 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 characterisation of isolates. *Avian Dis.* **40**: 900-907.

Morishita, T.Y., Lowenstine, L.J., Hirsh, D.C. and Brooks, D.L. (1996b). *Pasteurella multocida* in raptors: Prevalence and characterisation. *Avian Dis.* **40**: 908-918.

Mulbagal, A.N., Kulkarni, V.B. and Paranjape, V.L. (1972). Some observations on pasteurellosis in ducks. *Indian Vet. J.* **49**: 544-546.

Murata, M., Moriuchi, T. and Namioka, S. (1964). Studies on the pathogenicity of *Pasteurella multocida* for mice and chicken on the basis of O group. *Cornell Vet.* **54**: 293-307.

Murugkar, H.V. and Ghosh, S.S. (1995). *Pasteurella multocida* serotype A:1 in ducks in Tripura. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **16**: 109-111.

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

Namioka, S. and Murata, M. (1961). Serological studies on *Pasteurella multocida* I. A simplified method for capsule typing of the organism. *Cornell Vet.* 51: 498-507.

Neter, E. and Dryja, D.M. (1981). Use of penicillin and nafcillin discs as an aid to identification of *Pasteurella multocida*. *J. Med.* 12: 285-287.

Oberhofer, T.R. (1981). Cited by Rimler, R.B. and Rhoades, K.R. (1989).

*Ochiai, K., Yamanaka, T., Kimura, K. and Sawada, D. (1959). Inheritance of drug resistance (and its transfer) between Shigella strains and between Shigella and *E. coli* strains. *Nihon Iji Shimpo*, 1961, 34.

Okerman, I., Spanoghe, L. and De Bruycker, R.M. (1979). Experimental infections of mice with *Pasteurella multocida* strains isolated from rabbits. *J. Comp. Path.* 89: 51-53.

Orgam, A., Sayler, G.S. and Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *J. Microbiol. Meth.* 7: 57-66.

Pande A. and Singh, V.P. (1997). Role of plasmids in virulence in *Pasteurella multocida*. *Indian Vet. J.* **74**: 893-894.

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).

Perroncito (1878). Cited by Carter, G.R. (1967).

Pitt, T.L. (1994). Bacterial typing systems. The way ahead. *J. Med. Microbiol.* **40**: 1-2.

Plummer, D.T. (1988). An introduction to practical biochemistry. 3rd. Edn. Tata Mc Graw Hill Publishing Co. Ltd. New Delhi. pp. 314.

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

Purushothaman, V. (1990). Studies on salmonellae of poultry and related environment with special reference to their plasmids. Ph.D. thesis submitted to TANUVAS, Madras.

Rajini, R., Rao, A.S., Dhanalakshmi, K., Sarma, B.J.K. (1995). Studies on avian pasteurellosis in Andhra Pradesh. *Indian Vet. J.* **72**: 115-118.

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

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

Ratafia, M. (1988). Cited by Rhoades, K.R. and Rimler, R.B. (1989).

Rhoades, K.R. and Rimler, R.B. (1987). Capsular groups of *Pasteurella multocida* isolated from avian hosts. *Avian Dis.* **31**: 895-898.

Rhoades, K.R. and Rimler, R.B. (1989). Fowl cholera. "In Pasteurella and Pasteurellosis". Edited by Adlam, C. and Rutter, J.M., Academic Press, pp.95.

Rimler, R.B. and Rhoades, K.R. (1989). *Pasteurella multocida* "In Pasteurella and Pasteurellosis" Edited by Adlam, C. and Rutter, J.M. Academic Press, pp. 52.

Rochelle, P.A., Fry, J.C., Day, M.J. and Bale, M.J. (1985). An accurate method for estimating sizes of small and large plasmids and DNA fragments by gel electrophoresis. *J. Gen. Microbiol.* **132**: 52-59.

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

Sakurai, K., Kurihara, T., Matsuoka, T., Iijima, Y., Watanabe, F., Koeda, T. and Sawada, T. (1986). An outbreak of fowl cholera in green pheasants (*Phasianus colchicus*) in Japan. *Jpn. J. Vet. Sci.* **48**: 711-717.

Sambrook, J., Fritsch, E.T. and Maniatis, T. (1989). Molecular cloning. A Laboratory Manual. 2nd Edn.Cold Spring Harbor Laboratory, New York.

Sambyal, D.S., Soni, G.D., Sodhi, S.S. and Baxi, K.K. (1988). Characterisation of *Pasteurella multocida* (Serotype 1) from an outbreak of fowl cholera in ducks. *Indian J. Anim. Sci.* **58**: 1059-1060.

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

Schneider, L. (1948). Cited by Rimler and Rhoades (1989).

Sharma, R.N., Bhalla, N.P. and Lall, J.M. (1974). Observations on experimental infection of *Pasteurella septica* (avian) in domestic fowl. *Indian J. Anim. Health.* **113**: 105-109.

Sharma, K.N., Mehrotra, P.K. and Khanna, V.K. (1979). A note on characterisation and antibiotics sensitivity of Pasteurellae. *Indian J. Anim. Sci.* **49**: 142-145.

Sheela, Y. (1992). Characterisation of *Pasteurella multocida* isolates from rabbits. M.V.Sc thesis submitted to Kerala Agricultural University, Thrissur.

Sikka, V.K. (1992). In gel lysis analysis of plasmid profile and bacteria by submarine agarose gel electrophoresis. "In Proc. of Summer Inst. on Biotechnological Approaches to Diagnosis and Vaccine Development for Infectious Dis. of Livestock". Department of Vet. Microbiol. CCS, Hissar, June, 1-20.

Skeeles, J.K., Swafford, W.S., Wages, D.P., Hellwig, H.M., Slavik, M.F., Beasley, J.N., Houghten, G.E., Blore, P.J. and Crawford, D. (1983). Studies on the use of a long-acting oxytetracycline in turkeys: Efficacy against experimental infections with *Alcaligenes faecalis* and *Pasteurella multocida*. *Avian Dis.* 27: 1126-1130.

Smith, J.E. (1955). Cited by Smith, G.K. and Phillips, J.E. (1990).

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

Snipes, K.P., Hirsh, D.C., Kasten, K.W., Hansen, L.M., Hird, D.W., Carpenter, T.E. and Mc Capes, K.H. (1989). Use of an rRNA probe and restriction endonuclease analysis to fingerprint *Pasteurella multocida* isolated from turkeys and wildlife. *J. Clin. Microbiol.* 27: 1847-1853.

Stanisich, V.A. (1988). "In Methods in Microbiology" Vol.12. Edited by Grinsted, J. and Bennet, P.M. (1988). Academic Press. Quoted "In Topley and Wilson's Principles of Bacteriology, Virology and Immunity". 8th Edn. Vol.I. Edward Arnold London, Melbourne, Auckland. p. 172.

Sullivan, D.J.O. and Klaenhammer, T.R. (1993). Rapid mini preparation isolation of high quality plasmid DNA from Lactococcus and Lactobacillus species. *Appl. Environ. Microbiol.* 59: 2730-2733.

Targowski, S. and Fargowski, H. (1979). Carbondioxide requirement for the growth of a Pasteurella strain isolated from rabbits. *J. Clin. Microbiol.* **10**: 388-389.

Topley, W.W.C. and Wilson, G.S. (1936). Cited by Carter, G.R. (1967).

Trevisan (1887). Cited by Mannheim, H. (1984).

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

Williams, E.S., Runde, D.E., Mills, K. and Holler, L.D. (1987). Avian cholera in a gyrfalcon (*Falco rusticolus*). *Avian Dis.* **31**: 380-382.

Willshaw, G.A., Smith, H.R. and Anderson, E.S. (1979). Application of agarose gel electrophoresis to the characterisation of plasmid DNA in drug resistant enterobacteria. *J. Gen. Microbiol.* **114**: 15-25.

Windingstad, R.M., Duncan, R.M. and Thornburg, D. (1983). Outbreak of avian cholera on the wintering grounds of the Mississippi Valley Canada Goose flock. *J. Wildl. Dis.* **19**: 95-97.

Yadav, M.P., Sharma, V.D. and Sethi, M.S. (1977). An outbreak of fowl cholera due to *Pasteurella gallinarum* in Uttar Pradesh (India). *Avian Dis.* **21**: 323-325.

Yamamoto, J., Sakano, T. and Shimizu, M. (1990). Drug resistance and R-plasmids in *Pasteurella multocida* isolates from swine. *Microbiol. Immunol.* **34**: 715-721.

PLASMID PROFILE OF AVIAN STRAINS OF *Pasteurella multocida*

By
G. BALAKRISHNAN

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

1998

ABSTRACT

Four isolates of *P. multocida* (DC1, DC2, FC1 and FC2) from avian species (chicken and ducks) were subjected to various typing methods like biotyping, antibiogram and plasmid profiling. The pathogenicity of the isolates was also ascertained in mice, rabbits chicken and ducks.

By biotyping, the isolates were found to belong to two subspecies *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. All the isolates fermented xylose and none of them fermented dulcitol.

Antibiogram of the isolates was carried out and they were found to be resistant to furazolidone, metronidazole and nalidixic acid. Some of the isolates are also resistant to sulpha and trimethoprim.

All the isolates were pathogenic to mice on I/P injection. Duck isolates (DC1 and DC2) were found to be pathogenic to rabbits killing them on fourth and fifth day respectively.

The isolates DC1 and DC2 were lethal to orally infected chicks within 48 h of administration and *P. multocida* could be reisolated from these birds. The same isolates killed the S/C injected ducks within 24 and 48 h.

The chicken isolates obtained from apparently healthy birds were non pathogenic to rabbits, chicks and ducks.

Plasmid profiling revealed the presence of plasmids in three of the four isolates screened. One isolate was plasmid less and another isolate contained five plasmids. The molecular size of the plasmids ranged from 2.69 kb to 7.07 kb. One isolate did not contain plasmids at all. An isolate (FC2) which was not pathogenic to rabbits, chicks and ducks contained plasmids. Isolate FC1 which was resistant to six antibiotics did not possess plasmids. Hence plasmids obtained in this study may not be associated with either antibiotic resistance or pathogenicity in experimental animals.

A combination of two parameters such as plasmid profile and one of the typing methods, biotyping or antibiogram made it possible to differentiate the isolates. Thus, the plasmid profile analysis with any one of the typing methods could be used as an epidemiological tool in the differentiation of *P. multocida* strains of avian origin.