ISOLATION AND CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* FROM CATTLE IN PLATEAU STATE, NIGERIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN VETERINARY MICROBIOLOGY

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June, 2012

DECLARATION

I declare that the work reported in this dissertation was carried out by me in the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria, under the supervision of Prof. J.K.P. Kwaga, Prof. H.M. Kazeem, and Prof. N.D.G. Ibrahim. All information derived from the literature have been duly acknowledged in the text and a list of references provided. I declare that no part of this dissertation has been submitted elsewhere for a degree or diploma in any university.

Manasa Yohana SUGUN _____ _____

Signature **Date**

CERTIFICATION

This dissertation titled "**ISOLATION AND CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* FROM CATTLE IN PLATEAU STATE, NIGERIA"** by Manasa Yohana SUGUN meets the regulations governing the award of the degree of Doctor of Philosophy (VETERINARY MICROBIOLOGY) of the Ahmadu Bello University, Zaria, and is approved for the scholarly contribution to knowledge and literary presentation.

Prof. J.K.P. Kwaga, Chairman, Supervisory Committee	_____ Signature	_____ Date
Prof. H.M. Kazeem, Member, Supervisory Committee	_____ Signature	_____ Date
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Prof A. A. Joshua Dean, School of Postgraduate Studies Ahmadu Bello University, Zaria.	_____ Signature	_____ Date

DEDICATION

To my entire family.

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ABSTRACT

Pasteurella multocida is associated with haemorrhagic septicaemia in cattle and buffaloes, pneumonic pasteurellosis in sheep and goats, fowl cholera in poultry, atrophic rhinitis in pigs and snuffles in rabbits. Haemorrhagic septicaemia is caused by *P. multocida type* B: 2, B: 2, 5 and B: 5 in Asian countries and type E: 2 in African countries. The present research work was undertaken with a view to isolate and characterize *P. multocida* from cattle in Plateau state, Nigeria. This contemporary study was necessary as available information indicates that such similar studies were conducted about 3 decades ago. A total of 396 samples of tissues and nasal swabs from cattle were examined for the presence of *P. multocida*. The isolates were studied for their species using PM-PCR, different serotypes, the presence of plasmids and *in vitro* antibiotic sensitivity. Of the 396 samples tested 4.5 % were non-haemolytic, round, greyish, smooth and mucoid colonies on casein sucrose yeast agar (CSY) and blood agar, failed to grow on MacConkey agar, and produced no gas in glucose and were lactose negative. They were found to be coccobacillus, Gram negative and non-motile. All the isolates produced oxidase, catalase, were indole positive and reduced nitrate, but did not utilize simmons citrate. They fermented glucose, sucrose, mannitol and mannose but not maltose, arabinose, lactose, dulcitol, salicin, inositol and trehalose. The eighteen isolates were confirmed by Microbact GNB 24E and the supplied software version Microbact ^{TM} 200 identification package V2.03 (Windows ^{TM}). By the software interpretation package, the percentage probabilities of 12 isolates were above 75% and 6 others were below 75%. Two of the isolates were detected to have somatic antigen 3,4 and one isolate 2,5 while in the remaining fifteen, somatic antigens were not detected. There were no significance differences between sexes and age by fisher's exact test P >

0.05. In the distribution of *P. multocida* according to organs, lung, liver and spleen distribution were found to be significant by chi square P<0.05. Among the 18 isolates studied, (55.6%) were sensitive to sulphamethoxazole/trimethoprim, (44.4%) sensitive to gentamicin, amoxycillin/clavulanic and penicillin respectively. The % susceptibilities to other agents were: ciprofloxacin (38.9%), chloramphenicol (11.1%), oxacillin and vancomycin (5.6%) each and ampicillin (1.1%). All the isolates (100%) were resistant to tetracycline and erythromycin. Molecular characterisation of the isolates was carried out.The genotypic studies carried out included species-specific PCR assay, capsular grouping using multiplex capsular PCR typing system, determination of capsular groups A and B by PCR and determination of the carriage of plasmids by the isolates. All isolates produced *P. multocida* species-specific amplicons. Seventeen (94.4 %) of the isolates were identified as capsular group E and one (5.5 %) as capsular group F by multiplex PCR. The profiles of plasmids of each isolate were estimated by agarose gel electrophoresis. All the isolates harboured plasmids of 5kb. Three of the group E isolates had additional plasmid of 3kb, and one isolate (ka3) had a plasmid of 6kb; but none of the isolates carried all 3 plasmids. None of the eighteen isolates studied had capsular group A or B. The study confirmed the presence of the African capsular strain (E), but of greater interest is capsular group F that was identified for the first time in calves in Nigeria. *P. multocida* E: 3, 4 and *P. multocida* E: 2, 5 were identified amongst the isolates. These could redefine the vaccine strategy as the current vaccine used in Nigeria contain *P multocida* B: 3,4 and E: 2. However more work needs to be carried out in other parts of the country to gather more relevant information with regards to capsular and somatic types.

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ABBREVIATIONS

BHI	Brain heart infusion
cfu	Colony forming units
DNA	Deoxyribonuclic acid
RNA	Ribonucleic acid
IRP	Iron regulated proteins
KDa	kilodalton
bp	base pairs
ml	Milliliter
CLSI	Clinical Laboratory Standard Institute
OMP	Outer membrane protein
PCR	Polymerase chain reaction
USA	United States of America
CSY	Casein sucrose yeast agar
PM	*Pasteurella multocida*
REA	Restriction endonuclease analysis
REP	Repetitive Extragenic Palindromic
ERIC	Enterobacterial repetitive insertion consensus
FAGE	Field alternation gel electrophoresis
OIE	Office International des Epizooties
Rrna	Ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate-polycrylamide gel electrophoresis
HS	Haemorrhagic septicaemia
FAO	Food Agricultural Organization

WHO	World Health Organization
USSR	Union of the Soviet Socialist Republics
URT	Upper respiratory tract
BRD	Bovine respiratory disease
LD_{50}	Lethal dose
AGPT	Agar gel precipitation test
UK	United kindom
PFGE	Pulsed-field gel electrophoresis
RAPD	Random-amplified polymorphic DNA analysis
MLST	Multilocus sequence typing
WCP	Whole-cell–protein
IROMPs	Iron regulated outer membrane proteins
MCA	MacConkey agar
BA	Blood agar
TDA	Tryptophan deaminase
VP	Voges-Proskauer reaction
ul	Microliter
µg	Microgram
dNTP	deoxyribonucleotide triphosphate
$MgCl_2$	Magnesium chloride
AGID	Agar gel immunodiffusion
USDA	United States Department of Agriculture

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND INFORMATION

Nigeria currently has a livestock population of about 16.3 million cattle, 40.8 million goats, and 27 million sheep. Livestock and poultry production are the main economic activities of about 70 per cent of nigerians living in the rural and urban areas (Lombin, 2007). These animals suffer from large number of infectious and non-infectious diseases. Amongst them is Haemorrhagic Septicaemia (HS) of cattle and buffaloes caused by *Pasteurella multocida*. This organism, which is pathogenic for various animals and fowls, can be divided into 16 somatic serotypes. Certain serotypes are host specific while others are not. Consequently, different serotypes show different pathogenicity when tested in various hosts. The general and biochemical properties of the various strains are very similar, and from this point of view these organisms all belong to the single species, *P. multocida*. The organisms can be divided into two pathogenic groups; those causing a haemorrhagic septicaemia and those causing non-haemorrhagic septicaemia. *P. multocida* and *P. haemolytica* share several biochemical properties in common, but they differ with respect to indole production and haemolysis and there is no antigenic relationship between the two species (Namioka, 1978). Haemorrhagic septicaemia is prevalent in domestic cattle in Nigeria during the rainy season, especially in the marsh-land and swampy places (Kasali, 1972; Anosa and Isoun, 1975). It is an acute septicaemic disease principally affecting cattle and buffaloes which has emerged as a disease of great economic importance in South East Asia after the virtual eradication of rinderpest from that region. Besides this, pasteurellosis also causes great economic losses in sheep, goats, pigs, dogs, poultry, quails, ducks and sometimes in wild

animals such as tiger, lion, leopard, panther, etc (De Alwis, 1996). Over a century has passed since the first attempt by Louis Pasteur at immunisation against infection with gram negative facultative bacterium *Pasteurella multocida*, the organism which bears his name. During the past century, considerable research into the mechanism of immunity, host predilection, virulence and pathogenesis of *P. multocida* has resulted in only very small increase in our understanding of the organism. Untill recently there had been no extensive characterisation of this organism at the molecular level. The lack of genetic tools for use in *P. multocida* hindered investigation at a time when great inroads were being made into understanding the molecular basis of pathogenesis in many other bacterial pathogens. *Pasteurella multocida* is a small, non-motile, Gram negative coccobacillary rod, inhabiting the nasopharynx and gastro-intestinal tract of many wild and domestic animals and produces disease when the animals are under stress (De Alwis, 1996). *Pasteurella multocida* causes a number of diseases in various domestic and wild animals. The most important diseases are HS, septicaemic pasteurellosis (affecting sheep and goats), pneumonia, atrophic rhinitis, septicaemia (in pigs) and fowl cholera or avian cholera in chickin/turkey resulting in heavy economic losses. The organism is also known to be the causative agent of snuffles in rabbits and pasteurellosis in American bison, yak, deer, elephants, camels, horses, elk and other wild animals (De Alwis, 1996). The disease HS is characterised by a rapid course, fever, oedematous swelling in the head-throat-brisket region, swollen and haemorrhagic lymph nodes and presence of numerous subserous petechial haemorrhages (Carter and De Alwis, 1989). A conclusive diagnosis of the disease is based on history, clinical signs, necropsy findings and isolation of *P. multocida*. Blood smears and impression smears of the organs stained by Leishman's stain can be used for presumptive diagnosis by demonstration of bipolar organisms. *P. multocida* can be isolated

from clinical materials like heart, blood, liver, spleen, bone marrow or lungs immediately after death of the animals suspected of pasteurellosis. In living animals, blood or mucus from the nostrils or swabbing from the nasal cleft can be used for culturing. *P. multocida* grows best at temperature 35 ^{o}C to 37 ^{o}C. Growth on nutrient agar is supported by addition of five per cent bovine, horse or sheep blood. Colonies of *P. multocida* on blood agar are non-haemolytic, round, grayish in colour and can grow to about two mm in 48 h. The organism can be identified by classical microbiological tests particularly biochemical tests. The bacterial organisms over a period of time change their antibiogram patterns and develop resistance against commonly used chemotherapeutic agents. Thus for effective treatment, the *in vitro* susceptibility of bacterial organisms to different antibiotics must be carried out prior to the treatment (Okewole and Olubunmi, 2008). However, accurate and early diagnosis is the most effective tool to frame the strategy for control of HS. Conventional diagnostic system is not effective since it is time consuming and less sensitive as compared with the latest molecular techniques, such as Townsend *et al.* (1998) developed PCR based diagnostic method which is found very effective and sensitive. An improved vaccine such as DNA, oil adjuvanted and subunit vaccines is required for control of HS as most of the diseases resulting from these species are long standing problems that have not been adequately controlled (Anosa and Isoun, 1975). Their future control will depend on intense research into the epidemiology and basic biology of these pathogens and how they induce disease. There is dearth of information on the extent of HS in Nigeria.

1.2 STATEMENT OF RESEARCH PROBLEM

Available literature on HS in Nigeria is generally limited to the 60s and 70s (Perreau, 1961; Anosa and Isoun, 1975). This is a major challenge and there is need to expand the scope of information on the disease in Nigeria.

Haemorrhagic septicaemia caused by *P. multocida* is characterized by rapid course, making treatment in tropical animal production system impracticable (Bain *et al*., 1982). Economic losses occur from death of animals, weight loss, treatment cost, extra labour, and short fall in the supply of protein. The genotypic characterization of *P. multocida* from cattle has not been done in Plateau State, Nigeria. Therefore there is need to determine characteristics of *P. multocida* isolated from cattle in Plateau state.

Despite the application of advanced investigation and diagnostic techniques on both the organism and the affected animal species, diseases caused by *P. multocida* have continued to contribute to heavy losses in animal production as well pose a hazardous threat to human health worldwide (Patric *et al*., 2000; Ray, 2006). Haemorrhagic septicaemia has been classified under list B diseases by OIE, (2002) on the basis of distribution, zoonosis, the endemic nature and the great economic importance associated with the disease.

1.3 JUSTIFICATION

Haemorrhagic septicaemia is an acute disease principally affecting cattle and caused by 2 serogroups of *P. multocida;* B: 2 and E: 2. The disease is characterized by rapid course, making treatment impracticable (Bain *et al*., 1982). Economic losses occur from death of animals, weight loss, treatment cost, extra labour, short fall in the supply of protein, and unthriftness. An improvd vaccine is required for control of HS as most of the diseases resulting from this species are long standing problems that have not been adequately

controlled (Anosa and Isoun, 1975). Future control will depend on intense research into the epidemiology and basic biology of these pathogens and how they induce disease. There is a dearth of information on the prevalence of *P multocida* in cattle in Nigeria. Available literature on HS in Nigeria is generally limited to the 60s and 70s (Perreau, 1961; Anosa and Isoun, 1975). This is a major challenge and there is therefore the need to ascertain the current status of *P. multocida* in Nigeria, which may help in selection of strains for development of candidate vaccines.

1.4 RESEARCH QUESTIONS

1. Does *P. multocida,* the causative agent of haemorrhagic septicaemia occur in Plateau state?
2. Do *P. multocida* isolated from cattle in Plateau state harbor Plasmid DNA?
3. Are *P. multocida* isolated from Plateau state susceptible to commonly used antibacterial agents used in the environments?

1.5 AIM

This study was aimed at determining the phenotypic and genotypic profiles of *P. multocida* isolated from cattle in Plateau State, Nigeria.

1.6 SPECIFIC OBJECTIVES

1. To isolate, characterize and confirm, by Microbact 24E identification system, *P. multocida* from cattle in Plateau State
2. To carry out the antimicrobial susceptibility of *P. multocida* isolates
3. To identify *Pasteurella multocida* isolates using species-specific PCR primers

4. To determine the somatic serotypes of *Pasteurella multocida*
5. To carry out capsular grouping using multiplex PCR typing system
6. To determine the occurrence of plasmids among the isolates

CHAPTER 2

LITERATURE REVIEW

2.1 OCCURRENCE AND DISTRIBUTION OF HAEMORRHAGIC SEPTICAEMIA

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (Singh *et al.,* 1996). The disease has been recorded in wild mammals in several Asian and European countries (Carigan *et al*., 1991). In many Asian countries, disease outbreaks mostly occur during the climatic conditions typical of monsoon (high humidity and high temperatures), and also high rainfall. The disease is caused by *P. multocida*, a Gram-negative coccobacillus residing mostly as a commensal in the upper respiratory tract of animals. *Pasteurella multocida* serotype B:2 (6B) and serotype E:2 (6E) are the principal causes of Haemorrhagic septicaemiaa. Although serotype B2 has been mainly reported in Asian countries and B2 in African countries, both serotypes have been recovered from the disease in some African countries (Martrenchar and Njanpop, 1994, Carter and De Alwis, 1989, De Alwis, 1995). In wild animals, serotype B: 2, 5 are predominantly present. The association of other serotypes, namely A: 1, A: 3 with a HS-like conditions in cattle and buffaloes in India has been recorded (Kumar *et al*., 1996). HS has been erroneously and widely used as a synonym for shipping fever and other infections. The result has been that the disease has been mistakenly reported in South America and elsewhere. There was a similar confusion in the 1940s and the differences between the disease have been clarified (Carter, 1982). HS and shipping fever are two separate conditions caused by different bacteria (*Pasteurella multocida vs Mannheimia haemolytica*) (Carter and De Alwis, 1989). Unlike HS, shiping fever is not septicaemic nor does it cause multisystemic petechial haemorrhages (Carter, 1982). The presence of healthy animals

carrying the agent of HS has been documented in endemic areas of Asia, Africa and several middle Eastern countries (Carter and De Alwis, 1989). HS has been documented in wild and restricted-range bison (Heddleston *et al*., 1967), dairy cattle (Kradel *et al.,* 1969), beef calves (Blanchard *et al.*, 1993), and free-ranging elk (Franson and Smith, 1988; Roffe *et al*; 1993; Wilson *et al*., 1995). Expression of HS or any other disease is dependent on the interactions among the three components of the epidemiological host, agent and environment. Anything that affects a host's susceptibility to an infection, modifies an agent's pathogenicity, or alters environmental conditions that affect microbial survival, replication or transmission, will affect the course of the disease. The susceptibility to HS infection varies by host species. The B:2 serotype (Asian serotype) has been reported among pigs (*Sus* spp.), buffalo (*Bubalus* spp.), and elephants (*Elephas maximus*) in Sri Lanka and among healthy beef calves in California (Carter, 1982). The E:2 serotype (African serotype) has been reported among cattle in Africa. However, the B:2 and E:2 serotypes are non-pathogenic to dogs, chickens and ducks (Bain *et al*., 1982). One experiment in Sri Lanka showed that goats placed in close contact with buffalo clinically affected with B:2 serotype remained clinically normal and developed no carrier state or immune response (Wijewardana *et al*., 1986). The host's susceptibility to HS infection also varies with age, with greater susceptibility among young animals. HS is more likely to be fatal in younger than in older animals because of an absence of acquired immunity or because of poor physical condition (De Alwis and Vipulasiri, 1980). One extensive survey in Sri Lanka showed that 65% of all HS deaths among cattle and 77% of all HS deaths among buffalo were in animals less than two years of age (De Alwis and Vipulasiri, 1980). Another analysis of one outbreak showed the most susceptible age group to be six months to two years (De Alwis *et al*., 1976). The ability of haemorrhagic septicaemia-causing

Pasteurella multocida (HSPm) to induce disease depends on its virulence and pathogenicity. HSPm is highly virulent but can vary in its pathogenicity. For example, in one study, a highly virulent strain of HSPm, obtained from a bison that died in 1922, was injected into a 12-month-old heifer and the animal succumbed within 18 hours. But in this same study, one normal cow and one yearling were inoculated with comparatively large amounts of the same culture. Neither of these two animals reacted sufficiently to even show a rise in body temperature. Researchers were unable to determine why these two animals were immune to the disease caused by the organism presented this way (Gochenour, 1924). Environmental conditions such as geographic location, climate and husbandry practices can favour HSPm survival. Favourable environments can include husbandry practices that can stress the host and compromise its immune system. Webster (1924, cited in Rosen, 1981) showed that when physiological stresses, such as poor forage conditions and crowding on a winter range are applied to a herd, the host's resistance is lowered, the bacteria's virulence may increase, and clinical disease ensues. External environmental conditions that enable the organism to survive longer outside the host, and thereby facilitate transmission between animals also favour disease expression. HS has been usually associated with wet, humid weather, and an increased incidence has been recorded during wet seasons (De Alwis, 1992). The increased incidence has been attributed to the longer survival of the organism in wet conditions and waterways serving as a source of dissemination from carcasses and other infective material (De Alwis, 1989). The manner in which all three components of the epidemiological triad involving a susceptible host, a capable agent, and suitable environment-interact in expression of HS and the salient features of the cycle have best been described by De Alwis (1992). Haemorrhagic septicaemia occurs in Southern and Southeast Asia including Indonesia, Philippines, Thailand and Malaysia, in the Near and

Middle East, Southern Europe (including the U S S R.) and in North, Central, East and South Africa. (De Alwis, 1992). In the USA the disease was reported among bison in Natioal Parks in the years 1912, 1922 and 1967. Since then, apart from one explosive outbreak among young dairy cattle in New Jersey in 1969, the disease was not reported anywhere in the USA (Carter, 1982). The disease does not occur in Australia, Oceania, Japan, Canada and Western Europe. There was a reference to its occurrence in South America in the FAO-WHO-OIE, (1994), but no supporting literature on serotype identification was available for confirmation. Distribution of the disease beared some relationship to the type of animal reared and the system of management. This was amply borne out in the 65,000 km^2 island of Sri Lanka, having a variety of agroclimatic zones and cattle husbandry practices, where distinct enzootic and disease-free areas existed. In the hilly country where the climate was mild, and temperate breeds of cattle and their crosses were reared under intensive systems, HS was rare. In the dry plains, on the other hand, where indigenous cattle and buffaloes roamed freely, the disease was enzootic FAO-WHO-OIE, (1994).

2.1.1 Bovine pasteurellosis

Bovine pasteurellosis was known by a number of synonyms that were descriptive of the condition and were relevant in specific circumstances. The terms shipping fever, transit fever, bovine enzootic pneumonia, and bovine respiratory disease (BRD) complex were all very meaningful terms used to describe the disease (De Alwis, 1999). It was believed that in the United States, the losses to the beef and dairy industries from this disease complex were greater than the losses due to all other diseases put together. In the BRD complex, more than one species and serotype of pasteurellae were incriminated as playing a role that

was secondary to respiratory viruses and 'stress'. This was unlike HS, which was a primary pasteurellosis caused by specific serotypes of the species *P. multocida*. The pasteurellae associated with BRD were predominantly *M. haemolytica* type A, and *P. multocida* capsular serogroup A. There was no consistency in the somatic types involved. The pasteurellae that cause pneumonic pasteurellosis were carried in the upper respiratory tract (URT) of calves. In the case of *P. haemolytica* type A 1, the bacteria were not easily detected in the URT of healthy calves, but were shed and can be easily isolated in calves that were stressed in some way or affected with another concurrent infection. The URTs of stressed or otherwise diseased calves can be easily colonised by *P. haemolytica* A 1. In the case of *P. multocida*, no such relationship between stress and ability to colonise has been observed. The explosive multiplication of *M. haemolytica* that results from stress leads to two processes. Firstly, there was the invasion of the lungs, resulting in pneumonia. Secondly, there was excessive shedding resulting in dissemination of infection to healthy incontact calves. The exact mechanisms underlying the rapid proliferation were not completely understood. The most effective preventive method was good management and avoidance of stress. Vaccines were of limited use, since a multiplicity of other causative agents such as viruses were also involved. Vaccines against the pasteurellae involved will help to reduce the severity of the disease, since it was the secondary bacterial phase of the disease that contributes to both its severity and fatality. Treatment with appropriate antibiotics helps to reduce the severity of the disease and prevent death. In problem herds where the condition occurs frequently, knowledge of the antibiotic sensitivity patterns of the strains involved was useful (De Alwis, 1999). Radical changes in weather, including the advent of monsoon, debility caused by seasonal scarcity of the fodder and pressure of work (draught animals) were related to the explosive occurrence of the disease in certain parts of

the world (De Alwis, 1999), South Asia, where such conditions coincide, was the area of highest incidence. The disease also occured in Middle East and Africa, where environmental circumstances and predisposing conditions were not as clearly defined as in Southeast Asia.

2.1.2 Geographical distribution of haemorrhagic septicaemia

Haemorrhagic septicaemia is worldwide in distribution and endemic in most tropical and subtropical Africa and Asia (De Alwis, 1984; Carter and De Alwis, 1989). In Africa it has been reported in most countries (Mustafa *et al.*, 1978; Shigidi and Mustafa, 1979). The disease is found across Asia from the Middle East region to South Eastern Asian countries (Anon., 1979; De Alwis, 1984; De Alwis *et al.*, 1986). No confirmatory reports exist on the presence of the disease in Europe except Italy, Eastern European countries, one sporadic case in the UK (Johnes and Hussaini, 1982); and a case of HS in Danish deer (Aalbaek *et al.*, 1999). In the United States the disease has been reported as well (Dunbar *et al.*, 2000; Dyer *et al.*, 2001). The disease was reported in Australia, and Canada (Carter and De Alwis, 1989). HS, which is sometimes mixed with pneumonic pasteurellosis of cattle, is of great significance in central and South American countries (Carter and De Alwis, 1989). In sudan, the disease was reported from the Blue Nile province, Central Sudan, in 1939, and subsequently reported from Kassala province of Eastern Sudan; from Northern Kordofan province and Western Sudan, in 1943; from Upper Nile Province, Southern Sudan, in 1947 (Anon, 1933-1959). The disease was reported nearly from all parts of Nigeria, and had a seasonal prevalence as outbreaks usually occur after the onset of the rainy season (Mustafa *et al.*, 1978).

2.1.3 Seasonal distribution of haemorrhagic septicaemia

Haemorrhagic septicaemia is normally associated with wet, humid weather and in most countries, incidence increases early in the wet season. Exhaustive epidemiological studies in Sri Lanka have shown that the disease occurs at all times of the year but, whereas dry season outbreaks are contained, outbreaks occurring during the wet season tend to spread. This is presumably due to longer survival of the organism under moist conditions, and the movement of animals associated with the rains. Where annual seasonal outbreaks occur in endemic areas, only a small number of animals will die (De Alwis, 1992). In countries where systematic epidemiological studies have been carried out, it has become evident that outbreaks do occur throughout the year, but those occurring during wet seasons tend to spread presumably due to the longer survival of the organism under moist conditions (De Alwis, 1990; 1992). Saini *et al.* (1991) studied 26 outbreaks of HS which occurred from October 1989 to September 1990 in Punjab State and found that the disease was prevalent throughout the year. Of the 26 outbreaks recorded, twelve were in Ludhiana district, four each in Sangrur and Patiala districts, three in Faridakot and one each in Bhatinda, Ferozpur and Ropar districts. They correlated agro-climatic data and HS outbreaks recorded at Ludhiana. They found that the disease was prevalent throughout the year, but mostly coincided well with increased rainfall and environmental temperature. Bastinello and Jonker (1981) carried out study on the occurrence of septicaemia caused by *P. multocida* type E in cattle from South Africa. Haemorrhagic septicaemia caused by *P. multocida* type E was diagnosed at post-mortem examination in a bovine originating from South-West Africa. Ramrao and Roa (1990) studied the trends and differentials in the incidence of HS in Karnatala State during the period 1979-85. Jindal *et al.* (2002) also analyzed 26 recorded outbreaks of HS in various districts of Haryana from 1995 to 1999 and found that outbreaks occurred more often in winter than in rainy season and summer. Verma *et al.*

(2004) investigated eleven HS outbreaks during 1998 to 1999, the two year data revealed an increase of HS outbreaks. The HS was found to be positively correlated with high rainfall, humidity and low temperature. In Nigeria the first official reports of haemorhagic septicaemia was in 1925 from Northern States with Sokoto, Bauchi, Plateau and Adamawa provinces reporting outbreaks almost yearly and although the mortality was seldom high it was thought to be responsible for considerable loss throughout the country. The disease was first recognised in the Eastern Provinces in 1948 and by 1951 outbreaks were also reported in the West. Initially the disease was controlled by a formalinized broth culture vaccine of the Standard Asian Strain (Carter's Type B/Roberts Type 1) which proved very beneficial (Anon, 1925-1951). However, in 1961 there was an outbreak at the Obudu cattle Ranch in the East among vaccinated animals following the purchase of some cattle from the Cameroons. Investigation later showed that the causative organism was relatively unknown involving Cater,s Type E *Pasteurella multocida* which was distinctly different from the Standard strain Type B (Anon, 1925-1951). Haemorrhagic septicaemia was prevalent in domestic cattle in Nigeria during the rainy season, especially in marsh-land and swampy places. Reports of pasteurellosis in captive and wild animals in Nigeria were rare; the only mentioned case was a report in a Nigerian wild buffalo (Okoh, 1980). Increased incidence had been recorded during wet seasons. Clinical expression of HS had also been associated with changes in seasonal weather patterns or inclement weather that possibly reduced host resistance (De Alwis and Vipulasiri, 1980; Franson and Smith, 1988). Although outbreaks of HS were limited to winter months, it was observed that the relationship between incidence of HS and several climatic variables occureed in the tropics.

2.1.4 Transmission

P. multocida is transmitted by direct contact with infected animals and on fomites. Cattle and buffaloes become infected when they ingest or inhale the causative organism, which probably originates in the nasopharynx of infected animals. In endemic areas, up to 5% of cattle and water buffaloes may normally be carriers. The worst epidemics occur during the rainy season, in animals in poor physical condition. Stress factors such as a poor food supply are thought to increase susceptibility to infection, and close herding and wet conditions seem to contribute to the spread of the disease. *P. multocida* can survive for hours and possibly days in damp soil or water; viable organisms are not found in the soil or pastures after 2–3 weeks. Biting arthropods do not seem to be significant vectors (Brown and Torres, 2008).

2.1.5 Sources of haemorrhagic septicaemia infection

In general, *P. multocida* does not survive long enough outside the animal to become a significant source of infection, although survival may be longer in moist conditions. Some experiments have shown that the organism can survive in sterilized soil for 2-3 weeks (Bain *etal.*, 1982). It was shown in Malaysia, however, that when sterilized earth and mud from rice fields were artificially infected, the bacteria could not be recovered after a few hours exposure to sunlight (FAO, 1959). When deposited in mud where buffaloes wallow, the bacteria could not be recovered after 24 hours (Bain *et al.*, 1982). Carcasses dumped into rivers and waterways and carried downstream are often incriminated as a likely method of spread of the disease and it is believed that pasteurellae can survive in animal tissues, and perhaps in decomposing carcases, for a few days. Freshly contaminated pasture, bedding etc. may also be sources of infection. However, no permanent reservoirs of infection have been established outside the animal. Outbreaks of HS begin when clinically affected or,

more likely, carrier animals are introduced into the herd. Once an outbreak has occurred, decomposing carcasss not promptly buried or burnt serve as a source of infection. In experimental transmission, it has been found that large numbers of organisms - in the region of 10^7 to 10^{12} colony forming units (CFU) – are required to set up an infection by the natural routes (De Alwis *et al*., 1990). How an active carrier animal transmits such a large dose to an incontact animal is not completely understood. It can be speculated that organisms directly shed from a carrier may be more virulent than cultures grown *in vitro* and used in experimental transmission studies. Alternatively, unknown circumstances may alter the susceptibility of animals so that a smaller infecting dose can cause disease. When isolates from clinical cases and from latent carrier animals were compared, however, no difference in virulence was demonstrated as judged by the median lethal doses (LD50s) for mice (Wijewardana *et al*., 1986).

2.1.6 Routes of haemorrhagic septicaemia infection

It is believed that the natural routes of infection are by inhalation and/or ingestion, and successful transmission has been made experimentally using intranasal aerosol sprays or oral drenching. However, the dose required to produce clinical disease has not been consistent, and the the results obtained with a given dose are not always predictable. Subcutaneous inoculation of bacterial cultures grown in vitro, with doses ranging from 10^4 to 10^7 cfu, has produced more consistent results. In experimental transmission, the route of infection is loosely related to the course of the disease, the clinical syndrome and the extent of pathological lesions. Intranasal infection by aerosols and oral drenching results in a longer course of disease and more profound lesions; subcutaneous inoculation results in

rapid onset of disease, a shorter course and less marked pathological lesions (De Alwis, 1999).

2.1.7 Host range

Cattle and water buffaloes are the most susceptible animals (Francis *et al*., 1980; Myint *et al*., 1987). These two species are also the major reservoir hosts. Buffaloes are more susceptible than cattle (De Alwis, 1981). Bain (1963) confirmed experimentally the high susceptibility of rabbits and mice to *P. multocida* infection, while guinea pigs, pigeons, pigs and horses have moderate degree of susceptibility. The susceptibility is variable in sheep and goat, while fowl and dogs are not susceptible to HS causing *P. multocida* (Chandrasekaran *et al.*, 1981). Swine are also susceptible (Lungtenberg *et al.*, 1986). Highly fatal septicaemic pasteurellosis was reported in elephants (De Alwis, 1982), bisons (Carter, 1982) and deer (Jones and Hussaini, 1982; Eriksen *et al*., 1999).

2.1.8 Incubation period

The incubation period is usually 3 to 5 days but some animals can carry the organism for varying periods without symptoms. In experimental infections with lethal doses, cattle or buffalo develop clinical signs within a few hours and die within 18 to 30 hours (De Alwis, 1999).

2.1.9 Pathogenesis

Upon entry of the *Pasteurella* into the animal, it is believed that the initial site of multiplication is the tonsillar region. The outcome of this infection depends on an interaction between the virulence of the organism and its rate of multiplication in vivo; the specific immune mechanisms and nonspecific resistance factors of the host animal. Thus,

the dose of infection is a vital factor and if the organism overcomes the host's defence mechanisms, clinical disease will result. If the defence mechanisms dominate over the organism, what is described as an 'arrested infection' occurs, and the animal becomes an immune carrier. Such animals possess solid immunity, and the presence of large numbers of such immune animals following an outbreak of disease contributes to herd immunity' (De Alwis *et al*., 1996). There is currently no evidence that the HS-causing strains of *P. multocida* produce any exotoxins. It has however been observed that serotype B: 2 strains that cause HS produce hyaluronidase (Carter and Chengappa, 1981), whilst a few known B: 2 strains not associated with HS fail to produce this enzyme. It is equally true that other type B strains such as B: 3, 4, which are known to produce a syndrome indistinguishable from the typical HS, do not produce hyaluronidase (Carter and Chengappa, 1981), It is, therefore, uncertain whether this enzyme is of any significance in the pathogenesis of disease. It is significant that with some type B (B: 2) and type E strains (E: 2), disease can be predictably reproduced experimentally. Other type B strains (B: 3, 4, B: 1, B: 4) have been associated with sporadic outbreaks of disease, but pathogenicity cannot always be demonstrated experimentally (De Alwis, 1995). It is still uncertain whether E : 2 strains possess any specific virulence factors, and, if so, whether they are merely phenotypic characters or have a genetic base. Whatever the virulence factor(s), it is logical to expect it to allow the organism to initially multiply against the defence mechanisms of the body and then produce the lesions that are characteristic of the disease. At present, there are some circumstantial evidences to indicate what these factors might be (De Alwis, 1999). It has been found that the classical Asian serotype (B: 2) is capable of causing vacuolation and eventually lysis of macrophages, a property that will diminish phagocytosis and promote multiplication of invading bacteria (De Alwis, 1999). This activity of cytoplasmic

vacuolation leading to macrophage lysis and death has been demonstrated using HS-causing type B strains, but not with the non-HS B strains, in a model using mouse peritoneal macrophages and in *in vitro* studies using a mouse macrophage cell line (Shah *et al.,* 1996). Cytoplasmic vauolation has also been demonstrated using culture supernatants of B: 2 and E: 2 HS causing strains, but not for those of the non-HS strains. In the absence of a true exotoxin, it might be expected that free endotoxin would be found in the culture supernatants, a feature also common to other gram-negative bacteria. Using an ovine mammary neutrophil system and {^{3}H}- labelled type B strain of *P. multocida*. Muniandy *et al*. (1992) found that capsular polysaccharide extracts known to contain 20% lipopolysaccharides (LPS), potassium thiocyante extracts and Westphal type LPS extracts inhibited phagocytosis. These workers, (Muniandy *et al*., 1992), also found that when encapsulated cells and de-encapsulated cells were used, the percentage of de-encapsulated cells phagocytosed was significantly higher than when encapsulated cells of *P. multocida* were used. These observations indicated that HS-causing strains of *P. multocida* appeared to possess a factor in their capsule that inhibited the ability of phagocytes to engulf and destroy invading bacterial cells. It is well established that the endotoxins of gram negative bacteria consist predominantly of LPS. The toxic effects of the LPS of *P. multocida* were demonstrated by producing experimental HS in calves and pigs by different routes using type B strains (Rhoades *et al*., 1967). They also administered endotoxin prepared from type B strain to a calf. The symptoms and lesions produced in the calf given endotoxin resembled those of experimental infection. The pathogenicity of *P. multocida* is attributed to many factors, notably the capsule and *toxins*.

2.1.9.1 *Toxins*

The production of endotoxins in embryonic lung cells and vero cells has been successfully implemented for the study of the toxigenic effect of *P. multocida* (Pennig and Storm, 1984; Rutter and Luther, 1984). Variability of the toxigenic effect in experimental animal species was confirmed Rutter and Luther, 1984). Soluble and heat stable toxin of both avian and bovine strains were reported to be more lethal to rabbits than to mice (Adlam and Rutter, 1989). Purified toxins of *P. multocida* type 1 were later used experimentally in cattle and buffaloes through intravenous injection and were found to initiate malaise, fever and blood tinged diarrhoea (Bain, 1963). In general, the role of toxin produced by some strains of *P. multocida* in naturally occurring cases was reported in swine, goats and calves (Rutter, 1985; Musa *et al.,* 1972). Drucker (1977) and Nakai *et al*. (1984) reported a dermonecrotic effect of toxins in guinea pigs and rabbits. Another toxigenic effect of *P. multocida* is cytopathic effect attributable to the toxic reduction in DNA synthesis especially in rapidly dividing cells (Chanter *et al.*, 1986). The nature of toxigenic *P. multocida* is ascribed to a cell membrane structural component, lipopolysaccharide (LPS), which is the endotoxin fraction (Heddleston *et al*., 1967; Rebers and Rimler, 1984), besides a protein fraction (Rimler and Brogden, 1986). The chemical properties of *P. multocida* LPS-protein complex are similar to those of many Gram-negative bacteria endotoxins in addition to its immunogenic nature (Rimler and Brogden, 1986). The *P. multocida* capsular type D strain has been identified as causative agent of atrophic rhinitis in pigs and snuffles in rabbits. The *tox*A gene of *P. multocida* encodes the dermanecrotic toxin responsible for atrophic rhinitis. The *toxA* gene based PCR can be used for direct analysis of toxigenic *P. multocida* without additional hybridization. The assay appears to be the most sensitive and effective method for large scale analysis of nasal and tonsillar swabs (Kamp *et al*., 1996).

2.2 CLINICAL SIGNS

The disease occurs mainly in regions where husbandry practices are primitive and the animals are reared under free-range conditions. In such circumstances, the animals are not under constant observation and the only reported sign may be sudden death (Horadagoda *et al*., 1991). The classical clinical symptoms of HS may be characterized by three phases .The first phase is characterized by temperature elevation with anorexia and sometime salivation. The second phase is respiratory distress with profuse salivation and nasal discharge. The terminal phase is recumbency and death. Indeed, first hand descriptions of the clinical syndrome under natural conditions are scarce. Generally, the observed signs are temperature elevation, loss of appetite, nasal discharge, salivation and laboured breathing, with swellings in the submandibular region spreading to the brisket area and even down to the forelegs. Some descriptions of the syndrome arising from experimental transmission have been made (Horadagoda *et al.*, 1991). These include experimental transmission of the disease by subcutaneous inoculation of in vitro cultures, intranasal or oral transmissions, or natural infections occurring in animals housed in close contact with clinically affected animals, the last being the closest equivalent to naturally occurring disease (Horadagoda *et al.*, 1991). The duration of the clinical course of disease is highly variable. In experimental infections by the subcutaneous route, the clinical course lasted only a few hours. In experimental transmissions by natural routes (oral or aerosol) and in natural exposure experiments De Alwis, (1999) recorded a clinical course ranging from two to five days. Horadagoda *et al.* (1991) recorded a clinical course of 14-19 hours after experimental oral infection, 25-110 hours after aerosol infection and 19-70 hours after natural exposure. In field observations of five outbreaks of disease involving 37 buffaloes and 7 cattle, Saharee and Salim (1991) recorded a clinical course of 4-12 hours in per acute cases and 2-3 days in

acute and subacute cases. On a herd basis, the outbreaks usually occur very fast and do not persist for long. The observations of Saharee and Salim (1991) in west Malaysia indicated that 75% of outbreaks lasted for less than 15 days within a herd.

Post-mortem lesions following experimental inoculation were congested lungs with light petechial haemorrhages on the heart (De Alwis *et al*., 1975). In acute cases, wide-spread haemorrhages over the pleural surfaces and the parenchymal organs, oedematous and red-gray lobular inflamed lungs, oedematous lymph nodes, pericarditis with serous haemorrhages on the left ventricle and blood stained exudates were observed (Bastinnello and Jonker, 1981). In a case of HS in deer, the major clinical signs and peracute or acute pathological changes included extensive swelling of the head and neck, septic pneumonia, petechial and ecchymotic haemorrages on serous membranes and severely haemorrhagic adrenal gland and abomasum. Rhinitis and necrotic pharyngeal mucosa were common. Histologically, the most advanced lesions were in the nasal mucosa and pharynx. The swelling of the head and the neck had arisen from a diffuse cellulitis in the subcutaneous and intermuscular tissues. The earliest lesions in the lung included presence of large numbers of bacteria in the pulmonary capillaries, but various degrees of fibrinous exudation in the alveoli and infiltration with neutrophils were usually observed (Eriksen *et al*., 1999).

Dhanda and Nilakanthan (1961) reported the occurrence of paraplegia in 189 cattle from 28 villages in India following a vaccination campaign against rinderpest, where a total of 48,603 animals in 71 villages in the Andhra Pradesh were vaccinated. Of these animals, 135 died. The cerebrospinal fluid was collected from two animals, and *P. multocida* was isolated from samples. The authors typed the isolate as Roberts type I but it is now known that Roberts type I includes the Asian and African strains B:2 and E:2, respectively, as well

as the Australian strain 989, therefore not certain whether the report is one of an atypical syndrome caused by the Asian serotype, or one with a variant serological configuration. It is also significant that in this outbreak, there was no terminal septicaemia, a feature characteristic of infection with the Asian B: 2 serotype. Upon opening a carcass of an animal that has died of HS, the most obvious lesion is subcutaneous oedema - subcutaneous infiltration with yellow serosanguinous fluid, particularly in the submandibular and brisket regions. Subcutaneous petechial haemorrhages are also evident. There are also widespread petechial haemorrhages in the thoracic cavity, particularly on the base of the ventricles and the auricles. There may be excessive fluid in the pericardial sac and pericarditis with marked thickening of the pericardial wall may be present. The lungs may be congested with varying degrees of consolidation and with marked thickening of the interlobular septa. In the abdominal cavity, petechial haemorrhages are widespread in all tissues. De Alwis *et al*. (1975) observed that the pathological pictures depended upon the duration of the syndrome. In animals that died within 24-36 hours of experimental inoculation, the gross pathology was limited to widespread petechial haemorrhages and generalised congestion of the lungs. When the duration was 36-72 hours, haemorrhages were petechial or ecchymotic, and more pronounced. Fibrinous pericarditis was present. When the course was longer than 72 hours, there were extensive consolidations of the lungs, with marked lobulation, pleuritis, pericarditis with marked thickening of the pericardial wall and, in later stages, pleural adhesions (De Alwis *et al*., 1975). The pleura overlying the pneumonic lungs are sometimes thickened, forming a sheet of fibrin, often adhering to the coastal wall and pericardium (De Alwis *et al.,* 1975; Horadagoda *et al.,* 1991). Rhoades *et al*. (1967) found similar lesions, and **f**urther reported that the nature of the lesions depended on the route of infection. The clinical signs may broadly be divided into three phases as follows. *Phase* 1 is

dominated by increased temperature, loss of appetite, general apathy, and depression. If closely monitored, a rise in rectal temperature to 40-41°C is recorded, which lasts throughout the course of the disease, dropping to subnormal levels during the terminal phase, a few hours before death. *Phase* 2 is dominated by respiratory syndromes with an increased respiration rate (40-50/minute), laboured breathing, clear nasal discharge and salivation. Submandibular oedema may also begin to show during this phase. As the disease progresses, the nasal discharges become opaque and mucopurulent. Phase 3 is dominated by recumbency. The respiratory distress becomes more acute, the animal is recumbent, terminal septicaemia sets in and death follows: case fatality is nearly 100%. In many instances, there are varying degrees of overlap between the phases, and the shorter the course; the less distinct are the three phases. Unless a close vigilance is maintained, the first phase may be easily overlooked. The course of the disease arising from field reports is often shorter than experimental observations, probably due to the failure to detect the first phase under free-range conditions. In general, it has been observed that the disease is more acute and has a shorter course in buffaloes than in cattle (Graydon *et al*., 1993). In experimental subcutaneous inoculation, buffaloes died 24-31 hours after inoculation, whereas the time of death for cattle was 60 hours (Rhoades *et al*., 1967).

2.3 HAEMORRHAGIC SEPTICAEMIA-CAUSING *PASTEURELLA MULTOCIDA*

Haemorrhagic septicaemia (HS), a peracute bacteraemia of cattle and buffalo, caused by specific strains of *P. multocida* namely; B:2, B:2,5 and E:2 and E:2,5 in Asia and Africa, respectively (De Alwis, 1999). HS is endemic in most parts of tropical Asia, Africa and India and is considered to be one of the most economically important livestock diseases of South East Asia. While the serotypes of *P. multocida* causing HS principally affect cattle

and buffaloes, they have also been associated with disease in pigs, sheep and other animal species (Bain *et al*., 1982). Pathogens other than *P. multocida* have not been found to be associated with HS, although stress and adverse environmental conditions appear to precede the onset of disease. *P. multocida* has a unique capability to infect a wide range of animal hosts with a broad spectrum of diseases, namely HS, fowl cholera and infectious atrophic rhinitis of pigs. Numerous attempts have been made to correlate specific *P. multocida* serotypes with a particular disease, resulting in the development of five main serological typing systems. These systems use a variety of assay procedures and antigen preparations, which divide the *P. multocida* species into as few as three and as many as 16 serotypes (Heddleston *et al*., 1972). It was proposed by Carter and Chengappa (1981) to combine both the Carter (capsular) and Heddleston (somatic) serotyping methods, in order to standardize identification of *P. multocida* isolates. Despite this proposal, the complex and diverse nature of the typing systems, in addition to reports of 30-82% of isolates being 'untypeable' (Namioka and Bruner, 1963; Manning, 1982; Jones *et al*., 1988. Serological typing methods have also been found to provide limited characterization of isolates, and are not capable of differentiating strains to the extent required for epidemiological studies (Wilson *et al*., 1992). Analysis of genomic restriction patterns by ribotyping and field alternation gel electrophoresis (FAGE), has proved useful in differentiating bacterial strains that have been denoted as identical by all previous typing methods (Hector *et al*., 1992; Wei *et al*., 1992). Previous fingerprinting studies of *P. multocidu* isolates of avian and porcine origin demonstrated significant variability between strains of similar serotype (Snipes *et al.*, 1989; Gardner *et al*., 1994). Ribotyping was shown to correlate well with REA typing (Snipes *et al.*, 1989), and has the benefit of allowing easier interpretation by highlighting rRNA gene polymorphisms. FAGE has initiated renewed interest in microbial

genetics and genomic organization with its capacity to separate DNA fragments up to 10 megabase (Mb) pairs in length. An extensive review of the technique and its applications was published by Townsend and Dawkins (1993), illustrating its versatility and increasing importance in the field of molecular biology. The combination of FAGE and restriction endonuclease digestion of genomic DNA provide the added advantage of demonstrating DNA heterogeneity throughout the entire bacterial genome thereby giving a complete picture of genetic variation between strains. Since publication of the FAGE review, the spectrum of bacterial species analysed by FAGE has increased dramatically, and the technique has become an integral component of bacterial genetics and epidemiological studies Townsend and Dawkins (1993).

2.3.1 Distribution of serotypes

In Asian countries, the only serotype recorded was serotype 6:B, (Chandrasekeran *et al*., 1981). There was one report of a single isolation of a type D strain from what was believed to be a case of HS in Malaysia (Chandrasekeran *et al*., 1981). While type D in cattle is normally associated with a pneumonic syndrome, under certain circumstances it may produce a septicaemia, and the condition could be mistaken for classical HS.

2.3.2 History of *Pasteurella multocida*

Pasteurella multocida specific serotypes are the causative agents of Haemorrhagic septicaemia (HS) in cattle and buffaloes. The Family *Pasteurellacae* has three Genera: *Pasteurella, Actinobacillus,* and *Haemophilus*. According to current classification, the Family *Pasteurellaceae* includes a large group of gram-negative bacteria that are chemoorganotrophic, facultatively anaerobic and fermentative. *Pasteurella multocida* was named in honour of Louis Pasteur (Rimler and Glisson, 1997). Several other species that

exhibit complex phenotypic and genotypic relationships with these genera are also included. Most members of the family cause disease in mammals (including humans), birds and reptiles. On the basis of phenotypic similarities, more serotypes were added to the genus: *Mannheimia haemolytica* in 1932, *P. pneumotropica* in 1950, *P. gallinarum* in 1955, *P. ureae* in 1962 and the gas-producing *P. aerogenes* isolated from pigs in 1974 (Mutters *et al.*, 1985. More recent classification of the genus *Pasteurella* was based on genetic relationships, which have been determined by DNA: DNA hybridisation, rRNA: DNA hybridisation and 16S rRNA sequencing. On this basis, 7 species have been identified: *P. multocida* (with three subspecies: Multocida, Septica and Gallicida), *P. dagmatis, P. gallinarum, P. voluntium, P. stomatis, P. avium, P. langaa, P. anatis* and *Pasteurella* subspecies A and B (Mutters *et al.*, 1985; Bisgaard 1993). Several species that were previously classified under the genus *Pasteurella* have now been excluded as they were found to be genetically more closely related to the *Actinobacillus* group. These are *P. ureae, M. haemolytica* biotypes A and T, *P. testudinis, P. aerogenes* and *P. pneumotropica*, biotypes. Since the genus was redefined on entire basis, six new species have been included in the group: *P. granulomatis, P. caballi, P. bettii, P. lymphangitidis, P. mairi* and *P. trehalosi* (Bisgaard, 1994). Two members of the genus *Pasteurella* are of veterinary importance - *P. multocida* and *M. haemolytica*. These species cause a variety of disease syndromes (pasteurelloses) in agricultural, domestic and wild animals. *P. multocida*, is a gram-negative bacterium, and is the causative agent of a wide range of diseases in wild and domestic animals, birds and humans. *P. multocida*, the organism which is pathogenic for various animals and fowls can be divided into 16 serotypes. Certain serotypes are host specific, while others are not. Consequently, different serotypes show different pathogenicity when tested in various hosts. The general and biochemical properties of the

various strains are very similar, and from this point of view these organisms belong to the single species, *P. multocida*. The organisms can be divided into two pathogenic groups: those causing haemorrhagic septicaemia and those causing non-haemorrhagic septicaemia. *P. multocida* and *M. haemolytica* show several biochemical properties in common, but differ with respect to indole production, haemolysis, and there is no antigenic relationship between the two species. Human infections due to *P. multocida* are strongly associated with animal exposure and usually involve soft-tissue sites after animal bites or scratches (Francis *et al*., 1975). Respiratory tract infections and less frequently septicaemia, endocarditis, meningitis, peritonitis, or other unusual types of infection due to this organism have also been described (Weber *et al*., 1984; Koch *et al*., 1996). Thirteen patients were documented with *P. multocida* infections, diagnosed in the University hospital of Crete, Greece, between 1993 and 2004. Most patients (62%) were >70 years of age. Respiratory tract infections were most commonly encountered (61.5%), followed by soft-tissue infections (30.8%) and septicemia (7.7 %) (Koch *et al*., 1996). On the basis of information through participatory appraisal, haemorrhagic septicemia (HS) is considered to be among the five most important diseases of camel due to economic losses associated with it (Mochabo *et al*., 2005; 2006). An outbreak of a respiratory disease occurred in the dromedary population of Greater Cholistan desert, which was quite foreign to the locale (Mochabo *et al*., 2006).

2.3.3 Clinical diagnosis of haemorrhagic septicaemia

A clinical field diagnosis is based on the history, signs and lesions. The history includes the mortality pattern in relation to the surrounding circumstances, such as the previous occurrence of HS in the herd, whether it is an enzootic area, the age group and species affected, and vaccination history. Mortality rates can vary from 5% in herd of cattle in a n

enzootic area with regular annual epizootics, to near 100% among cattle and buffaloes in a disease- free arear experiencing a sporadic outbreak after 10 years (De Alwis, 1984). When a suspected outbreak of haemorhagic septicaemia (HS) is reported, a provisional diagnosis could be made based on clinical signs and if carcases are available, on the gross pathological lesions seen on postmortem examination. An investigation of relevant epidemiological parameters is also helpful. It is also important to consider other diseases prevalent in the locality that could account for the clinical signs observed, lesions and the number and pattern of death observed (De Alwis, 1999).

2.3.4 Laboratory diagnosis of haemorrhagic septicaemia

Biochemically, strains of *P. multocida* causing HS are not different from other members of the group, and they can be differentiated only by serology. The earliest serological tests to gain some acceptance were the passive mouse protection test, the plate agglutination test of Little and Lyon (1943), and the mouse protection test of Roberts (1947). Roberts (1947) defined 4 different serological types, designated types I-IV. All HS strains belonged to type I. Carter (1955, 1961) devised an indirect haemagglutination test and identified 4 different types, designated A, B, D and E, based on th e capsular antigens. The limitations of both techniques became evident when Namioka and Murata (1961) described 11 somatic types. There were 2 somatic types within Roberts ' type I or Carter's type B, one of which caused HS (type 6) and the other did not (type 11). Namioka and Murata (1961) also described a simplified capsular typing technique. Heddleston *et al.* (1972), described an agar gel precipitation test (AGPT) using a heat-stable antigen and identified 16 different types of *P. multocida*. These types are sometimes also referred to as somatic types, presumably because the HS serotypes belonging to Heddleston’s type 2 corresponded closely to

Namioka's somatic type 6. Haemorrhagic septicaemia is caused by *Pasteurella multocida* type B: 2, B: 2, 5 and B: 5 in Asian countries and type E: 2 in African countries (Rajeev *et al*., 2011). *Pasteurella multocida* has five capsular serotypes i.e. type A, B, D, E and F (Rajeev *et al*., 2011). Tentative diagnosis of the disease is mainly based on the clinical signs, symtoms and post mortem findings. Confirmatory diagnosis is done by isolation and identification of causative agent. A variety of laboratory diagnostic techniques have been developed over the years for pasteurellosis and used routinely in the laboratory. Among these techniques, a molecular technique of diagnosis is most important. This technique not only gives diagnosis, but it also provides information regarding capsular type of *Pasteurella multocida*. Techniques which are used for molecular diagnosis of haemorrhagic septicaemia are PCR based diagnosis, Restriction endonuclease analysis (REA), Ribotyping, Colony hybridization assay, Field alternation gel electrophoresis (FAGE) and detection of *Pasteurella multocida* by Real Time PCR. Among these techniques real time PCR is most sensitive and specific (Uhl *et al*., 2002; Rajeev *et al*., 2011). Two typing systems for serotyping of *Pasteurella multocida* isolates are adopted. One for capsular typing by Carter's IHA system and other somatic typing by the method of Namioka and Murata (1961) or Heddleston *et al*. (1972).

2.3.5 Molecular diagnosis of haemorrhagic septicaemia

Rapid slide agglutination test is performed on slide for rapid diagnosis; in which floccular agglutination appears within 30 seconds in the positive cases. Indirect haemagglutination test is carried out for the determination of capsular types of *P. multocida*. With development in biotechnological techniques for the detection of nucleic acid, the identification and characterization of etiological agents has become quick, easy and

accurate (Dutta *et al*., 2005). Because culture conditions can influence the expression of morphology, sugar fermentation patterns and serological properties, phenotyping is found not to be a stable and reliable method for strain identification and subtyping of *Pasteurella* spp. (Hunt *et al.,* 2000) and *Mannheimia* spp. (Angen *et al.*, 2002) in a clinical or epidemiological context. Molecular identification bypasses these disadvantages, and will further improve accuracy of characterization (in pure and/or mixed cultures), speed of detection, determination of taxonomic position, and understanding of intra-species genetic relationships (Hunt *et al.*, 2000). Because this methodology requires extended laboratory equipment and experience, the currently most applied alternative approaches are species-specific PCRs and molecular fingerprinting (Olive and Bean, 1999; Aarts *et al.*, 2001). The first type of approach consists of species specific PCR that amplify unique DNA sequences. They have been successfully developed for the *toxA* gene (Nagai *et al.*, 1994), *psl* gene (Kasten *et al.*, 1997), and KMT1 region (Townsend *et al.*, 1998) in *P. multocida*, and are used predominantly in clinical specimens for diagnostic purposes. Townsend *et al.* (2001) combined several of such regions (*cap* loci) to develop a multiplex capsular PCR typing system which is able to discriminate the five capsular types of *P. multocida*. The second approach, molecular fingerprinting, provides a unique 'signature' of a bacterial strain and has been used for the identification of microorganisms in a broad microbiological context. In recent years, genotypic methods of bacterial identification have proved beneficial in overcoming some limitations of traditional phenotypic procedures. Nucleic acid-based assays allow the detection of organisms directly from clinical samples or from small amounts of cultured bacterial cells, thus dramatically improving the sensitivity and decreasing the time required for bacterial identification. PCR has been particularly useful in this regard, with the use of primer sequences designed to facilitate identification at any

level of specificity: strain, species, genus, or members of a domain (Relman and Persing, 1996).

2.3.6 Epidemiology of haemorrhagic septicaemia/*Pasteurella multocida*

Gupta (1962) found that the detection of carrier animals was related to incidence of HS. During an outbreak, he found 7.5 per cent of clinically normal in-contact animals to be carriers whereas 40 days later he was unable to detect any in the same herd. Mustufa *et al.* (1978) reported that the presence of HS causing *P. multocida* in the nasopharynx of a small proportion of apparently normal cattle and buffaloes had documented for a long time. Healthy cattle carriers in association with an outbreak of HS were found to constitute 44.4 per cent of the population in comparison with 3.89 per cent, 5.5 per cent and nil in herds unassociated with the disease. Healthy carrier rate was always higher in calves less than two years old than in adult cattle. Shigidi and Mustufa (1979) isolated 42 strains of *P. multocida* from different outbreaks of HS from healthy cattle in Sudan and carried out biochemical characterization. Chandrasekaran *et al*. (1981) isolated 155 strains of *P. multocida* from different outbreaks of HS throughout Malaysia. This organism was also isolated from the lymph nodes of the upper respiratory tract of nine out of 800 healthy cattle and buffaloes examined in the state of Perak. Jones *et al.* (1988) isolated *P. multocida* in pure culture from subcutaneous and periocular tissue of 12 calves in UK having periocular oedema of such severity as to cause prolapse of the eye balls. Those isolates were found to be extremely virulent to mice. Dwivedi and Sodhi (1989) isolated 19 *P. multocida* strains (4 each from cattle and buffaloes, 6 from sheep and 5 from poultry) in Punjab State. Jindal *et al.* (1996) studied HS outbreaks among buffaloes in Haryana. They demonstrated bipolar organisms in blood smears and isolated *P. multocida* from buffaloes

suffering from the disease. Jindal *et al.* (2002) isolated *P. multocida* in Haryana State from blood samples of affected, untreated cattle and buffaloes and from the tracheal swabs, lung tissues and heart blood at post-mortem examination. Kapoor *et al*. (2004) cultured 470 samples for bacterial isolation in Himachal Pradesh, which were collected from 53 cattle, 23 buffaloes, 104 sheep, 83 goats and 207 rabbits. They recovered 11 isolates of *P.multocida* including two from cattle and four from buffaloes. Javia (2004) isolated 13 *P. multocida* from animal and avian origin in the laboratory. He collected 89 samples of different types. Out of total 13 isolates three were animal origin. Verma *et al.* (2004) collected samples from dead as well as live animals suspected for HS from 11 outbreaks in Haryana State and isolated *P. multocida* B: 2 organisms. Genotypic methods provide tools to investigate the transmission dynamics of *P. multocida* at farm and regional levels. Pulsed-field gel electrophoresis (PFGE) and PCR based methods such as random-amplified polymorphic DNA (RAPD) typing are highly discriminatory and useful for investigation of disease outbreaks (Blackall and Miflin, 2000; Dziva *et al*., 2008). Multilocus sequence typing (MLST) (Subaaharan *et al*., 2010) has advantages over PFGE and RAPD typing in terms of reproducibility and portability, allowing global analysis of strains. However, the use of housekeeping genes may limit discriminatory power and reduce its utility for investigations of disease outbreaks or in farm level epidemiology (Hotchkiss *et al.*, 2011). The molecular epidemiology of *P. multocida* has rarely been studied at the farm level in cattle (Subaaharan *et al*., 2010). PFGE was more discriminatory than RAPD, but confirmed results with respect within farm homogeneity or heterogeneity of strains, whereas MLST was not discriminatory enough for farm level epidemiology. As in other host species, either several strains or one dominant strain of *P. multocida* may exist within farms, with evidence for a role of management factors such as movements onto the farm in

the number of strains detected (Hotchkiss *et al.*, 2011). *P. multocida* comprises five capsular serogroups of 16 somatic serotypes (Frank, 1989; Heddleston *et al.*, 1972). In cattle, *P. multocida* serogroups B and E are the pathogens of haemorrhagic septicaemia, and serogroup A is mainly associated with respiratory disease (Frank, 1989). *P. multocida* A3 is the second most commonly isolated bacterium from fibrinous pneumonia in beef cattle. In epidemiology and other fields of applied microbiology, reliable methods for the identification and characterisation of bacterial strains within a particular genus and species are primary requirements. Several methods have been used to differentiate bacterial strains, including those of *P. multocida*. Traditional phenotypic methods, such as outer-membrane-protein (OMP) subtyping, whole-cell-protein (WCP) analysis, serotyping and biotyping (Akhtar and Eley, 1992) are not sensitive enough for strain differentiation. Restriction endonuclease analysis (REA) of whole genomic DNA has been useful in the genetic characterisation of bacterial strains (Murphy and Robinson, 1993) but its limitations have led to the use of *Escherichia coli* ribosomal RNA (rRNA) as a broadspectrum probe (ribotyping) for both epidemiological and taxonomic purposes. Townsend *et al.* (1997) used ribotyping and field alternation gel electrophoresis (FAGE) to differentiate *P. multocida* isolates derived from haemorrhagic septicaemia.

2.3.7 Sero-surveillance of haemorrhagic septicaemia in cattle and buffaloes

Study on sero-surveillance of haemorrhagic septicemia in cattle and buffaloes in district Malakand Pakistan was reported by (Asadullah *et al.*, 2006). The average geometric mean titre (GMT) was recorded for HS in buffaloes in the range of 4.12 to 46.98, while those in cattle were recorded in the range of 4.45 to 46.40. In young buffalo calves, incidence, mortality and morbidity rates were observed to be 22.25%, 21.19% and 95.25%,

respectively. In adult buffaloes, morbidity, mortality and case fatality rates were 5.49%, 1.65% and 30%, respectively (Asadullah *et al*., 2006). In case of young cattle calves, morbidity, mortality and case fatality rates were recorded as 3.94%, 1.77% and 45%, respectively. While in case of adult cattle, morbidity, mortality and case fatality rates were recorded as 2.51%, 0.39% and 15.79%, respectively (Asadullah *et al*., 2006). During the investigation the incidence, mortality and case fatality due to haemorrhagic septicaemia were found greater in the young calves as compared to the adult in both buffaloes and cattle (Asadullah *et al*., 2006). Study on the sero-prevalenous on haemorrhagic septicaemia had been carried out in other region of the world as well as some areas of the Punjab province in Pakistan (Zyambo *et al*., 1986; Dutta *et al*., 1990; Molina *et al*., 1994; Zaka, 1998). Epidemiological studies reported over a period of thirteen years (1974-1986) in India indicated that mortality-wise, HS was placed first and morbidity-wise, second as compared to four other epizootic diseases namely, foot and mouth disease, rinderpest, anthrax and black quarter (Dutta *et al*., 1990). Many states in India were marked as high risk zones. About 26 outbreaks were recorded in Punjab State from 1989 to 1990 (Saini *et al*., 1991). Similarly, outbreaks of haemorrhagic septicaemia (HS) was recorded in Sri Lanka, the Middle East, Africa and South Asia (De Alwis and Vipulasiri, 1980; FAO, 1989; Lane *et al*., 1992). The morbidity rate reported was 6.40 and the mortality rate was 6.28 per hundred thousand of bovine population (Dutta *et al*., 1990). A wide range of mortality rate (5 to 90%) was reported in different outbreaks and seasons in India, Nepal and the Philippines (FAO, 1991). Most of the outbreaks were managed by medical treatment alone and resulted in poor survival rate.

2.3.8 Plasmid DNA of *Pasteurella multocida*

Salmah, (2004) estimated the size and number of plasmids for each isolate by agarose gel DNA, ranging in size from 1.5 to 3.6 Mda and there was no presence of large plasmids in all isolates. All isolates of *P. multocida* serotype B were found to contain common plasmids. Plasmid has been used in several studies in order to learn more about the pathogenicity and virulence mechanism (Shivshankara *et al*., 2000) of the *P. multocida*. Plasmid profile analysis is a useful tool in epidemiological studies (Salmah, 1997; 2000; 2004). Various studies have been done on *P. multocida* plasmid; in one such study a cryptic plasmid of *P. multocida* related to its protein has been reported by McGee and Bejcek, (2001). Elsewhere plasmid DNA has been used as a probe to identify the species by Zhao and Aoki (1992). Several plasmids that were isolated from complement-resistant *P. multocida* or *E. coli* were evaluated for phenotypic markers. Plasmid p2267, isolated from a tetracycline-resistant, complement-resistant fowl cholera field isolate of *P. multocida* (PM2267), was used to transform a K-12 *E. coli* (C600); this resulted in increased complement resistance, which was eliminated by curing. Either of two plasmids (p1870 or p70-1) isolated from *P. multocida* and *E. coli*, respectively conferred an increase in complement resistance and invasiveness to turkey epithelial cells when expressed (Margie and Richard, 1995). No detectable differences in major outer-membrane proteins, capsule, or carbohydrate fermentation were found to be associated with the acquisition of the above plasmids.

2.3.9 *In vitro* antimicrobial sensitivity

The variety of compounds nowadays available to control bovine pasteurellosis is substantial. Examples are beta-lactam antibiotics such as aminopenicillins, clavulanic acid and extended spectrum cephalosporins (cefquinome, ceftiofur), tetracyclines,

oxytetracycline, doxycycline hygrochloride, potentiated sulfonamides (trimethoprim/sulfonamides), macrolides (tylosin, tilmicosin, and tulathromycin), aminoglycosides (gentamicin), aminocyclitols (spectinomycin), lincosamides (lincomycin), phenicols (florfenicol), and second generation fluoroquinolones (marbofloxacin, danofloxacin and enrofloxacin). Antibiotic sensitivity testing showed that all *P. multocida* isolates were susceptible to beta-lactams, quinolones, chloramphenicol, tetracycline and trimethoprim/sulfamethoxazole (Athanasia *et al.*, 2005). However, decreased susceptibility and antimicrobial resistance is frequently reported for many of these agents in *Pasteurella* and *Mannheimia* organisms isolated from pneumonic lungs of calves (Watts *et al*., 1994; Kehrenberg *et al*., 2001). The ultimate goal of antimicrobial therapy in the context of bovine septicaemic pasteurellosis is to achieve a concentration in the lungs (or blood in case of haemorrhagic septicaemia) that has an inhibitory effect on target bacteria such as *M. haemolytica* and *P. multocida*, and the clinical recovery of the affected animal is enhanced. The incidence of microbial resistance against chemotherapeutic agents is increasing all over the world mainly due to indiscriminate use of the agents. Thus the sensitivity of an organism to different chemotherapeutic agents must be carried out prior to the treatment. Dao *et al.* (1973) carried out antibiotic sensitivity test for *P. multocida* of calf origin. They found that the isolates were sensitive to chloramphenicol and furazolidone and resistant to chlortetracycline, penicillin and oxytetracycline. Karaivanov (1983) isolated 330 *P. multocida* strains from fowl cholera and from mammals with pneumonia and tested them against various antimicrobial agents. Karaivanov (1983) found 100 % isolates were sensitive to ampicillin, chloramphenicol, erythromycin, tetracycline and gentamicin, 86.6 % to furazolidone, 74.2 % to neomycin, 82.2 % to spectinomycin, 52.7 % to kanamycin, 23.7 % to streptomycin and 32.7 % to novobiocin. Isolates of intermediate sensitivity were

also observed from mammals with pneumonia as follows: 67.3 per cent to novobiocin, 62.4 % to streptomycin, 47.3 % to kanamycin, 25.8 % to neomycin, 13.4 % to furazolidone and 11.8 % to spectinomycin. Verma and Saxena (1987) studied 35 isolates, of *Pasteurella* cultures for their antibiogram against antimicrobial agents. They found that the organisms were resistant to tetracycline, sulpha drugs and penicillin-G. Sambyal *et al.* (1988) subjected three isolates of *P. multocida* to *in vitro* drug sensitivity test and observed that all the isolates were highly sensitive to chloramphenicol, nitrofurantoin and tetracycline, moderately to gentamicin and resistant to neomycin, ampicillin, trimoxazole, erythromycin and streptomycin. Waltman and Horne (1993) studied resistance patterns of 176 isolates of *P. multocida* of avian origin to oxytetracycline, chlortetracycline, neomycin, gentamicin, triple sulfa, sulfamethoxazole and trimethoprim, erythromycin, penicillin-G, ampicillin, nitrofurantoin, nalidix acid and chloramphenicol. All the isolates were found to be highly susceptible to all the antimicrobial agents tested, except the sulfonamides. Dimri *et al.* (1994) isolated *P. multocida* from an outbreak of pneumonia in sheep and carried out antibiotic sensitivity test. They found that the isolates were resistant to tetracycline, ampicillin and sensitive to gentamicin, kanamycin and streptomycin. Gupta *et al.* (1996) studied drug resistance of 39 field isolates of *P. multocida* and found that the highest percentage of sensitivity was observed with gentamicin and chloramphenicol (90%) followed by nitrofurantoin (72%) and oxytetracycline (62%), while the highest percentage of resistance was observed with bacitracin (100%), followed by ampicillin (82%), cephalexin (67%), penicillin-G (51%) and streptomycin (49%). Das and Bhagwan (1997) checked the resistance pattern of two isolates of *P. multocida*. The isolates were sensitive to chloramphenicol, gentamicin, penicillin, kanamycin, trimethoprim, deoxycycline, and erythromycin but resistant to oxytetracycline, bacitracin and polymyxin-B. Intermediate

sensitivity was observed to streptomycin, tetracycline and neomycin. Harmansdorfer and Bauer (1998) checked the resistance pattern of 221 (89 bovine and 132 porcine) *P. multocida* isolates against 16 antibiotics. They found that over 90% of porcine *P. multocida* were sensitive to penicillin-G, ampicillin, cephalexin, polymyxin-B, enrofloxacin, chloramphenicol and florfenicol while bovine strains were at least 90 % sensitive to oxacillin, erythromycin, gentamicin and sulfamethaxazole-trimethoprim. Aye *et al*. (2001) carried out antibiotic sensitivity of *P. multocida* of turkey origin. They found that the majority of the isolates were susceptible to amikacin, ampicillin, ceftiofur, cephalothin, enrofloxacin, florfenicol, entamicin, neomycin, novobiocin, oxacillin with 2% NaCl, sarafloxacin, tilmicosin and trimethoprim with sulphadiazine and resistant to clindamicin, penicillin-G, tiamutin and tylosin. Balakrishnan and Mini (2001) isolated and studied four strains of *P. multocida* of avian origin for drug resistance pattern. The isolates were found susceptible to oxytetracycline, pefloxacin, tetracycline, streptomycin and resistant to furazolidone, metronidazole and nalidixic acid. Moderate sensitivity were observed to chlorampenicol, gentamicin, kanamycin and penicillinG. Jonas *et al.* (2001) recovered nine isolates of *P. multocida* from avian cholera outbreaks in Indonesia. Jonas *et al.* (2001) characterized these isolates by antimicrobial susceptibility pattern and found that all the isolates were susceptible to ampicillin and trimethoprim. Of the nine isolates, seven (78%) were susceptible to doxycycline and gentamicin Jonas *et al.* (2001). Verma *et al*. (2004) checked the resistance pattern of 11 *P. multocida* isolates against 10 antibiotics. Majority of these isolates were sensitive to enrofloxacin, gentamicin and choramphenicol but moderately sensitive to pefloxacin and ciprofloxacin and resistat to sulphadimidine, oxytetracycline, streptomycin, amoxycillin and tetracycline (Verma *et al.*, 2004). Sharma *et al*. (2004) performed antibiogram on 52 strains of *P. multocida* from pigs. Among the

antibiotics tested cephalexin (100%) was found to be highly effective, followed by norfloxacin (96.15%), gentamicin (88.46%), sulphadiazin (65.38%) and chloramphenicol (63.46%). Howevere, the isolates were highly resistant to kanamycin (88.47%) and lincomycin (92.31%). Javia (2004) isolated 13 *P. multocida* isolates of animal and avian origin and all were subjected to *in vitro* antibiotic sensitivity in the laboratory. All the isolates were sensitive to enrofloxacin, flumequine, chloramphenicol and cephalexin while 12 isolates were sensitive to pefloxacin, six were sensitive to ciprofloxacin and four were sensitive to amoxicillin Javia, 2004 and all the isolates were found resistant to sulphadiazine. Patel (2004) performed *in vitro* antibiotic sensitivity of ten isolates, all the isolates (100%) were sensitive to enrofloxacin, flumequine, chloramphenicol and tetracycline, while nine isolates (90%) were sensitive to norfloxacin and cephalexin, and two isolates (20%) were sensitive to penicillin-G. All the isolates were found resistant to sulphadiazine while four isolates (40%) were intermediate to penicillin-G.

2.3.9.1 *Treatment*

As HS is a primary bacterial disease with no other biological agents involved, treatment may appear simple using the wide range of antibiotics currently available. In reality, however, treatment is constrained by a host of practical considerations. It has been found in practice that animals can only be cured if they are treated in the very early stages of the disease. However, as the disease occurs mainly in situations with primitive husbandry practices most field cases escape detection in the early stages, thus rendering treatment ineffective (De Alwis, 1999). In organized farms, however, a practical method of achieving early detection and successfully treating animals is to check the rectal temperatures of all incontact animals regularly once an outbreak has been detected. Any animals showing an

increased temperature can be separated and treated with a course of an appropriate antibiotic. Although not documented, there is considerable information from reports of field outbreaks indicating that antibiotic treatment in the terminal stages accelerates death. This may be because administration of antibiotics to an animal with septicaemia precipitates an endotoxin shock due to release of free endotoxin from the killed bacteria (De Alwis, 1999). Due to hunger, infrequent watering, shipping, exposure to harsh weather conditions, sudden changes in temperature and atmospheric conditions, there is a possibility of the pH dropping to 7.3, 7.2, or, even as low as 7.1, and, as the optimum pH for the growth of the *Pasteurella bovisepticus* is 7.2, it has an opportunity under those circumstances to grow and cause haemorrhagic septicaemia. Alkalization method using sodium bicarbonate solutions for treatment of haemorrhagic septicaemia has been reported (De Alwis, 1999).

2.3.9.2 *Control and prevention*

Haemorrhagic septicaemia can be eradicated with quarantines, movement controls, tracing of contacts, euthanasia of infected and exposed animals, and cleaning and disinfection of the premises. *P. multocida* is susceptible to most common disinfectants, as well as to mild heat (55°C/131°F). In endemic areas, this disease is mainly prevented by vaccination. The removal of carriers from the herd is also helpful. Management to keep the animals in good condition can reduce the risk of clinical signs and/or transmission of the organism. Animals should not be crowded or stressed, especially during wet weather. Antibiotic treatment is effective only if it is started very soon after the onset of clinical signs (OIE, 2009).

2.4 WHOLE CELL PROTEIN PROFILING OF *PASTEURELLA MULTOCIDA*

Eight *P. multocida* isolates, collected from different localities of Pakistan, were characterized by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

technique (Nawaz *et al.,* 2006).. After sonification, the bacterial proteins were separated by centrifugation. Proteins from sonicated supernatant were salted out by ammonium sulfate (Nawaz *et al.,* 2006). Sonicated supernatant, as well as ammonium sulfate precipitated proteins, were analyzed. Molecular weights of proteins were determined from graph between Rf value and log of molecular weight. The ammonium sulfate treated samples showed fewer bands with low molecular weights of 54, 45, 42, 40 and 20.5 kDa, while total 31 visible bands were observed in sonicated supernatants ranging from 126 to 11 kDa. The common protein bands in both preparations were of 54 and 23 kDa molecular weight (Nawaz *et al.,* 2006). Ireland *et al*. (1991) analyzed proteins profiles by using sonicated *P. multocida* strains of serotype 1 isolated from HS cases. They found complex protein profiles with a large number of bands. Patterns obtained by staining with coomassie blue were similar. Srivastava (1998a) reported 12 polypeptides ranging from 120 to 30 kDa in sonicated cells. He observed some protein bands only in sonicated cells like 120, 105, 81, 60 and 62 kDa, while some only in 54, 52, 50, 45, 40 and 30 kDa.

2.4.1 Outer membrane proteins of *Pasteurella multocida*

In all countries where septicaemic pasteurellosis occurs, vaccinations are considered as an effective means of controlling this disease (De Alwis, 1996; Chandrasekaran *et al*., 1994). Local isolates are usually used for vaccine preparation. Initially, simple bacterins were used for immunization (Chandrasekaran *et al.,* 1994. Additionally, precipitated alum or aluminium hydroxide gel vaccines were manufactured (Chandrasekaran *et a.l*, 1994). The use of the vaccine with an oil adjuvant (water-oil emulsion) with a dense bacterin and mineral oil as water and oil phases, respectively, were advantageous in controlling this disease (Muneer *et al.,* 1994). Also several authors immunized cattle with live attenuated

vaccines (Chengappa *et al*., 1989; De Alwis and Carter, 1980). Outer membrane proteins of many Gram-negative bacteria are known to function as protective antigens. In *E. coli* the OMPs were first reported to serve as a receptor for high iron binding system responsible for providing protection in turkeys against colisepticaemia (Bolin and Jensen, 1987). Later, protective ability of outer membrane proteins (OMPs) was also demonstrated with *Pseudomonas aeruginosa* (Sokol and Woods, 1986) and *Shigella* species (Adamus *et al.*, 1980). Among pasteurellae, OMPs from *P. haemolytica* have been found to be associated with immunity in cattle against pneumonic pasteurellosis (Morton *et al.*, 1996). Strains of *P. multocida* have been reported to possess membrane proteins which are protective to rabbits (Lu *et al.,* 1991) and poultry (Zhang *et al.,* 1994; Confer *et al.,* 2001). Few reports considered the immunogenic properties of OMPs from *P. multocida* strains belonging to B: 2. 5 (Borkowska-Opacka and Kedrak, 2002). Srivastava (1998) studied the role of OMPs from this serotype in providing protection of animals against *P. multocida* infection. Mice immunized with whole cell vaccine showed at challenge a survival rate of 84% whereas in mice given OMPs vaccine, it was 67% (Srivastava 1998). Though the level of protection against OMPs was lower than the whole cell vaccine, still it was lower than the acceptable level, i.e. 67%. None of the nonimmunized control mice resisted the challenge. Mice immunized with whole cell vaccine gave the evidence that OMPs based vaccine was protective to laboratory animals. This was further confirmed by employing the passive mouse protection assay. Eighty percent of the mice treated with antisera against whole cells or OMPs were protected against the challenge infection. Similar results were also observed in cattle Srivastava, (1998). It was concluded that a vaccine consisting of *P. multocida* cells grown under iron-restriction may be more effective than the vaccine prepared from cells grown conventionally. Also Kennet *et al.* (1993) observed protection of mice vaccinated

with OMPs from *P. multocida* type B: 2. They reported that OMPs prepared in iron-deficient medium afforded better protection against experimental challenge exposure than did OMPs prepared in iron-sufficient medium. Subunit vaccines comprising OMPs from *P. multocida* serotype B:2 were used by Pati *et al.* (1996) to immunize buffalo calves and immunoblotting suggested that proteins of 44, 37 and 30 kDa were the major immunogens. Antibody production started from day 7 after vaccination and ELISA titres rose significantly at day 14 Pati *et al.* (1996). The titres further increased at day 21 but remained unchanged at day 26 Pati *et al.* (1996). All buffalo calves given OMPs vaccine survived challenge with virulent *P. multocida*. Results suggested that OMPs were protective and could be used in vaccines against HS. The occurrence of antibodies against specific outer membrane proteins detected by immunoblotting and ELISA in the sera of immunized cattle argued for a beneficial immunogenicity of the vaccines. *Pasteurella multocida* (serotype B: 2) may cause primary infections called (HS) in older cattle. This microorganism serotype A: 3 is involved in the etiological diseases of the respiratory system in calves. The outer membrane proteins (OMPs) play a significant role in the pathogenesis of pasteurellosis (Dubreuil *et al.*, 1992). Several OMPs are immunogens and the antibodies produced against these OMPs demonstrate a strong protective action. Such antigens may be used as components of subunit vaccines. The immunogenicity of selected OMPs of *P. multocida* was demonstrated in rabbits, calves and chickens (Zhang *et al.*, 1994; Dabo *et al.*, 1997; Confer *et al.*, 2001). Moreover, the protective action of OMPs of serotype B:2 against HS was reported (Pati *et al.*, 1996). Numerous authors provided evidence that *P. multocida* strains grown *in vivo* and *in vitro* on an iron-restricted medium produce surface antigens with protein properties and a high molecular weight; these antigens are recently considered iron regulated outer membrane proteins (IROMPs) (Choi

et al., 1991). Apart from detailed mechanisms of their action, the proteins participate in a complex system of iron uptake from the host cells and are regarded as one of the factors of bacterial virulence (Choi *et al*., 1991). These antigens used as immunogens revealed immune properties against pasteurellosis caused by various *P. multocida* strains (Srivastava, 1998b). The antibodies against IROMPs may block significant receptors and prevent bacteria from iron uptake (Ruffolo *et al*., 1998). Srivastava (1998a) demonstrated an enhanced production of 84 kDa protein by the strain of serotype B: 2 cultured in iron-restricted media. The vaccines prepared using the culture of such strains stimulated the increased production of antibodies in mice, rabbits and cattle as compared to the vaccines with antigens obtained from the strains grown under normal conditions. On the other hand, Kennett *et al*. (1993) found that the vaccination of cattle with the IROMPs of serotype B: 2 markedly increased protection against experimental infections with the strains causing HS.

2.5 IMMUNOLOGY OF HAEMORRHAGIC SEPTICAEMIA INFECTION

Bain, (1954) found that some cattle examined in Thailand were naturally immune to HS. The importance of this finding in the epidemiology of HS was not realised at the time, and it was concluded that about 10% of cattle and buffaloes in Asia may be naturally immuned to HS. Investigations made in Sri Lanka in the late 1970's (De Alwis, 1982) indicated that the proportion of naturally immuned animals varied from herd to herd , and from time to time in the same herd. This phenomenon was related to recent occurrence of HS in the herd or locality. While a few instances of cattle naturally immuned to HS have been found in Australia (Bain, 1979) and in Chad (Perreau, 1961 cited by Bain, et *al*., 1982) where natural exposure was not evident, sufficient evidence has been deduced by De Alwis (1982) in a study of the immune status of cattle exposed to HS, that subclinical or arrested

infection is the chief source of naturally acquired immunity in enzootic countries. In situations where no vaccination is practised, it is clear that the morbidity and the mortality patterns are largely governed by the proportion of naturally immuned to non immuned animals at the time of the outbreak. Thus, in enzootic areas where frequent outbreaks occur, the proportion of naturally immuned animals is high, and consequently only a small number of hitherto unexposed susceptible animals die. When the disease occurs after a long interval, the proportion of non immuned susceptible animals is high, and explosive outbreaks occur. *P. multocida* is a primary or, more frequently a secondary invader in pneumonia of cattle, swine, sheep, goats and other species (Carter and Wise, 2003). Following an epizootic of haemorrhagic septicemia, surviving clinically normal in-contact animals carry in their nasopharynx virulent *P. multocida* (Carter and Wise, 2003). Such animals could excrete the organism in their nasal secretions and constitute a source of infection to susceptible animals (Hiramune and De Alwis, 1982). Vaccination against *P. multocida* is applied only when the outbreak of haemorrhagic septicemia occurs. At such situation, if vaccination is to be accomplished, all populations of cattle or all herds will not be covered. Therefore, the majority of HS antibody titers that were found may be due to natural infections. On the other hand, indirect haemagglutination (IHA) test titer was indicative of natural exposure and confers solid immunity, whereas the IHA response to vaccination was poor (De Alwis and Sumandasa, 1982). The sensitivity of IHA test was low to reveal the vaccination induced immunity. In a group of vaccinated animals, immuned to direct challenge by *P. multocida* serogroup B, only 27% showed IHA antibody (De Alwis and Sumandasa, 1982; De Alwis *et al*., 1990). Antibodies against serogroups B, E and A were demonstrated by IHA, respectively in 93%, 98% and 98% of c alves tested in USA (Swada *et al*., 1985).

2.5.1 The capsule

Polysaccharide capsules are found on the surface of a wide range of bacteria. In Gram negative bacteria, the capsule lies outside the outer membrane and is composed of highly hydrated polyanionic polysaccharides (Moxon and Kroll, 1990; Roberts, 1996). Capsule has a significant role in determining access of certain molecules to the cell membrane, mediating adherence to surfaces and increasing tolerance to desiccation. Furthermore, the capsule of pathogenic bacteria generally impairs phagocytosis and may reduce the action of complement-mediated killing (Finlay and Falkow, 1989; Moxon and Kroll, 1990). Thus, capsule is likely to be a major virulence determinant and indeed genetically defined acapsular mutants have been shown to have reduced virulence in a number of organisms including: *Vibrio vulnificus, Streptococcus pyogenes, Staphylococcus aureus and Actinobacillus pleuropneumoniae* (Zuppardo and Siebeling, 1998; Thakker *et al*., 1998; and Ward *et al*., 1998). Capsule has been implicated in virulence in *P. multocida* as encapsulated strains have been shown to be more virulent (Jacques *et al*., 1993) and able to resist complement-mediated killing (Snipes and Hirsh, 1986; Hansen and Hirsh, 1989). However, no genetically defined acapsular strains have been constructed to allow unequivocal demonstration of the *P. multocida* capsule as a virulence determinant. *P. multocida* strains can be separated into serogroups A, B, D, E and F based on the antigenicity of the capsule (Carter, 1967; Rimler and Rhoades, 1987) and serotypes 1-16 based on LPS antigens (Heddleston *et al*., 1972). *P. multocida* M1404 belongs to serotype B:2 and although, nothing is known of the chemical structure of its capsule, the *P. multocida* B:6 capsule has been shown to contain D-mannose, D-galactose and L-arabinose at a ratio of 2.0:0.8:0.5, but no chemical linkage structure has been determined (Muniandy *et al*., 1992). Experiments with purified *P. multocida* B: 6 capsular extract was reported to

have indicated non-toxic, non protective in mice and rabbits but has significant antiphagocytic activity, although it was noted that the extract used contained small amounts of nucleic acid and protein contaminants (Muniandy *et al.*, 1992). The nucleotide sequences of capsule biosynthetic loci which conform to the *Escherichia coli* group II K antigen type have been completely determined in a number of species and also from many serotypes within E. coli. The genetic organisation of the loci is highly conserved, with genes being organised into three functional regions (Roberts, 1996). Region 1 and 3 (designated Regions C and B in *Neisseria meningitidis*) encode proteins involved in transport of the polysaccharide (Roberts, 1996). These include proteins involved in phospholipid substitution of the polysaccharide and those involved in inner membrane, periplasmic and outer membrane translocation. There is significant conservation of the proteins involved in polysaccharide export both within different serotypes of the same species and between species (Roberts, 1996). Region 2 (or Region A in *N. meningitidis*) contains genes involved in the biosynthesis of the polysaccharide capsule and this region is serotype specific. Each serotype expresses a different type of polysaccharide and therefore requires a different subset of genes to synthesise the specific sugar polymer (Roberts, 1996). The entire capsule biosynthetic locus of a fowl cholera strain of *P. multocida* A: 1 has been cloned and its nucleotide sequence determined (Chung *et al*., 1998). The capsule has been implicated in the virulence of *P. multocida*. Boyce *et al.* (2000a) have identified and determined the nucleotide sequence of the *P. multocida* B2 capsule biosynthetic locus. Wild-type, mutant and complemented strains were tested for virulence by intraperitoneal challenge infection of mice; the presence of the capsule was shown to be a crucial virulence determinant Boyce *et al.* (2000a). Following intraperitoneal challenge of mice, acapsular bacteria were removed efficiently from blood, spleen and liver while wild-type bacteria multiplied

rapidly Boyce *et al.* (2000a). The acapsular bacteria were readily taken up by the murine peritoneal macrophage, but the latter ones were significantly resistant to phagocytosis. Both wild-type and acapsular bacteria were resistant to the complement in bovine and murine sera (Boyce *et al*., 2000b). Limited information is available on antigens of *P. multocida* that stimulate immunity in cattle. In haemorrhagic septicaemia, capsular antigen, LPS or LPS-protein complex, and outer membrane proteins, including iron-regulated outer membrane proteins, are effective immunogens for serogroups B and E (Boyce *et al.*, 2000b).

2.5.2 Vaccination

Some control is achieved with alum-precipitated or oil-adjuvanted killed whole-cell vaccines injected subcutaneously (S.C.), but these vaccines had the disadvantage of providing only short-term immunity and require annual administration for effectiveness (De Alwis, 1992; Chandrasekaran *et al*., 1994). The oil-adjuvanted vaccines have the added disadvantage of high viscosity, which makes them unpopular among field users, although improved oil-adjuvan-ted vaccines with lower viscosities have been described (Shah *et al*., 1997). However, all such vaccines suffer from a requirement for high numbers of inactivated cells (10^{10} to 10^{11} cells) and consequent problems of reactogenicity. Live attenuated vaccines in general have the advantage of a natural route of entry into the host, which allows targeting of immunostimulatory factors to the same sites of the immune system that occur in the natural infection, but for live strains to be used as vaccines, the mode of attenuation should be well defined.

2.5.3 Public health significance of *P. multocida*

There are no reports of human infections with *P. multocida* serotypes B: 2 and E: 2; however, other serotypes of this organism do infect humans, and precautions should be taken to avoid exposure (De Alwis, 1999).

2.5.4 Economic importance of haemorrhagic septicaemia

Haemorrhagic septicaemia (HS) is a disease of utmost economic importance particularly in Asia and to a lesser extent in Africa. In Asia the susceptible animal population consists of 432 million cattle and 146 million buffaloes, which constitute 30% and 95% of the world's cattle and buffalo population, respectively (De Alwis, 1984). In India where the production of milk is highest in Asia, around 50% of the milk comes from the more susceptible buffaloes. In Asia as a whole, the contribution of the buffaloes to the milk production is 37% (De Alwis, 1984).. Most of the cattle and buffaloes are used as draught animals in the rice fields, and rice is the staple diet in many countries. Thus, the high population of buffaloes in Asia, the high susceptibility of buffaloes to HS and the high case fatality, all point to the significance of the economic losses due to the disease (FAO, 1994). In India, for four decades it was found that HS accounted for 46–55% of all bovine deaths (Dutta *et al.*, 1990). During the twelve year period of 1974 to 1986, it accounted for 58.7% of the aggregate deaths due to five endemic diseases, viz. foot-and-mouth disease (FMD), rinderpest, blackquarter, anthrax and HS (Dutta *et al.*, 1990). In an active surveillance study in Sri Lanka, it was shown that in the 1970's, around 15% buffaloes and 8% cattle in the HS endemic areas died of the disease annually (Dutta *et al.*, 1990). During the same period, the passive reporting systems recorded only 1200 to 1500 deaths a year in a cattle and buffalo population of approximately 2.5 million (De Alwis and Vipulasiri, 1980). Other countries in South Asia also ranked HS as the most economically important infectious dis-

ease or the most economically important bacterial disease. Pakistan reported that 34.4% of all deaths in susceptible stock was due to HS (Sheikh *et al*., 1994). With a cattle and buffalo population of 17.7 and 18.8 million respectively, the annual economic losses due to HS have been estimated at 1.89 billion rupees (350 million USD) (Chaudhry and Khan, 1978; FAO, 1979; Sheikh *et al*., 1994). In the South-East Asian region, countries such as Indonesia, Malaysia, Thailand, Myanmar, Laos, Cambodia and the Philippines rank HS high among the economically important diseases of cattle and buffaloes (Patten *et al*., 1993). In Myanmar it is reckoned that 50% of the government's effort in animal disease control is directed towards HS (Patten *et al*., 1993). Malaysia, with a relatively small population of 735 000 cattle and 186 000 buffaloes, estimated the animal losses due to HS to be 2.25 million Malaysian Ringit (0.85 million USD) (FAO, 1979). Most estimates of losses take into account only direct losses, i.e. value of animals that die of HS. A true estimate of losses should take into account a variety of factors which constitute indirect losses including the following:

(i) Loss of productivity – milk, meat, draught power, and cost of alternate sources of draught power.

(ii) Impairment of the reproductive potential of the animals.

(iii) A reliable differential diagnosis as there is tendency during the Monsoon to attribute any mortality to HS. Thus a possible over-estimation of these losses should be taken into account. However, it must also be borne in mind that reported losses constitute only a fraction of the actual losses. This is bound to be so since HS is a disease that occurs in situations where husbandry practices are poor and therefore disease reporting system will also be poorly developed. In a study in Bangladesh, an attempt was made to compute the

economic losses resulting from three important endemic diseases, anthrax, blackquarter and HS (Patten *et al*., 1993). It was found that the direct losses which took into account the market value of the animals that died and the cost of treatment was 2.3 million US dollars (Ahmed, 1996). Their computation of indirect losses took into account the value of the rice that would have been produced from the land left uncultivated as a result of loss of draught power. These losses amounted to 148 million USD annually due to these three diseases (Ahmed, 1996). It may therefore be concluded that no accurate estimates are available on the actual deaths due to HS, and the available information on direct and indirect economic losses is incomplete. The available information, however, suggests that HS is a disease of considerable economic importance in the Asian Region and other parts of the world. Haemorrhagic septicaemia is rooted in Africa and Asia with regular sporadic or massive seasonal outbreaks resulting in tremendous losses. There is no definite statistical data to estimate its actual economic impact. As economically important disease, HS represents one of the most serious diseases of livestock; it causes great losses in buffaloes and cattle, which are vitally important for the rural economy in many countries (Bain, 1963; Bain *et al*., 1982). There are many integrated factors that affect the incidence of HS; these include clinical, immunological and epizootiological factors (Carter and DeAlwis, 1989).

2.5.5 *Pasteurella multocida* Polymerase Chain Reaction (PM-PCR) diagnosis

PM-PCR techniques play a critical role in the clinical laboratory as rapid and specific method for detection of microorganism which has provided remarkable advances in the diagnosis of infectious agents, particularly in cases where the presence of organism is having significance (Relman and Persing, 1996). Townsend *et al.* (1998) developed PCR assay for species and type specific identificatin of *P. multocida* isolates and found that the

primer pair designed from the sequences of the clone 6b (KTT72 and KTSP61) specifically amplified a DNA fragment from HS causing type B isolates (B: 2, B: 5, and B: 2, 5) of *P. multocida* and got approximately 590 bp amplified product size. These primers were unable to amplify DNA from other *P. multocida* serotypes, other *Pasteurella* species, other members of the *Pasteurellae* family, or unrelated bacteria. They also reported that no product was amplified from type B *P. multocida* isolates possessing somatic serotypes other than type 2, type 5, or type 2, 5. Townsend *et al.* (1998) also developed PM-PCR by the primers KMT1T7 and KMT1SP6 and found an amplification product of approximately 460 bp from all strains of *P. multocida*, from the three *P. multocida* subspecies reference strains and from *Pasteurella canis* biotype 2. Townsend *et al.* (1998) also reported that these *P. multocida* specific PCR (PM-PCR) provided a rapid, sensitive method for detection of clinically infected birds with *P. multocida*. Polymerase chain reaction (PCR) amplification preformed directly on bacterial colonies or a culture represents an extremely rapid, sensitive method of *P. multocida* identification (Townsend *et al.,* 1998). Neuman *et al.* (1998) compared PCR and microbiological techniques for the detection of *P. multocida* in swab samples from dogs. They reported that both sensitivity and specificity of the PCR compared with the culture were 100% and the PCR detected *P. multocida* within 24 hrs which was not influenced by the presence of other bacteria in the samples. Neuman *et al.* (1998) reported that PCR is more reliable technique for detection of *P. multocida* than other microbiological techniques. Lee *et al.* (2000) carried out PM-PCR based on the method of Townsend *et al.* 1998) with minor modification by using 12 strains of *P. multocida* originating from poultry in Australia and Vietnam. They reported that use of selective medium and the PCR assay were highly sensitive in detecting 100 cfu from colony contents after experimental oral infection. Lee *et al.* (2000) found 460 bp amplicon from all the

serotypes (A: 1, A: 3, and A: 4) which were used throughout the study. Dutta *et al.* (2001) carried out species specific PCR (PM-PCR) assay using 18 Indian isolates of *P. multocida* of various serotypes and nine other organisms.They revealed an amplified product of approximately 460 bp for all the *P. multocida* isolates, while the other organisms were negative for the amplicon. They also found that these PCR techniques can be easily carried out by using genomic DNA, culture lysate, direct bacterial colony from plate as well as from contaminated cultures. Townsend *et al.* (2000) reported that *P. multocida* produced an amplified product of 460 bp size using primer KMT1SP6 and KMT1T7. Shivshankara *et al.* (2001) carried out serotype B specific PCR assay by using primers KTSP61-KTT72 for diagnosis of HS. They found that these primers specifically amplified a product of approximately 590 bp from HS causing type B isolates of *P. multocida*. These primers were unable to amplify DNA from other *P. multocida* serotypes, other *Pasteurella* species, other members of *Pasteurellaceae* family or unrelated bacteria. Anupama *et al.* (2003) carried out study to evaluate PCR for identification of virulent strains of *P. multocida* using primers IPFWD and IPREV. They found that these primers would specifically amplify *P. multocida* serotype B: 2 and also found that the PCR techniques using the above primers can be used as tool to differentiate virulent and avirulent *P. multocida*. Javia (2004) tested 13 field isolates of *P. multocida* along with one vaccine strain (P52) and five other bacterial cultures by PM-PCR in the laboratory, and revealed an amplified product of approximately 465 bp size for all the *P. multocida* isolates while other bacterial cultures did not. Patel (2004) tested reisolated colonies of *P. multocida* along with *E. coli* (negative control) by PM-PCR in the laboratory, which revealed amplified product of approximately 465 bp size of all the *P.multocida* isolates while *E.coli* culture could not.

2.5.6 Detection of *P. multocida* by real time PCR

This is the latest method for detection of *Pasteurella multocida* in field sample.This a more highly sensitive and specific test than PM PCR and Multiplex PCR (Rajeev *et al*., 2011). A colony lift hybri-dization assay using a commercially available multicolour detection kit was recently developed for rapid and simultaneous detection of toxigenic *Pasteurella multocida* and *Bordetella bronchiseptica* (Register *et al*., 1998). The major advantage of this assay is the ability to screen the suspect colonies in primary isolation plate, there is no need of pure cultures (Rajeev *et al*., 2011). It can analyze large number of samples in a very short period.

2.5.7 Molecular typing of *P. multocida*

Pasteurella multocida is a heterogeneous species that produces septicemic or respiratory diseases in domesticated and wild animals (Rimler and Rhoades, 1989). Considerable variation has been observed among strains with respect to host, predilection, pathogenicity, carbohydrate fermentation, colonial morphology, and antigenic specificity (Carter and Chengappa, 1981). Serological differences were also observed in the type-specific capsular antigen of *P. multocida* (Carter, 1961). This resulted in the development of the indirect hemagglutination test, which now recognizes five distinct capsular serogroups (serogroups A, B, D, E, and F) (Rimler and Rhoades, 1989). Until recently, little was known of the composition of capsular material from *P. multocida* serogroups other than that of serogroup A (Carter, 1958; Carter and Annau, 1953). Nuclear magnetic resonance studies confirmed that the major polysaccharide component of the capsule was hyaluronic acid (Rosner *et al.*, 1992). The capsular materials of serogroups D and F have been identified primarily through the action of mucopolysaccharidases (Rimler, 1994). On the basis of the decapsulation

profiles of *P. multocida* by these enzymes, it was proposed that serogroups D and F produced capsular material that contained heparin and chondroitin sulfate, respectively (Rimler, 1994). The production of a chondroitin or chondroitin-like polysaccharide capsule by *P. multocida* serogroup F was recently confirmed by carbohydrate analysis (DeAngelis and Padgett-McCue, 2000). The monosaccharide analysis of a serogroup B *P. multocida* strain determined that the purified capsular polysaccharide was composed of arabinose, mannose, and galactose in a ratio of 0.5:2.0:0.8. The chemical composition of the serogroup E capsule remains unknown. The biosynthetic loci of the complete capsules of *P. multocida* serotypes A: 1 and B: 2 was identified with gene identification within the serogroup-specific region 2 of both loci (Chung *et al.,* 1998; Boyce and Adler, 2000). Two genes within this region of the B:2 locus, *bcbA* and *bcbB*, were similar to *Escherichia coli wecB* and *wecC*, which catalyze the conversion of UDP-*N*-acetylglucosamine to *N*-acetyl-d-mannosaminuronic acid (Boyce and Adler, 2000). The presence of these homologues in the serogroup B *cap* locus suggests that the mannose identified as the major type B capsular component (Boyce *et al.,* 2000a). Region 2 has also been partially cloned and sequenced from serogroup F, with the identification of a glycosyltransferase involved in elongation of chondroitin polymers (DeAngelis and Padgett-McCue, 2000). A detailed review of the composition, function, and genetics of the *P. multocida* A:1 and B:2 capsules was published (Boyce *et al.,* 2000b). The lack of genetic knowledge regarding the capsular material of serogroups D, E, and F and the increasing need for a simple, DNA-based capsular typing method provided the impetus for the investigatio. Oligonucleotide primers designed during sequencing of the biosynthetic loci of the capsules of serogroups A and B were used to determine the nucleotide sequences of the region 2 genes from the remaining three capsular serogroups Serogroups D, E, and F (Boyce *et al.,* 2000a). Serogroup-specific

sequences were then identified for use as primers in a multiplex PCR assay and sequence analysis of the biosynthetic locus of the capsule of an organism can lead to a greater understanding of its capsular polysaccharide composition and can provide a genetic basis for the serological differences observed between strains (Boyce *et al* 2000b). (Chung *et al.,* 1998) identified components responsible for the synthesis of hyaluronic acid, consistent with hyaluronic acid being the principal component of the type A capsule. However, genetic analysis of the serogroup B biosynthetic locus revealed only three gene products with similarity to proteins known to be involved in polysaccharide biosynthesis, while six gene products had no similarity to known proteins (Boyce *et al.,* 2000a). The structure of the type B capsule remains unknown. Until recently, very little was known about the compositions of the serogroup D and F capsular polysaccharides, with even less known about the serogroup E capsular polysaccharide (Boyce *et al*., 2000a).

2.5.8 *Pasteurella multocida* type B PCR

The PCR amplification can also detect the serotype B specific *P. multocida* directly from HS causing type B and has been found to be 100 % specific for type B serotypes of *P. multocida* isolates. Serotype B cultures with any combination of somatic antigen are identified by the amplification of a 620 bp fragment with the following primers:

KT SP61: 5'- ATC CGC TAA CAC ACTCTC- 3'

KTT72: 5'- AGG CTC GTT TGG ATTATG AAG- 3' (Townsend *et al*., 1998; 2001).

2.5.9 *Pasteurella multocida* type A specific PCR

Pasteurellosis has long been recognized as a disease of major economic importance among livestock and birds. Its confirmation is sometimes difficult due to varied clinical signs and

time-consuming laboratory procedures. It has been observed that the detection of *P. multocida* in clinical material is greatly accelerated by the use of PCR technique. The advantages of the PCR compared with existing tests include speed, sensitivity, specificity and simplicity. It does not require culture or laboratory animals and is, therefore, safer as a result of the avoidance of handling live bacteria. Serogroup A PCR method was designed to amplify the *hyaC–hyaD* gene of *P. multocida*. This gene was an ideal amplification target for PCR because hyaluronic acid is a principal component of type A capsule (Chung *et al*., 1998; Townsend *et al*., 2001). The capsules of several bacteria including *P. multocida* are important virulence factors (Moxon and Kroll, 1990; Boyce and Adler, 2000a). The presence of hyaluronic acid in type A strains has been documented (Chung *et al*., 1998) and the entire capsule biosynthetic locus of *P. multocida* has been sequenced and the components responsible for the synthesis of hyaluronic acid, which is a principal component of type- A capsule, identified (Chung *et al.*, 1998). Targeting a gene encoding hyaluronic acid could enable the design of primers that amplify the target molecule specific to isolates of *P. multocida* bearing capsular type A. Primers for typing of serogroup A strains which cause a number of infections in livestock and poultry have been reported to be useful in specific identification of isolates.

The forward primers RGPMA5: 5'- AATGT TTG CGATAG YCC GTTAGA- 3'and reverse RGPMA6: 5'- ATTTGG CGC CATATC ACAGTC- 3'gives PCR amplicon size of 564 bp which confirms the presence of *P. multocida* serotype A (Gautum *et al*., 2004).

2.6 MULTIPLEX PCR FOR *PASTEURELLA MULTOCIDA* CAPSULAR TYPING

A multiplex PCR assay is a rapid alternative to the conventional capsular serotyping system and used for capsular type determination. The serogroup specific primers used in this assay

were designed following identification, sequence determination and analysis of the capsular biosynthetic loci of each capsular group. The multiplex capsular PCR assay is highly specific and its result correlates well with conventional capsular serotyping results with the exception of those for some serogroup F strains (Townsend *et al*., 1998; 2001). The capsular typing of all the isolates can be determined by multiplex PCR using capsular types A, B, D, E and F specific primers.

2.6.1. REP- PCR and ERIC- PCR

Repetitive Extragenic Palindromic (REP) and Enterobacterial Repetitive Insertion Consensus (ERIC) PCR have been developed for the characterization of *P. multocida* isolates. REP elements (33 to 40 base pair repeats) are present in 500- 1000 copies accounting for up to 1% of the genome (Stern *et al*., 1984) and are present in a wide range of bacteria (Olive and Bean, 1999). As the REP elements are distributed widely across the genome, it produces a multiple banding pattern. ERIC- PCR has been successfully used to differentiate strains of *Pasteurella multocida* (Olive and Bean, 1999). The visual analyses of banding patterns were in range of 100- 900 bp. The band patterns provide DNA fingerprints which allows distinction between species and between strains within species.

2.6.2 Ribotyping

Ribotyping in conjunction with REA has been widely used to characterize and differentiate the *P. multocida* isolates (Blackall *et al.*, 1995). REA followed by additional hybridization with a labeled DNA probe made it easy to read the banding pattern and give the necessary interpretation. The probe may be labeled either by radio active or non- radioactive materials. rRNA probe is widely accepted for hybridization and subsequent interpretation (Blackall and Miflin, 2000).

2.6.3. Restriction endonuclease analysis (REA)

Restriction endonucleases cleave the DNA at specific nucleotide sequences and produce a set of DNA fragments which, upon electrophoresis separate into a characteristic banding pattern or fingerprint of the respective genome. Restriction endonuclease analysis has been successfully used as a tool for differentiation of strains in a variety of bacterial infections including that caused by *P. multocida*. Several restriction enzymes (HhaI, HpaII, SmaI, BglII, PstI, EcoRI) have been taken into consideration for characterization of the different isolates of *Pasteurella multocida* (Zhao and Aoki, 1992).

2.6.4 Status of *P. multocida* infection in Nigeria

By conventional microbiological methods, investigation was carried out by (Ugochukwu, 2008) in Nsukka and Enugu areas of Enugu State, Nigeria to determine the percentage frequency of occurrence of *P. multocida* from caprine pneumonic lungs. Four of the isolates were characterized as *P. multocida.* Inspite of the low percentage frequency (1.14%) of isolation of *P. multocida* in that study, attention was drawn to the pathogenic potential of this organism for goats and other livestock in Nigeria (Ugochukwu, 2008). An outbreak of haemorrhagic septicaemia caused by *P. multocida* was reported in which six out of seven holstein bulls, about 2 years old, died in the University of Ibadan Teaching and Research Farm in the rainy season of 1971 (Anosa and Isoun, 1975). Careful analysis of the outbreak indicated that it might have been precipitated initially by climatic stress associated with the onset of heavy rainfall, a marked drop in atmospheric temperature and an equally marked increase in relative humidity which occurred in June, and exacerbated later by combinations of chronic debilitating infections including streptothricosis, trypanosomosis, anaplasmosis, and strongylosis (Anosa and Isoun, 1975). Although the buffalo is

indigenous to West Africa, in Nigeria it is now confirmed to the game reserve at Yankari and occasionally as specimens in zoos such as the one at Jos. This probably means that it is not a serious reservoir of infection for domestic cattle in Nigeria (Kasali, 1972). A case of haemorrhagic septicaemia in an African buffalo (Syncerus nanus) was described in Nigeria for the first time. It occurred in a buffalo in captivity at the zoo in Jos. Robert' s Type 1 organism were recovered (Kasali, 1972).

An outbreak of pasteurellosis caused by *Pasteurella multocida* was reported in six elands (*Taurotragus oryx*), a gnu (*Connuchaetes taurinus albojubatus*), a zebra (*Equus burchelli*), five kangaroos (*Macropus rufus*), an ostrich (*Struthio camelus camelus*) and a bateleur eagle (*Terathopius ecaudatus*) in Kano Zoological Garden towards the end of the rainy season in 1978 (Okoh, 1980)

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY AREA

This study was carried out in Plateau state, north central zone of Nigeria. Plateau state of Nigeria lies between latitude 80^0 22 and 100^0 24 North and longitude 80^0 32 and 80^0 32 East with a total land area of 26899 square kilometres (RIM, 1993). It is bordered in the North West by Kaduna state, in the North East by Bauchi state, in the South West by Nasarawa state and in the south East by Taraba state (Fig 3.1). The state has temperate

climatic condition with average temperature of 25 ^{0}C, while the annual rainfall varies from 131.75cm in the southern part to146 cm on the Plateau (RIM, 1993). The state falls largely within the northern savannah zone which consists mainly of short trees, grasses and plateau type mosaic vegetation. The human population was estimated at 3.5 million people in 2007 (RIM, 1993), with over 30 ethnic groups, and a large percentage of the population are engaged mostly in farming. Livestock production, which accounts for 25% agriculture of the state is under the traditional management system, although there are few commercial farms, and the cattle population of the state is estimated at about 1.5 million (RIM, 1993).

3.1.1 Sample size

Asuming the prevalence of 50 % of HS for Plateau state, a total of 384 samples were caculated using the formula ($N = Z^2pq/d^2$) outlined by (Thrushfield, 1997), where N = Sample Z =, the appropriate value from the desired confidence (1.96), P= Prevalence from previous study, q = 1-prevalence and d= Allowable error, 0.05.

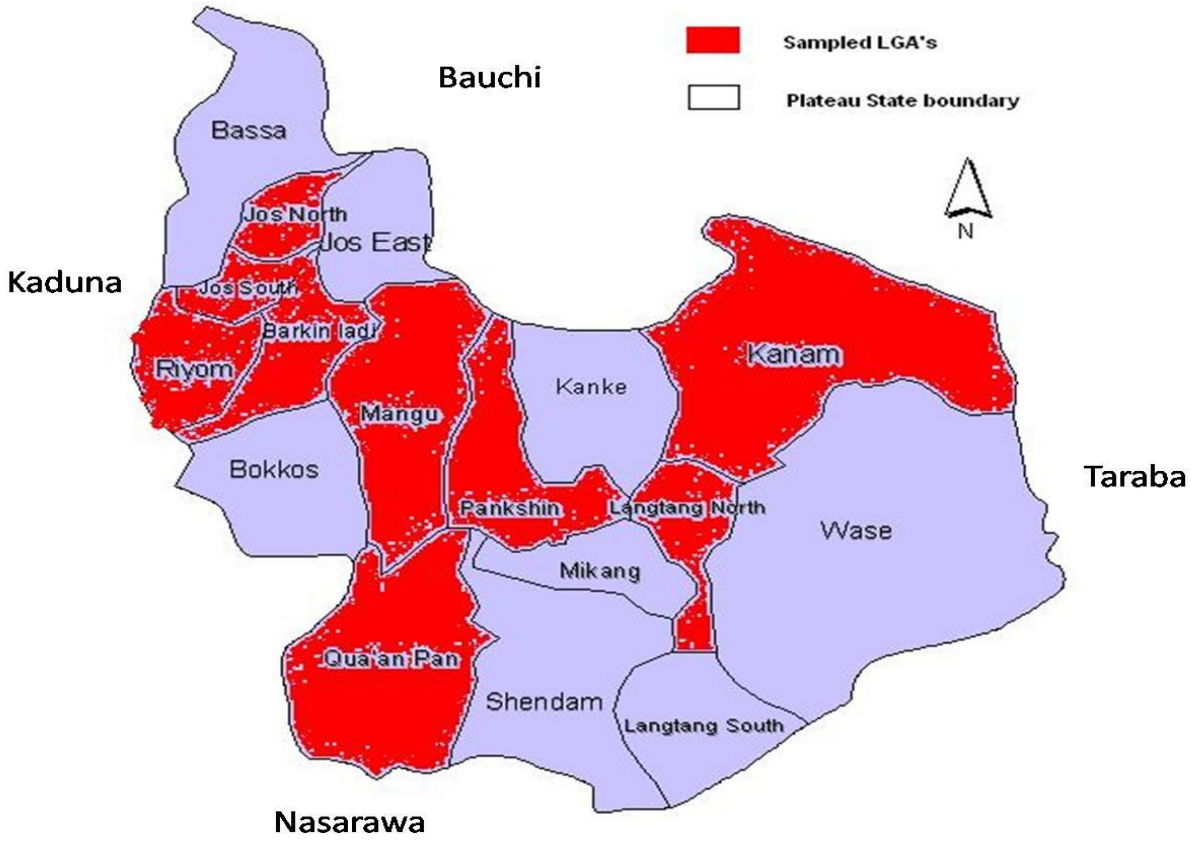


Figure 1: Area of study (Plateau state) From Wikipedia, the free encyclopedia, 2010.

3.1.2 Sampling methods

Three hundred and ninety six samples including, lungs, liver and spleen, from ninety one animals slaughhtered in abattoir/slaughter slabs and nasal swabs from two hundred and twenty one live animals were collected from cattle in the nine Local Government Areas of Plateau state using purposive sampling technique according to Portney and Watkins (2007). The criteria used were; haemorrhages, congestion and necrosis in tissue samples and physical conditions in live animals. The age (Young and Adult), Sex (Male and Female), breeds (Exotic and Local) of the animals were recorded. The samples were placed in sterile plastic bottles, kept in a Coleman box containing ice and transported to the National Veterinary Institute Vom laboratory for examination.

3.2 CULTURAL EXAMINATION OF SAMPLES

Nasal and throat swabs were cultured indirectly by inoculating into 5 ml of brain heart infusion broth (BHI), incubated at 37°C for 24 h aerobically and then streaked onto Casein Sucrose Yeast (CSY) Agar (selective media) (OIE, 2008). The tissues were cultured directly on to CSY agar by disinfecting the surface with a hot spatula and cut upened using a sterile scizures and with a sterile wire loop, we picked and streaked on the CSY incubated at 37^0C for 24 h and then subcultured on MacConkey agar MCA with salt (Biotec laboratories) UK, which was used as primary culture media for preliminary isolation of the organisms from the samples according to methods described by Quinn *et al.* (1994). The isolates which failed to grow on MCA were presumed to be *P. multocida.* Single non-haemolytic colonies of these isolates were picked from primary culture and re-streaked on fresh Blood agar (BA) plate and incubated at 37 ^{0}C for 24 h to obtain single colonies of pure culture of the isolates. The cultures so obtained were subjected to Gram's staining to check for purity of the growth and morphology of the organisms. The cultures were then

transferred onto nutrient agar slants for storage, pending further identification by biochemical tests and other characterization.

3.2.1 Identification of *P. multocida* by conventional biocheimical test

Further tests included: Oxidase, Catalase, Indole, Citrate utilization, Nitrate reduction and fermentation of glucose, mannitol, sucrose, mannose, maltose, arabinose, lactose, dulcitol, salicin, inositol and trehalose as per methods described by Cowan and Steel (1985) with modification of using CSY agar.

3.2.2 Oxidase test

Standard oxidase discs (oxoid) were used to perform the test. The loopful of culture from single colony was used to make an impression on the disc. Immediate development of blue colour was considered as positive.

3.2.3 Catalase test

This test was performed by taking 2-3 drops of three per cent H_2O_2 on a clean grease free glass slide and single colony was mixed with the help of wire loop. Immediate formation of gas bubbles was considered as positive test.

3.2.4 Indole test

Few drops of xylene were added in a two day old growth of the isolates in 2 ml of tryptone water and mixed thoroughly to dissolve and about 0.2 drops of kovacs reagent was added. Pink layer of xylene was considered as positive reaction.

3.2.5 Citrate utilization

Slant of simmon's citrate agar (Oxoid) was inoculated with culture and incubated at 37 ^{0}C for four days. Growth with a development of intense blue colour of the medium was considered as a positive reaction.

3.2.6 Nitrate reduction

Add few drops of few days' old culture in peptone water containing 0.1 percent potassium nitrate and incubated at 37 ^{0}C for two days. Presence of nitrate was detected by adding approximately 1 ml of sulfanilic acid and 1 ml of alpha naphthylamine reagent to nitrate broth culture. Development of a distinct red colour (which may turn to brown rapidly) was considered as positive test.

3.2.7 Carbohydrate fermentation tests

Fermentation reactions of ten sugars viz; glucose, mannitol, sucrose, mannose, maltose, arabinose, lactose, dulcitol, inositol, and lactose were studied. One percent of each sugar (Oxoid) in peptone water base with one percent Andrade' s indicator was used. Few drops of one day growth of the isolates in peptone water were inoculated into each sugar and a thin layer of sterile liquid paraffin was spread over the medium. Tubes were incubated at 37 ^{0}C 48 hrs and readings were recorded after 24 hrs. Production of pink red colour was considered as positive. Proper controls were always kept for each of the biochemical tests performed.

3.3 IDENTIFICATION OF *P. MULTOCIDA* USING MICROBACT GNB 24E

The organisms were further confirmed using Microbact GNB 24 according to the manufacturer recommendations (Oxoid). Before testing, all isolates were streaked on blood agar containing 5% bovine blood and incubated at 37^{0}C for 24 h. To prepare the test

inoculum, sterile normal saline (5 ml) was dispensed into each test tube. Using a sterile loop, 1-3 isolated colonies of the culture were picked and emulsified in the saline, which was mixed thoroughly and incubated at 37 ^{0}C for 4 hours to obtain a homogeneous suspension. The wells of the individual substrates set were exposed by cutting end tag of the sealing strip and slowly peeled backward. The plate was placed in a holding tray and using a sterile Pasteur pipette, four drops of the bacterial suspension were added to each well set. The substrates underlined on the holding tray were overplayed with 1 drop of mineral oil, i.e. wells 1, 2, 3, 20 and 24. The inoculated wells were sealed with adhesive seal. The specimen number was written on the end tag with a marker pen. The plates were incubated at 37 ^{0}C for 48 hours. The plates were removed from the incubator after 48 h, the adhesive seal peeled and Nitrate, Kovacs, Voges- Proskauer (VP) and Trytophan Deaminase (TDA) reagents were added to wells 7, 8, 10 and 12 respectively. The results were read and interpreted as stipulated by the manufacturer and organisms identified using the software version Microbact ^{TM} 200 identification package V2.03 (Windows ^{TM}). *Pasteurella multocida* B: 3, 4 standard vaccine strain was used as control.

3.3.1 *In vitro* Antimicrobial Sensitivity of *P. multocida* Isolates

Susceptibility testing was performed using the standardised single disk diffusion method of Bauer *et al*. (1966) on Mueller-Hinton agar (Schering Corporation, Bloomfield, NJ, USA) plates. The agar was enriched by the addition of 5 % horse serum commercially prepared. The medium was prepared and poured into petri dishes at a depth of 5–6 mm. The agar was allowed to set at room temperature and then refrigerated for use within 24 hours. The selection of antibiotics was based largely on the most commonly used antibiotics against Gram negative bacteria available in the market and previous studies. The

following antibiotics were used for the study; Amoxycillin/clavulanic acid (30 µg), Tetracycline (5 µg), Chloramphenicol (30 µg), Gentamicin (10 µg), Oxacillin (5 µg), Vancomycin (5 µg), Streptomycin (10 µg), Ciprofloxacin (5 µg), Erythromycin (5 µg), Sulphamethoxazole/trimethoprim (25 µg), Penicillin (10 i/u), Ampicillin (10 µg). All antibiotics were supplied by Oxoid. Combinations of 12 antibiotic disks were used with no more than 6 disks per plate. Then the plates were incubated at 37 ^{0}C for 24 h. The inhibition zone around each disk was measured indepently and compared with standard interpretative charts: Clinical Laboratory Standards Institute (CLSI, 2009). Zone size for each antimicrobial agent was measured independently before comparison. *Pasteurella multocida* vaccine whose strain B: 3.4 was used as control.

3.3.2 *Pasteurella multocida* specific PCR was carried out as follows to identify the isolates

Template DNA was prepared according to manufacturers instruction (ZR Fungal/Bacterial DNA mini prep) and 5 µl was added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 3.2 pmol of each primer and 0.5 µl *Taq* DNA polymerase. *P.-multocida*-specific

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

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

Cycling parameters for a Corbett FTS-320 thermocycler were as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. The reaction was held at 4°C until required for electrophoresis; 5 µl of each sample was electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 h. PCR products

along with 100 bp DNA ladder were loaded with 3 µl DNA loading buffer, electrophoresed in two per cent agarose gel. PCR products were visualized as a single pink fluorescent band under the UV light. The gel was stained with 1% ethidium bromide and DNA fragments were viewed by UV transillumination. The PCR technique is based on the protocol described by Townsend *et al.* (1998).

3.3.3 *Pasteurella multocida* type A specific PCR

Template DNA was prepared according to manufacturers instruction (ZR Fungal/Bacterial DNA mini prep) and 5 µl was added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 3.2 pmol of each primer and 0.5 µl *Taq* DNA polymerase *P. multocida* type A specific primers.

RGMA5FWD: 5'-AAT-GT-TTG-CGA-TAG-TCC-GTT-AGA-3'
RGPMA6REV: 5'-ATT-TGG-CGC-CAT-ATC-ACA-AC3'

Cycling parameters for a Corbett FTS-320 thermocycler were as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. The reaction was held at 4°C until required for electrophoresis; 5 µl of each sample was electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 h. PCR products along with 100 bp DNA ladder were loaded with 3 µl DNA loading buffer, electrophoresed in two per cent agarose gel. PCR products were visualized as a single pink fluorescent band under the UV light. The gel was stained with 1% ethidium bromide and DNA fragments were viewed by UV transillumination. The PCR amplification yields a product of 564 bp. The technique was based on the protocol described by Townsend *et al.* (1998).

3.3.4 *Pasteurella multocida* type B specific PCR

Template DNA was prepared according to manufacturers instruction (ZR Fungal/Bacterial DNA mini prep) and 5 µl was added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 3.2 pmol of each primer and 0.5 µl *Taq* DNA polymerase *P.-multocida* type A specific primers.

KTT72 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'
KTSP61 5'-ATC-CGC-TAA-CAC-ACT-CTC-3'

Cycling parameters for a Corbett FTS-320 thermocycler were as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. The reaction was held at 4°C until required for electrophoresis; 5 µl of each sample was electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. PCR products along with 100 bp DNA ladder were loaded with 3 µl DNA loading buffer, electrophoresed in two per cent agarose gel containing ethidium bromide. PCR products were visualized as a single pink fluorescent band under the UV light. The gel was stained with 1% ethidium bromide and DNA fragments were viewed by UV transillumination. Somatic antigen typing antisera was prepared in chickins. The PCR technique was based on the protocol described by Townsend *et al.* (1998).

3.3.5 Somatic typing of isolates

Determination of somatic serotypes by the agar gel immunodiffusion (AGID) test (Heddleston *et al.*, 1972) was performed. The isolates were typed using the procedure of

Hendleston *et al.* (1972), and the source of antisera used was from chicken. A panel of of 16 reference antibodies made against (Henddleston *et al.*, 1972) reference serotypes 1-16 was used in the typing procedure. Antigen and antisera controls were used in each test.

3.3.6 Antigen preparation

The 18 to 24 hour growth from a heavily seeded blood agar plate was suspended in 1 ml of 0.85% NaCl solution containing 0.3% saturated solutions of formaldehyde. The suspension of cells was heated in a resistant glass test tube in water bath at 100 ^{O}C for one hour. The cells were sedimented by bench minor centrifugation at 5000 g for 10 minutes at room temperature, and the supernatant was used as the antigen in the gel diffusion precipitin test within 24 hrs of preparation. Antiserum was sourced from National Veterinary Services Laboratories, USDA, Ames, Iowa, USA.

3.3.7 Preparation for agar gel

The agar gel consisted 0.9% special agar- noble (Difco), 8.5% NaCl and 0.01% sodium azide in distilled water. Six wells were created by a template. Gel was cast on glass slide using 5 ml of molten agar. Thereafter 0.01 ml of antigen preparation was placed in the center well and equal volume of antisera in the outer wells of the melted agar. The test plates/slides were incubated at 37 ^{0}C in a humid chamber and result was recorded after for 24 - 48 h.

3.3.8 Somatic serotyping of isolates

The isolates were shipped to the National Veterinary Services Laboratories, USDA, AMES, IOWA, USA for somatic typing: The isolates were typed using the procedure of Henddleston *et al.* (1972). A panel of of 16 reference antibodies made against Henddleston

reference serotypes 1-16 was used in the typing procedure. Antigen and antisera controls were used in each test.

3.4 *PASTEURELLA MULTOCIDA* MULTIPLEX CAPSULAR PCR TYPING SYSTEM

Capsular serogroup-specific sequences were used as primers in multiplex PCR-typing system (Boyce *et al*., 2000). The *P. multocida* specific primers were included as an internal control for species identification.

Table 3.1. **Multiplex capsular typing of *Pasteurella multocida* primers.**

		Capsular type	Expected amplicon size
CAPA-FWD	5-TGC-CAA-AAT-CGC-AGT-GAG-3'	A	1044 bp
CAPA-REV	5'-TTG-CCA-TCA-TTG-TCA-GTG-3'		
CAPB-FWD	5'-CAT-TTA-TCC-AAG-CTC-CAC-C-3'	B	760 bp
CAPB-REV	5-GCC-CGA-GAG-TTT-CAA-TCC-3		
CAPD-FWD	5,-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC-3'	D	657 bp
CAPD-REV	5,-CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG-3'		
CAPE-FWD	5'TCC-GCA-GAA-AAT-TAT-TGA-CTC3'	E	511 bp
CAPE-REV	5,GCT-TGC-TGC-TTG-ATT-TTG-TC3'		
CAPF-FWD	5'AAT-CGG-AGA-ACG-CAG-AAA-TCA-G-3'	F	851 bp
CAPF-REV	5,-TTC-CGC-CGT-CAA-TTA-CTC-TG-3'		
KMT117	5'ATC-CGC-TAT-TTA-CCC-AGT-GG-3'	*P multocida*	460 bp
KMT1SP6	5,-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'	Specie specific	

3.4.1 *PCR condition*

Template DNA was added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl2, 3.2 pmol of each primer and 1 u *Taq* DNA polymerase. Initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds; with a final extension at 72°C for 5 minutes (Boyce *et al.*, 2000).

3.5 Plasmid DNA detection

Isolates were grown overnight at 37 ^{o}C in 3 ml BHI broth for plasmid isolation. Plasmid DNA was extracted by using Plasmid DNA extraction Kit (Fermentas Life Sciences Germany), in which the procedure was carried out according to the manufacturer's instructions. The presence of plasmid DNA after extraction was detected by agarose gel electrophoresis.

3.5.1 Agarose gel electrophoresis

Plasmid DNA was resolved by electrophoresis in submerged horizontal agarose slab gel (0.9%) in tris-borate buffer (TBE). The required amount of agarose (Sigma Chemical Co., USA) was dissolved in tris-borate buffer (pH 8.4) by boiling. The DNA ethidium bromide complex in the in the gel were visualized on a 302 nm UV transilluminator. This was then photographed with a camera fitted to the UV transilluminator. Gloves were worn at all times when handling solution and agarose slab gels containg ethidium bromide, as it is a powerful mutagen and carcinogen.

3.6 STATISTICAL ANALYSIS

The data was analysed using Fisher's Exact test and chi square. Microsoft excel was used to determine prevalence in order to generate descriptive statistic.

CHAPTER 4

RESULTS

4.1 ISOLATES OF *PASTEURELLA MULTOCIDA*

Of the 396 lungs, liver, spleen, nasal and throat samples tested, 18 isolates from the samples were Gram negative rods, non-haemolytic, round, grayish, smooth and mucoid colonies on Casein sucrose yeast agar (CSY) and blood agar. They were small, coccobacilli and non-motile. They were oxidase, catalase and ornithine decarboxylase positive, were urease negative and fermented glucose without production of gas, mannitol and sucrose positive but not lactose and showed no growth on MacConkey agar, reduced nitrate to nitrite, were indole positive and did not utilise citrate. They did not fermented maltose, arabinose, lactose, dulcitol, salicin, inositol and trehalose. Jos North had the highest percentage positive samples of 13.5 %, followed by Kanam L.G.A with 9%. Barkin ladi had a percentage positive sample of 6.8 %, Jos south 4.5%, Mangu 4.4% and Quanpaan 2.2%. Langtang North, Ryom and Pankshin did not have a positive sample (Table. 4.1).

The presence of *P. multocida* were examined from the nine local government areas according sex. It was found that presence of the organism based on sex, male 6 (2.8) and female 12 (6.7) had no significance difference usingt fishers extract test $P > 0.05$ (Table. 4.2).

In the age distribution, young 8 (7.1) and old 10 (3.5) had no statistical significance by the statistical test, fishers exact tet $P > 0.05$. (Table. 4.3).

There was significance difference in organ distribution (lungs, liver, and nasal swabs) $P < 0.05$. (Table. 4.4).

All the eighteen isolates tested by microbact 24E (oxoid) were confirmed to be *P. multocida* using the supplied software version Microbact ^{TM} 200 identification package V2.03 (Windows ^{TM}). The percentage probabilities of 12 isolates were above 75% and 6 others were below 75% by the software interpretations package. The cut off percentage by the software was 75% and above. Microbact gives percent probability of the organism being *P. multocida* above or below 75%. Quanpaan LGA QP had (99.35%) positive isolates from lung sample, followed by Jos north, Jn18 97.56%, Jn12 92.75 %, Jn14 99.19%, and Jn5 85.44 % had positive isolates above 75% from lungs except Jn6 (66.16%) from liver and Jn3 (69.77%) from lung had positive isolates below 75%. Barkin ladi Bld3 (96.23%), Bld15 (77.46%) and Bld10 (97.14%) from spleen, liver and lung respectively had positive isolates above 75%. Kanam L.G.A, Ka2 (75.40%) and Ka3 (93.53%) positive isolates were above 75% isolated from lungs. Ka4 (56.58%) and Ka5 (42.96%) isolated from liver were below 75%. Jos south L.G.A Jst2 (97.14%) was isolated from liver had positive isolates above 75%, and Jst8 isolated from lung had percentage positive sample below 75% was isolated from lung. In Mangu L.G.A Mg7 (91.45%) isolated from lung with percentage positive isolate above 75%, Mg14 (48.00%) isolate from spleen was below 75% (Table: 4.5).

The results of the antimicrobial susceptibility testing study showed a high level of resistance and multiresistance among the isolates tested. The isolates were commonly resistant to tetracycline (100%) and erythromycin (100%). Among the 18 isolates tested, 13(72.2%) were sensitive to sulphamethoxazole/trimethoprim. The sensivity of the isolates to streptomycin were 61.1%. Sensitivity of isolates to amoxycillin/clavulanic acid was 44.4%, ciprofloxacin were sensitive to 38.9%, a similar result was observed in

chloramphenicol, and it was sensitive to 33.3%. Sensivity to other antimicrobial agent, ampicillin 27.8 %, penicillin 10 iu, oxacillin and vancomycin had sensitivity of 5.6% each and 94.4% each to the isolates (Table.4.6).

Out of the eighteen isolates only 3 could be typed by the Hendleston *et al.*, (1972) scheme. Two of the isolates from lungs in Jos North LGA, Jn3 and Jn18 contain the same somatic antigens of 3,4. One isolate Jn5 isolated from lung in Jos north LGA contains somatic antigen of 2, 5. The other isolates could not be typed by the Henddleston *et al.* (1972) scheme (Table 4.7).

Eighteen isolates of *P. multocida* were examined for the presence of plasmid DNA. All isolates indicated presence of one or more plasmid. All isolates of *P. multocida* tested harboured a plasmid of about 5kb. Three isolates had an additional plasmid of 3kb and one isolate Ka3 harboured a plasmid of 6kb. (Table 4.8).

The identity of the strain of *P. multocida* were further confirmed by PCR amplification of the gene with size of 460 bp. Plate I and II show the amplicon sizes of the gene fragments amplified by PCR. All the samples gave positive bands for species specific amplicon. The set of primers, KMT1T7 and KMT1SP6 were species specific and amplified the expected products from all isolates of *P. multocida.*

Plate V and plate VI had no band for *P. multocida* type B using *P. multocida* type B specific primers. It amplified only the species specific fragment used as internal control in the test.

The groups of the capsular strains of *P. multocida* were identified using multiplex capsular PCR together with the species specific primers to serve as internal control of the reaction. *P. multocida* species specific with size of 460 bp. Capsular group E with a size of 511 bp and a unique capsular type F with size of 851 bp were observed (Plate III and IV).

Agarose (0.9%) gel electrophoresis showing plasmid DNA extracted from of *P. multocida* serotypes. Plasmids were detected in 18 isolates tested. The most common plasmid size to all the isolates contains size of 5kb. One isolate contain size of 6 kb and three of the isolates 3kb (Plate VII).

Table 4.1: Local government area based percentage of positive samples collected

from cattle in Plateau state.

LGA	Male	Female	Young	Old	Lung	Liver	Spleen	Nasal	+ ve	% +ve
BL	26	18	15	29	4	0	8	32	3	6.8
LN	24	20	14	30	4	8	5	27	0	0.0
Ry	19	25	12	32	8	9	8	19	0	0.0
JS	19	25	31	13	10	5	6	23	2	4.5
JN	17	27	32	12	8	12	10	14	6	12.6
PNK	32	12	32	12	4	3	2	35	0	0.0
KN	26	18	31	13	3	3	10	28	4	9.0
MG	25	19	36	8	6	2	11	25	2	4.4
QP	28	16	35	9	3	15	8	18	1	2.2
Total	216	180	158	238	50	57	68	221	18	40.5

Key

LGA= Local government area

BL= Barkin Ladi

LN= Langtang north

RY= Ryom

JS=Jos south

JN= Jos north

PNK= Pankshin

KN= Kanam

MG= Mangu

QP= Quanpan

Table 4.2: Sex distribution of *P. multocida* isolates from nine local government areas of Plateau state.

Sex	No. of samples tested	No +ve (%)
Male	216	6 (2.8)
Female	180	12 (6.7)
Total	396	18 (4.5)

Fisher's Exact Test $P > 0.05$.

Table 4.3: Age distribution for *P. multocida* isolates from nine local government areas of Plateau state.

Age	No. of samples tested	No +ve (%)
Young	112	8 (7.1)
Old	284	10 (3.5)
Total	396	18 (4.5)

Fisher's Exact Test P > 0.05.

Table 4.4: Organ distribution of *P. multocida* isolates from nine local government areas of of Plateau sate.

Organs	No of samples tested	No +ve (%)

Lungs	50	6 (2.8)
Liver	57	12 (6.7)
Spleen	68	2 (2.9)
Nasal swabs	221	0.0
Total	396	18 (4.5)

$X^2 = 14.83$ df = 2. $P < 0.05$

Table 4.5. Percentage probability of *P. multocida* isolates with Microbact 24 E system.

S/N	Specimen No./Isolate No.	Specimen type	OCT INDEX	% Probability
1	BL3	Spleen	515632744	96.23
2	JN12	Lung	517632660	92.75

3	JN14	”	537736600	99.19
4	JN18	”	526733060	97.56
5	KA2	”	517622664	75.40
6	KA3	”	517732560	93.56
7	JN5	”	517232720	85.44
8	JST2	Liver	517722700	97.14
9	QP	Lung	517720700	99.35
10	BL15	Liver	517722760	77.46
11	MG7	Lung	577737500	91.45
12	BL10	”	517722700	97.14
13	JN6	Liver	517632764	66.16
14	JN3	Lung	517231760	69.77
15	KA4	Liver	513622742	56.58
16	JST8	Lung	517732750	48.00
17	KA5	Liver	517722764	42.96
18	MG14	Spleen	517732750	48.00

Table 4.6: Antibiogram data of 18 *Pasteurella multocida* isolated from cattle in Plateau state.

Antimicrobial agent	Concentration (µg/disk)	No. (%) of strains resistant n=18	No. (%) of strains sensitive n=18
SXT	25 µg	5(27.8)	13(72.2)

S	10 µg	7(38.9)	11(61.1)
AMC	30 µg	10(55.5)	8(44.4)
CN	10 µg	10(55.5)	8(44.4)
CIP	5 µg	11(61.1)	7(38.9)
C	30 µg	11(61.1)	7(38.9)
AMP	10 µg	13(72.2)	5(27.8)
P	10 i/u	17(94.4)	1(5.6)
OX	5 µg	17(94.4)	1(5.6)
VA	5 µg	17(94.4)	1(5.6)
TE	5 µg	18(100)	0(0.0)
E	5 µg	18(100)	0(0.0)

AMC=Amoxycillin/clavulanic acid, TE=Tetracycline, C=Chloramphenicol, CN=Gentamicin, OX=Oxacillin, VA=Vancomycin, S=Streptomycin, CIP=Ciprofloxacin, E=Erythromycin, SXT=Sulphamethoxazole/trimethoprim, P=Penicillin, AMP = Ampicillin.

Table 4.7: Somatic and capsular serotypes of *P. multocida* isolates from cattle in Plateau State.

Specimen No.	Specimen	Somatic type	Capsular type
BLD3	Spleen	Untypable	E
BLD9	Spleen	//	E

BLD10	Lung	//	E
JST2	Liver	//	E
JS8	Lung	//	E
JN6	Liver	//	F
JN5	Lung	2,5	E
JN3	Lung	3,4	E
JN12	Liver	Untypeable	E
JN14	Lung	//	E
JN18	Lung	3,4	E
KA2	Liver	Untypeable	E
KA3	Liver	//	E
KA4	Liver	//	E
KA5	Lung	//	E
MG4	Lung	//	E
MG7	Lung	//	E
OT2	Lung	Untypeable	E

Table 4.8: Plasmid profiles of 18 *P. multocida* isolated from Cattle in Plateau state.

S/No.	*P. multocida* isolates	Estimated sizes of plasmids harboured by isolates		
		6 kb	5 kb	3 kb
1	Ka2	_	+	+
2	Q2	_	+	-
3	JN18	_	+	_
4	Mg7	_	+	+
5	Mg4	_	+	_
6	JN6	_	+	_

7	JN14	_	+	_
8	Bld10	_	+	_
9	Bld9	_	+	_
10	Bld3	_	+	_
11	JST2	_	+	_
12	JN5	_	+	-
13	Ka5	_	+	-
14	Ka3	+	+	-
15	Jst8	-	+	_
16	JN12	_	+	+
17	Ka4		+	_
18	JN3	_	+	_

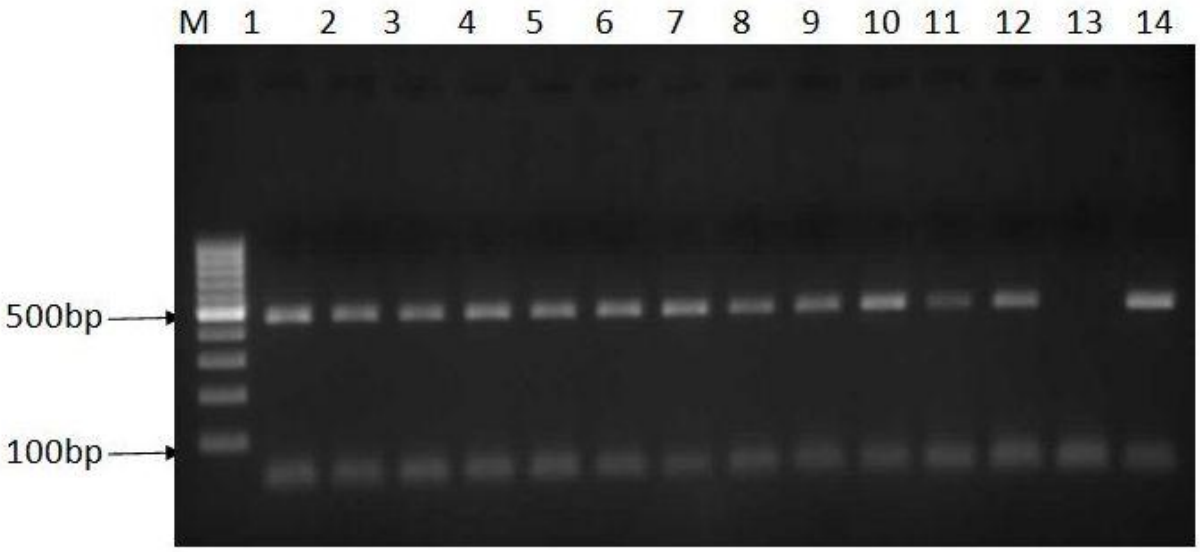
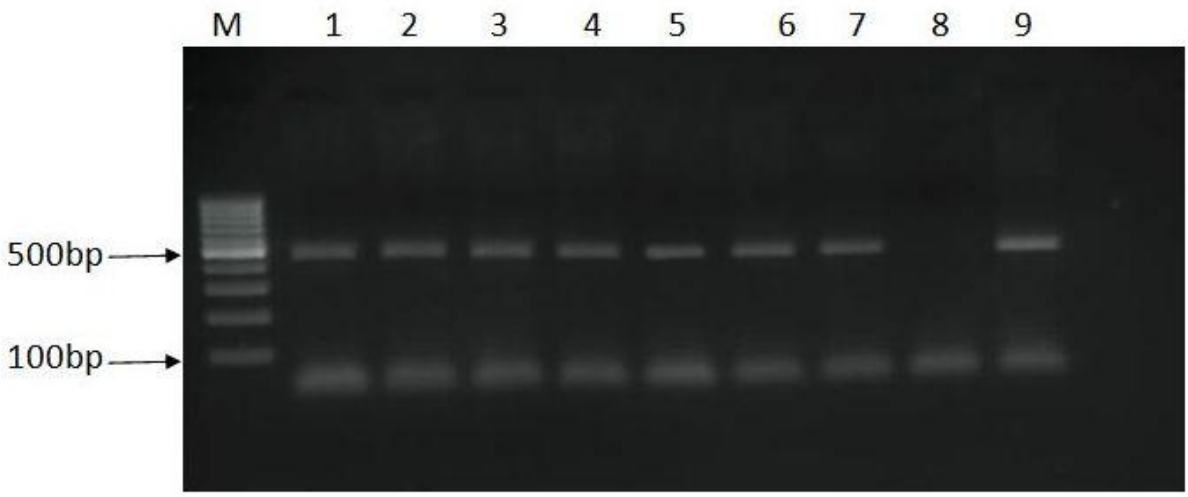


Plate I: Species specific PCR.

Amplicons of *P. multocida* with size of 460 bp (Species specific). Lane 1- Bld3 isolate, lane 2- Bld9 isolate, lane 3- Bld10 isolate, lane 4-Jst2 isolate, Lane 5-JS8 isolate, lane 6 Jn3 isolate, lane 7-Jn5 isolate, lane 8- lane 9- Jn12 isolate, Lane 10- Jn14 isolate, lane 11- Jn18 isolate, lane12 -Ka2 isolate, lane13-negative control, lane 14- positive control. lane M- 100 bp DNA molecular marker (Fermentas®).



Plat II: Species specific PCR.

Amplicons of *P. multocida* with a size of 460 bp (Species specic). Lane 1- Ka3 isolate, lane 2- ka4 isolate, lane 3- ka5 isolate, lane 4-Mg4 isolate, Lane5- Mg7 isolate, lane 6- Ot2 isolate, lane 7- Jn6 isolate, lane 8- a negative control, lane 9- positive control and lane M- 100 bp DNA molecular marker (Fermentas®).

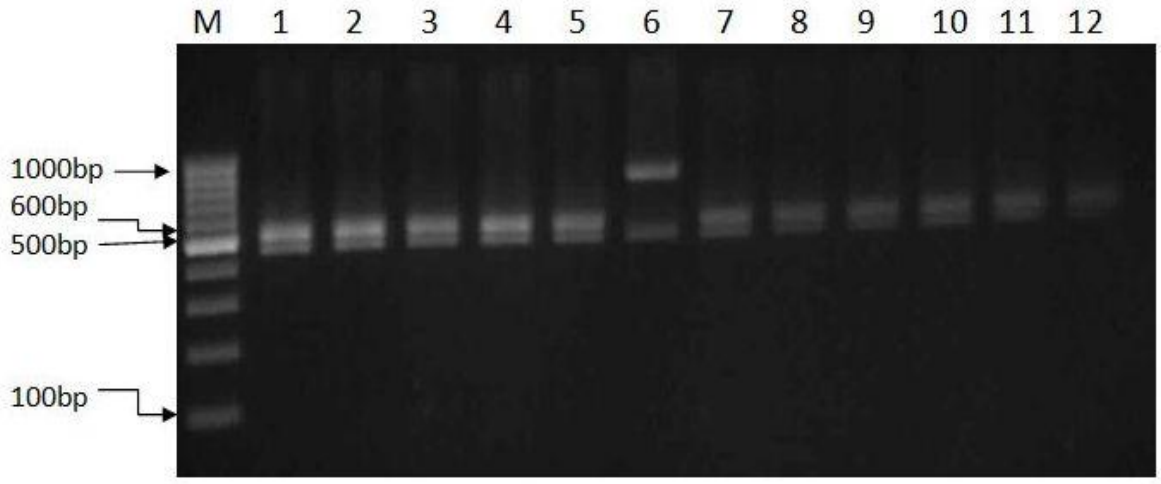


Plate III: Multiplex PCR capsular typing of *P. multocida*.
Amplicons sizes of 460 bp (species specific), 511 bp (capsular group E) and 851 bp (capsular group F) were obtained. Lanes 1- 5; 7 - 12 are capsular type E isolates. Lane 6 is capsular type F isolate. Lane 1- Bld3 isolate, lane 2- Bld 9 isolate, lane 3-Bld10 isolates, lane 4- Jst 2 isolate, Lane 5-JS8 isolate, lane 6- Jn3 isolate, lane 7- Jn5 isolate, lane 8-Jn6 isolate, lane 9- Jn12 isolate, Lane10-Jn14 isolate, lane11-Jn18 isolate, lane12-Ka2 isolate, and lane M- 100 bp DNA marker (Fermentas®).

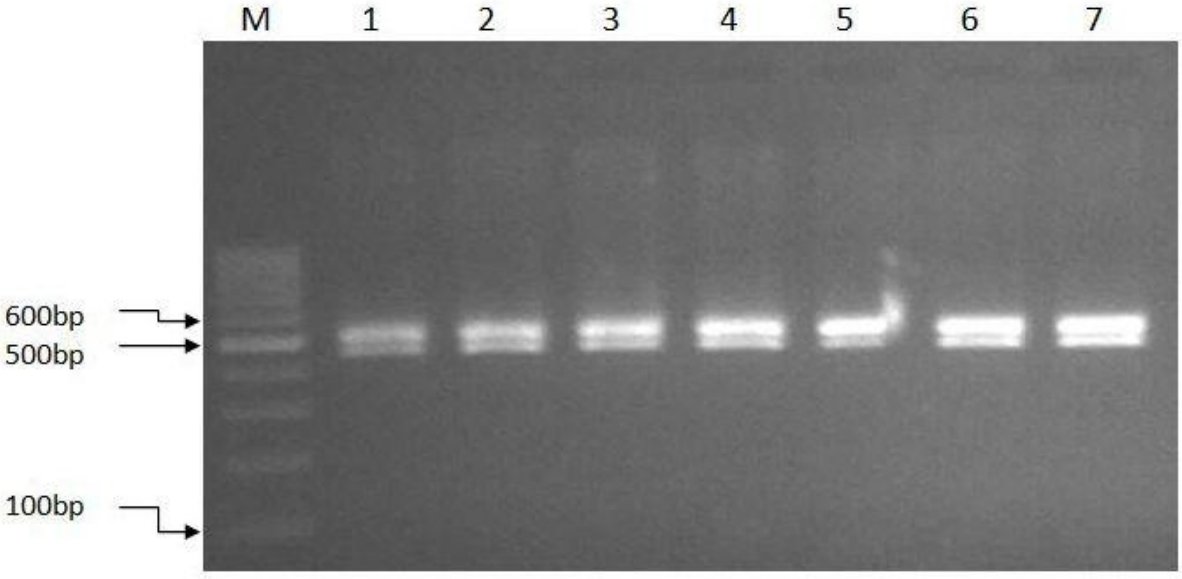


Plate IV: Multiplex capsular typing of *P. multocida*.
Amplicons sizes of 460 bp (species specific), 511 bp (capsular group E). Lane 1- Ka3 isolate, lane 2- ka4 isolate, lane 3- ka5 isolate, lane 4- Mg4 isolate, Lane5-Mg7 isolate, lane 6- Ot2 isolate, lane 7-Jn6 isolate and lane-M, 100 bp DNA molecular size maker (Fermentas®).

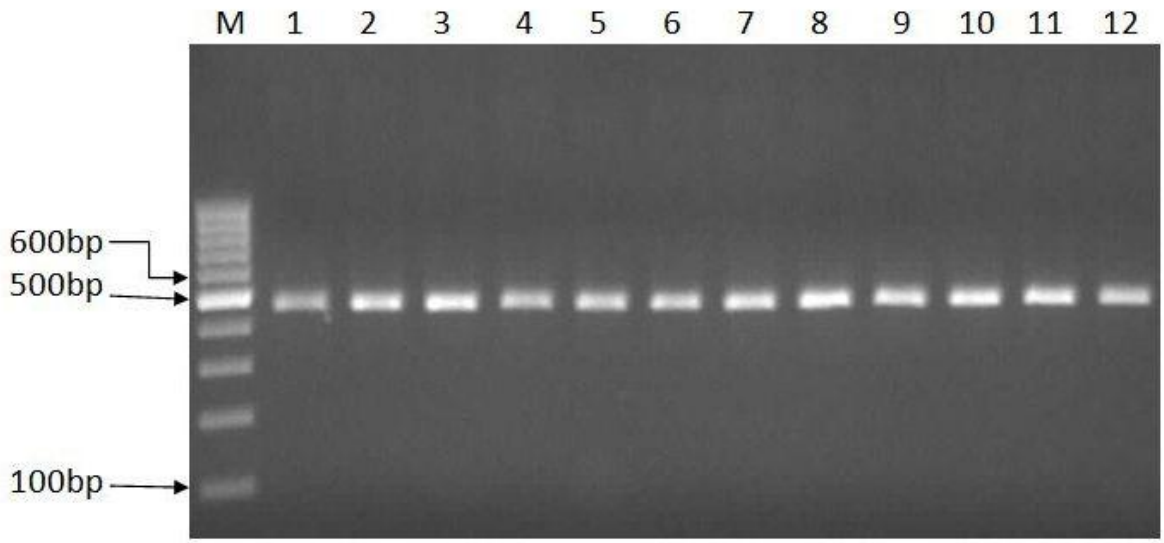


Plate V: Agarose gel electrophoresis for capsular group B amplicon. Amplicons of *P. multocida* with a size of 460 bp species specific not 620 bp expected for group B. Lane 1 Bld3, lane 2 Bld 9, lane 3 Bld 10, lane 4 Jst 2, Lane 5 JS 8, lane 6 Jn 3, lane 7 Jn 5, lane 8 Bld 10, lane 9 Jn 12, Lane 10 Jn 14,c, lane 11 Jn 18, lane12 Bld 15, and lane M, 100 bp DNA size marker (Fermentas®). This is to show that the isolates were all negative for capsular type B amplicons.

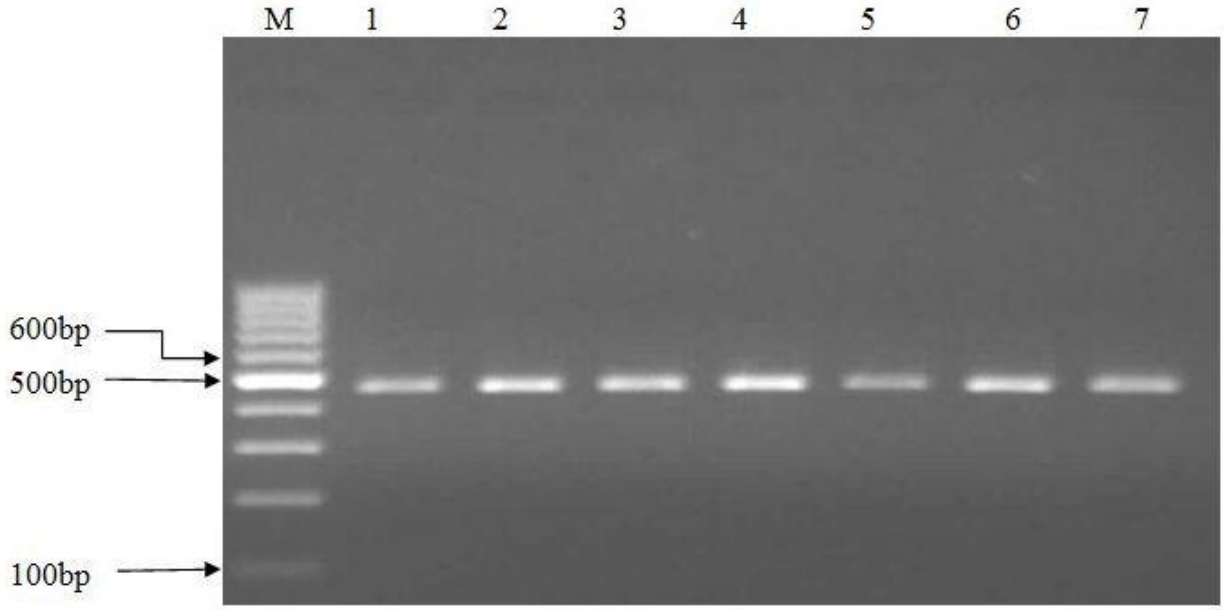


Plate VI: Agarose gel electrophoresis for capsular group B amplicon. Amplicons of *P. multocida* with of 460 bp not 620 expected for group B. This gel shows Lane 1 Ka3, lane 2 ka4, lane 3 ka5, lane 4 Mg4, Lane5 Mg7, lane 6 Ot2, lane 7 Jn6, and lane M, 100 bp DNA size marker (Fermentas®). This is to show that the isolates were all negative for capsular type B amplicons.

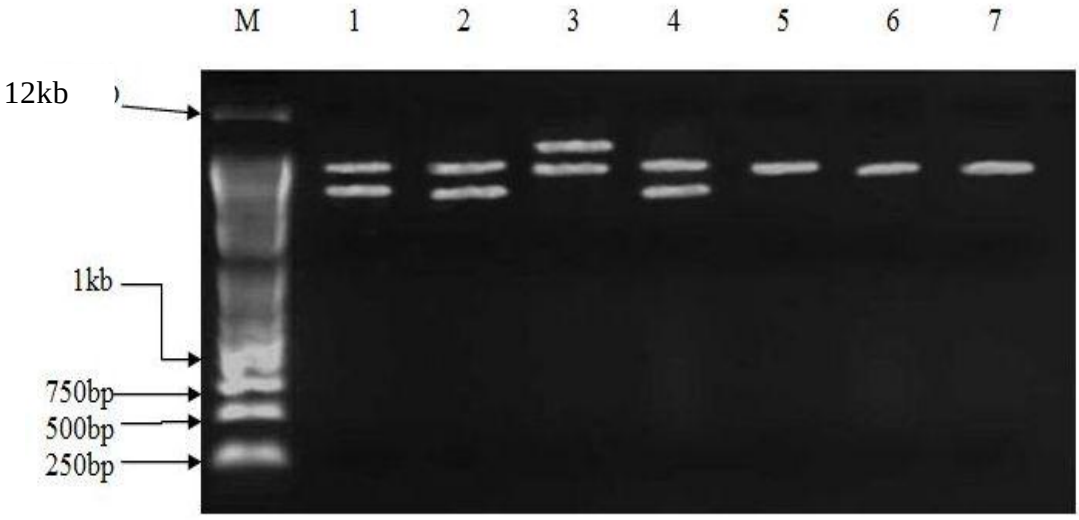


Plate VII: Plasmid Profile of *P. multocida* isolates from cattle.
Lane M – 1kb DNA molecular weight marker (Invitrogen®). Lane 1- Ka2 isolate, lane2- Ot2 isolate, lane3- Jn18 isolate, lane4- Mg7 isolate, lane 5- Mg4 isolate, lane 6-Jn6 isolate, lane7- Jn14 isolate. The band sizes of 6.0kb, 5.5kb and 3.0kb plasmids were isolated.

CHAPTER 5
DISCUSSION

This study is an attempt to define the current status of HS causing *P. multocida* isolates in LGA of Plateau state. These contemporary studies were aimed at unraveling or defining current spectrum of *P. multocida* serotypes in cattle in Nigeria which is lacking. Available information indicate that such similar studies were conducted about 3 decades ago. The study confirmed the presence of the African capsular strain (E), but of greater interest is a capsular group F strain which was identified for the first time in Nigeria. There was no statistical significance in sex and and age with regard to *P. multocida* distribution ($P > 0.05$). However, there was a significant difference in the distribution according to organs ($P < 0.05$). This might be because of the high affinity the organism has for lungs and the respiratory system of animals. *P. multocida* E: 3, 4 and *P. multocida* E: 2, 5 were identified amongst the isolates. *Pasteurella multocida* setrotype B:2 (6:B) and E:2 (6:E) are the principal causes of HS (Carter and De Alwis, 1989). Although serotype B:2 has been mainly reported in Asian countries and E:2 in African countries, both serotypes have been recovered from the disease in some African countries (Martrenchar and Njanpop, 1994; De Alwis, 1995; Kumar *et al*., 2004). All the isolates harboured a plasmid of 5 kb, three of the isolates had an additional plasmid of 3 kb, and one isolate had a third plasmid of about 6 kb. *P. multocida* is an important pathogen causing a number of diseases in various domestic and wild animals. The most important diseases are HS and septicaemic pasteurellosis in cattle and buffaloes, pneumonia and septicaemic pasteurellosis in sheep and goats, pneumonia, atrophic rhinitis and septicaemia in pigs and fowl cholera or avian cholera in poultry resulting in heavy economic losses (De Alwis, 1996). The present study recorded noticeable variation in the distribution and occurrence of *P. multocida* infections in different local government areas of the state. As evident from the study *P. multocida* the causative agent of haemorrhagic septicaemia occurred throughout the period of study. This

is in contrast to the perception that HS caused by *P. multocida* is mostly precipitated during the rainy season (Khrea, 1979). Moreover, *P. multocida* is known to have a wide distribution, particularly in tropical countries in Asia and Africa (Benkirane and De Alwis, 2002). All the 18 isolates from this study were found to exhibit typical cultural, morphological and biochemical characteristics as reported by previous workers in (Holt *et al*., 1994). None of the isolates exhibited any deviation from the typical characteristics. However, Waltman and Horne (1993) observed variability in oxidase, whereas variability in glucose fermentation was observed by Kumar *et al.* (1996).

In clinical aspects, antibiotic sensitivity assay serves as a guide to choose the correct antibiotic to be used in the field (Coates and Hoops, 1980). Bacterial organisms over a period of time change their antibiogram patterns and develop resistance against commonly used chemotherapeutic agents. In the present study, eighteen *P. multocida* isolates were tested for their sensitivity against twelve commonly used antibiotics. Upon testing of the 18 isolates, 8(44.4%) were found to be sensitive to gentamicin, amoxycillin, and streptomycin, respectively. All the isolates (100%) were found to be resistant to tetracycline and erythromycin and 13(72.2%) sensitive to Sulphamethoxazole/trimethoprim. Similar finding was reported by Dao *et al.* (1973), Karaivanov (1983), Sambyal (1988), Waltman and Horne (1993), Rajini *et al.* (1995), Das and Bhagwan (1997), Rahman (1997), Javia (2004) and Patel (2004) with same agent. However, Gupta *et al*. (1996) and Sharma *et al*., (2004) found 90 % and 63.46 % of the isolates sensitive to chloramphenicol, respectively. Gentamicin was found to be effective against 44.4 % of the isolates which is at variance with the findings of Verma (1991), Dimri *et al.* (1994), Das and Bhagvan (1997) and Rahman *et al*. (1997) where they all reported 100 % susceptibility to this agent. Aye *et al.*

(2001) found the majority of isolates sensitive to gentamicin and moderate sensitivity to gentamicin was noted by Sambyal *et al.* (1988). Gupta *et al.* (1996) and Jones *et al.* (2001) also found 90 % and 78 % of the isolates sensitive to gentamicin respectively. In this study, none of the isolates was found to be sensitive to tetracycline. Ampicillin was found effective against 72.2 per cent of the isolates, while Verma (1991) observed 85.7 % of the isolates susceptible to ampicillin. However, Aye *et al.* (2001) and Jonas *et al.* (2001) observed 100 % isolates to be sensitive to ampicillin. Sambyal *et al. (*1988) and Dimri *et al.* (1994) reported that ampicillin was not effective on any of the isolates. In the present study, 94.4 % of the isolates were resistant to penicillin-G, which is in accordance with the observation of Aye *et al.* (2001). In the present study, 38.9 % of the isolates were found resistant to streptomycin and 100 % of the isolates were found resistant to erythromycin. Similar results were obtained by Sambyal *et al.* (1988) and Das and Bhagwan (1997), while Dimri *et al.* (1994) and Gupta *et al.* (1996) found 97.14 %, 100 % and 49 % isolates sensitive to streptomycin and erythromycin respectively. Resistances to most of the drugs tested in this study may not be unconnected with the fact that these inexpensive drugs are widely available from distributors and can be purchased easily from vendors without a prescription in Nigeria. There is therefore the need to legislate and enforce laws to limit the prescription and dispensation of antibiotics to only qualified professionals. Also the susceptibility of the isolates to Sulphamethoxazole/trimethoprim and streptomycin having less resistance among the isolates tested in this study may be suggestive of fact that they are less abused in the environment and may be used for the treatment of *P. multocida* causative agent of HS (Sambyal, 1988). There is also need to educate the cattle owners on the dangers of indiscriminate use of drugs.

The development of molecular tools has paved the way for rapid and specific identification of infectious agents, thus PCR was used for identification of *P. multocida*. During the present study all the isolates of *P. multocida* and the B2 vaccine strain used as control were positive for species specific amplified product of aproximately 460 bp size using primers KMT1SP6 and KMT1T7 (Townsend *et al.,* 1998). These findings confirmed the results obtained by Townsend *et al.* (2000); Lee *et al.* (2000), Dutta *et al.* (2001), Javia (2004) and Patel (2004), who reported the specificity of this primer pair for all *P. multocida* isolates. Townsend *et al.* (1998) reported that the primer pair gave amplification from all strains of *P. multocida* (all serotypes and capsular serogroups), the three subspecies *i.e. P. multocida* subsp. *multocida*, *P. multocida* subspp. *gallicida*, *P. multocida* subspp. *septica* and also *P. canis* biotype 2. Dutta *et al.* (2001) also carried out PM-PCR using various serotypes of *P. multocida*. It is evident from present results and earlier related reports, that PM-PCR provides rapid identification of *P. multocida* isolates irrespective of serotypes and can be used as rapid diagnostic method specific for detection of *P. multocida* infection.

Presumptive identification of HS-causing type-B-specific *P. multocida* is also possible by PCR amplification (Brickell *et al*., 1998; Townsend *et al*., 1998). Comparative analysis with the *Haemophilus influenzae* Rd genome indicates that DNA regions amplified in both assays reside in close proximity, yet slight differences in specificity are evident. To date, the HS-causing type-B-specific PCR (Townsend *et al*., 1998) remains 100% specific for HS-causing type B serotypes of isolated *P. multocida*. Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a 620 bp fragment with the KTSP61 and KTT72 primers. In the present study similar pair of primers were used in the reaction added to species specific primers KMT1T7 and

KMT1SP6 which served as an internal control did not ampify any fragment. That confirmed the isolates they do not belong to capsular group B.

Previous reports on HS associated *P. multocida* serotypes from Plateau state is scarce. It is accepted by those working on HS in cattle and buffalo that the disease is caused specifically by *P. multocida* having the group B or E capsule and somatic serotype 2 or 5 antigen. *P. multocida* organisms with the B: 2 or B: 2, 5 formulae are occasionally isolated from other species (i.e., swine and deer) in which they produce clinical signs and pathologic lesions that are similar to those seen in cattle and buffaloes. Recent evidence indicates that Hemorrhagic septicemia in wild ruminants such as deer and elk can also be produced by *P. multocida* with the antigenic formula B: 3, 4. However, either the incidence of these organisms is rare or they are only rarely reported (Wilson *et al*., 1992). *P. multocida* isolates of serogroup E are unique in many respects. All previously reported serogroup E isolates have possessed only somatic antigens 2 and 5. The lipopolysaccharides of these organisms produce identical patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Rimler, 1990). Additionally, these organisms have never been isolated outside Africa. In the present study, the serotypes of only 3 isolates were detected: E: 3, 4, E: 2, 5, and E: 3, 4. The remaining 15 isolates were somatically untypeable. These isolates for which the somatic antigens were not detected might belong to serotypes not included in the 16 reference serotypes or have lost their somatic antigens (Henddleston *et al*., 1972). The multiplex PCR for detection of capsular sergroups detected capsular group F isolated from lung of calf which is being reported for the first time in cattle in Nigeria. This result was similar to that of Catry *et al*. (2004), who detected *P. multocida* capsular group F in the lung tissue of male Holstein-Friesian calf (age-2 weeks) that had fatal peritonitis using

multiplex PCR. Outbreaks caused by capsular group F are usually characterized by fibrinous peritonitis and mortality, which are hitherto unreported features of *P. multocida* capsular type F infection.

Eighteen *P. multocida* were examined for the presence of plasmids. All the *P. multocida* isolates showed the presence of 5 kb plasmid DNA. Three of the isolates (16.7 %) had an additional plasmid of 3 kb and one (ka3) had second additional plasmid of 6 kb. These results are similar to the report by Jamal *et al*. (2005), who reported the presence of plasmids in all *P. multocida* isolates with profiles of 2 plasmids with sizes of 3.0 and 5.5 kb. In the present study the strain with capsular group F (JM6) carried 1 plasmid of 5 kb only which agree with Catry *et al.* (2004) that reported *P. multocida* capsular group F carried a single plasmid of 5.2 kb only. The finding of this study will help to further investigate whether any of these plasmids are related to virulence mechanism (pathogenicity) of *P. multocida*. The multiple resistance observed in this study might have been encoded by the various plasmids genes encountered in the study.

Chapter 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

The present research was carried out with a view to isolating and characterizing *P. multocida* from cattle in Plateau state. The isolates were identified by cultural,

morphological and biochemical characters. The isolates were studied for their *in vitro* antibiotic sensitivity. The PM-PCR based detection was also carried out for the identification of the isolates. On Blood agar the colonies were non-haemolytic, round, grayish, smooth or mucoid and did not grow on MCA. The isolates were Gram negative coccobacillary rods. All the isolates were found to be positive for oxidase, catalase, indole production, nitrate reduction and fermentation of glucose, mannitol, sucrose and mannose. They were negative for citrate utilization and fermentation of maltose, arabinose, lactose, dulcitol, salicin and trehalose. *In vitro* antibiotic sensitivity of the isolates was performed against twelve commonly used antibiotics. Among the 18 isolates studied, (44.4%) were sensitive to gentamicin and amoxycillin/clavulanic acid respectively. The % susceptibilities to other agents were: chloramphenicol (38.9%), sulphamethoxazole/trimethoprim (72.2%), penicillin (5.6%), ciprofloxacin (38.9%), ampicillin (27.8%), oxacillin (5.6%) and vancomycin (5.6%). All the isolates (100%) were resistant to tetracycline and erythromycin. The genotypic studies carried out included species-specific PCR assay, capsular grouping using multiplex capsular PCR typing system and determination of the carriage of plasmids by the isolates. All isolates produced *P. multocida* spcies-specific amplicons. Seventeen (94.4 %) of the isolates were identified as capsular group E and one (5.5 %) as capsular group F by multiplex PCR. The profiles of plasmid of each isolates was estimated by agarose gel electrophoresis. All the isolates harboured a plasmid of about 5kb. Three of the group E isolates had additional plasmid of 3 kb, and one isolate (ka3) had a plasmid of 6kb; but none of the isolates carried all 3 plasmids. This study is an attempt to define the current status of HS causing *P. multocida* isolates in nine local government of Plateau state. This contemporary study was necessary as available information indicates that similar studies were conducted about 3 decades ago. The study confirmed the presence

of the African capsular strain (E), but of greater interest is capsular group F that was identified for the first time in a calf in Nigeria. *P. multocida* E: 3, 4 and *P. multocida* E: 2, 5 were identified amongst the isolates. These could redefine the vaccine strategy as the current vaccine used in Nigeria contain *P. multocida* B: 3,4 and E: 2. However more work needs to be carried out in other parts of the country to gather more relevant information with regards to capsular and somatic types.

6.2 CONCLUSIONS

The results of this study have contributed to knowledge on the biology of *P. multocida* associated with HS and could lead to the development of improved strategies for the control of HS.

The highlights of the present study are:

- Variation in the sensitivity to different antibiotics by the isolates showed the necessity of *in vitro* antibiotic sensitivity before treatment. It also emphasizes the need for judicious selection of antibiotic for effective treatment.
- The study confirmed the preponderance (17) of the African capsular strain (E), but of interest is the isolation for the first time in Nigeria of capsular group F.
- *P. multocida* E: 3, 4 and *P. multocida* E: 2, 5 were identified among the isolates. These could redefine the vaccine strategy as the current vaccine used in Nigeria contain *P multocida* B: 3,4 and E: 2.
- All the isolates were found to carry plasmids of various sizes.

6.3 RECOMMENDATIONS

The following recommendations have been suggested based on the study undertaken in Plateau state.

- Hygienic measures such as the control of movement of animals from diseased areas and the proper disposal of carcasses and other infected materials, and the adoption of improved husbandry practices, where possible, be carried out in order to reduce the incidence and spread *P. multocida* causative agent of HS.
- Although the current vaccine used , developed nearly 4 decades ago using strains B3,4 and E 2, has been of great benefit, there may be need to add the unique capsular strain F, and E, somatic antigens 2,5, and 3,4.
- Continued efforts should be directed towards improving the methods for rapid diagnosis and complete serological typing of *P. multocida* in regional laboratories.
- More work needs to be carried out in other parts of the country to gather more relevant information with regards to capsular and somatic types.

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