PHENOTYPIC, GENOTYPIC AND PATHOGENICITY STUDIES OF *PASTEURELLA MULTOCIDA* ISOLATED FROM CHICKENS IN JOS OF PLATEAU STATE, NIGERIA

BY

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ZARIA, NIGERIA

FEBRUARY, 2013

DECLARATION

I declare that the work in this Dissertation entitled **"PHENOTYPIC, GENOTYPIC AND PATHOGENICITY STUDIES OF *PASTEURELLA MULTOCIDA* ISOLATED FROM CHICKENS IN JOS OF PLATEAU STATE, NIGERIA"** was performed by me in the Department of Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria, under the supervision of Dr. M. A. Raji, Professor S. B. Oladele and Professor P. A. Abdu.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work was previously presented for another degree or diploma at any University.

Dashe Yakubu Gunya Dogonyaro. _____ Signature _____ Date

(Name of student)

CERTIFICATION

This dissertation entitled **"PHENOTYPIC, GENOTYPIC AND PATHOGENICITY STUDIES OF *PASTEURELLA MULTOCIDA* ISOLATED FROM CHICKENS IN JOS OF PLATEAU STATE, NIGERIA"** by Yakubu Gunya Dogonyaro DASHE meets the regulation governing the award of the degree of Doctor of Philosophy in Veterinary Medicine of Ahmadu Bello University Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

I wish to dedicate this work to God Almighty and the Dashe's family.

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ABSTRACT

Sporadic outbreaks of fowl cholera have been experienced from time to time in Jos, Nigeria despite vaccination against the disease. This study was conducted from November, 2010 to October, 2011 to determine the phenotypic and genotypic profile of *Pasteurella multocida* isolated from chickens in Jos, Nigeria. A total of 400 oro-pharyngeal swabs from apparently healthy chickens and 2,000 samples consisting of bone marrow, heart, liver, lungs and spleen (400 each) were collected from 400 clinically sick chickens and analyzed bacteriologically for *Pasteurella multocida.* Swab from each sample was cultured on 7% defibrinated sheep blood, MacConkey and casein sucrose yeast agar. Presumptive colonies of *Pasteurella multocida* were subjected to biochemical tests. Isolates identified by biochemical tests were further subjected to Microbat 24E test and *Pasteurella multocida* specific Polymerase Chain Reaction. Multiplex PCR typing using specific primers for capsular types CAP A, CAP B, CAP D, CAP E and CAP F was carried out on all *Pasteurella multocida* isolates. Biochemical tests identified *Pasteurella multocida* in 1.0% of clinically sick chickens. The monthly frequency of isolation of *P. multocida* showed that the bacterium was isolated mostly between the months of July and October. *Pasteurella multocida* was isolated from 5(1.3%) apparently healthy birds. *Pasteurella multocida* serotypes A: 1, A: 3 and A: 4 were identified in Jos, Nigeria. Polymerase Chain Reaction recognized only 12 *P. multocida* isolates out of 25 isolates, showing a specific amplicon of 460 base pairs. The amplification of capsular genes in these 12 isolates by multiplex PCR showed band sizes of 1,044 base pairs signifying that all the 12 *P. multocida* isolates belong to capsular group A. Disk diffusion test was employed to assess the sensitivity of all *P. multocida* isolates confirmed by PCR. Ciprofloxacin and gentamicin were highly effective (100%) against the twelve *P.*

multocida isolates. High rates resistance by *P. multocida* isolates were recorded for ampicillin and amoxicillin/clavulanate acid among others. The gross lesions in chickens infected with *P. multocida* serotypes A1 and A3 were prominent keel, congested heart, liver, kidneys and lungs. Histopathologically, moderate to severe lymphocytic, heterophilic and macrophage cellular infiltration were noticed in the lungs and heart of chickens. In Japanese quails, congested heart, liver and lungs were seen. Cellular infiltrations were noticed in the lungs and heart of infected Japanese quails. Pathogenicity study indicated that *P. multocida* serotypes A: 1, A: 3 and A: 4 caused (100%) mortality in mice. Moderate cellular infiltrations were noticed in the lungs and heart of mice. In conclusion, it was observed that *P. multocida* serotypes A: 1, A: 3 and A: 4 were identified in Jos metropolis and they caused mortality in experimentally infected chickens, Japanese quails and mice. This indicates that the antigenic component of the fowl cholera vaccine which is capsular group A and serotype 1 that is produced in the country may be limited in scope; it is therefore recommended that the vaccine should be re-validated by incorporating the prevailing serotypes of *P. multocida* in Nigeria such as serotypes A: 3 and A: 4 in order to widen the protection margin.

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LISTS OF ABBREVIATIONS

AI	-	Avian influenza
AGDT	-	Agar gel diffusion precipitation test
AGID	-	Agar gel immunodiffusion
Bp	-	Base pair
BHI	-	Brain heart infusion
BA	-	Blood agar
Cap A	-	Capsular type A
Cap B	-	Capsular type B
Cap D	-	Capsular type D
Cap E	-	Capsular type E
Cap F	-	Capsular type F
CDC	-	Centers for Disease Control and Prevention
Cfu	-	Colony forming unit
DNA	-	Deoxyribonucleic acid
dNTP	-	deoxynucleotide triphosphate
EMB	-	Eosin Methylene blue
FAO	-	Food and Agriculture Organization
H_2S	-	Hydrogen sulphide
H_2O_2	-	Hydrogen peroxide
H_o	-	Null hypothesis
GNB	-	Gram negative bacilli
LPS	-	Lipopolysaccharide

MCA	-	MacConkey Agar
Ml	-	Millilitre
NA	-	Neuraminidase
OIE	-	Office International Des Epizooties
OMP	-	Outer membrane protein
PACE	-	Pan-African Control of Epizooties
PCR	-	Polymerase Chain Reaction
Pm 70	-	*Pasteurella multocida 70*
Psl	-	P6-like-protein
Rho	-	Rhodopsin
TAE	-	Tri- acetate
tRNA	-	Transfer ribonucleic acid
Tsh	-	Temperature sensitive haemagglutinin
UV	-	Ultra-violet
µl	-	Microlitre
µg	-	Micogram
WHO	-	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Poultry has been adjudged the largest group among domesticated livestock in Nigeria with estimated population of about 140 million (FAO, 2000). The poultry industry consists of four production systems, sector 1, 2 and 3 characterized by moderate to high bio-security and sector 4 poultry system with very low bio-security; which is owned by families, often under the control of the wife and children of the household and consisting of up to 20 birds of mixed ages (Mathewman, 1975; Adene and Oguntade, 2006). The introduction of sector 2 poultry production methods to complement sector 4, which is traditional 'backyard' poultry raising has been of critical importance in many parts of the world including Nigeria. This system of production was overwhelmingly embraced by poultry farmers because of its short incubation period and high returns.

Poultry offers the quickest supply of animal protein to man, and provides comparatively faster returns to investment than cattle, small ruminants or pigs (Etukudo and Adegboye, 1983; Molokwu *et al*., 1988). In spite of the enormous economic gains derivable from sector 2 production system, many farmers in developing countries are skeptical about its adoption on the ground of its high input requirement. Nevertheless, modern poultry production has witnessed a rapid transformation during the past decades due to the introduction of modern intensive production methods, new breeds, improved biosecurity and preventive health measures. This system of production places high emphasis on proper health hygiene and management and requires only a small but very skilled labour-force (FAO, 2000).

Poultry production still accounts for major part of all animal protein produced in developing countries and is an integral component of nearly all rural, peri-urban and urban households (Wosu, 1992). Rural poultry sector contributes 68.5% of the national meat supply; while11.4% is from commercial chickens and 19.7% from other poultry (Wosu, 1992; Musa *et al*., 2009). It also constitutes a significant proportion to the rural as well as the national economy (FAO, 1999). Despite the unquantifiable economic gains from this industry, it often suffers disease induced losses due to sustained challenges from environmental factors, viral, bacterial, protozoan, parasitic and fungal infections among others. In Nigeria, a great loss in poultry production as a result of these agents still remains one of the major constraints hindering the success and growth of the poultry industry, where disease prevention and control measures are rare and high mortality are common even in vaccinated flocks in some cases. The epizootic of fowl cholera in the world poses a serious threat to poultry (Pandey, 1992; Ambali *et al*., 2003). The aetiologic agent of fowl cholera, *Pasteurella multocida* is not host specific and has demonstrated the capacity to infect and cause severe illness and death in livestock. Fowl cholera has often occurred as a sporadic disease in many parts of the world including Nigeria with grave economic consequences on livestock and humans (Ambali, 2003). Fowl cholera is widely identified as one of the major diseases of economic importance wherever intensive poultry production occurs; particularly in the United States of America (Rimler *et al.*, 1998). However, the disease has significant impact in less intensive systems. For example, fowl cholera is one of the main diseases seen in Mexican village chickens (Gueye, 1999). Fowl cholera is endemic in regions of Asia and Africa where it causes immense economic losses to the poultry industry due to cost of treatment, weight loss and

mortality (Townsend *et al*., 1997). Despite considerable research on fowl cholera in domestic and wild birds, the source of new infections in the flocks is still uncertain (Muhairwa, 2000).

1.2 EPIZOOTIOLOGY OF FOWL CHOLERA

Fowl cholera outbreaks have been reported in both domestic and wild birds in many parts of the world, since the causative agent was isolated in 1881. Fowl cholera is a contagious bacterial disease that affects both domestic and wild birds (Glisson *et al*., 2003; Kwon and Kang, 2003). It is often transmitted by wild birds and other domestic birds, and spreads by contamination of feed or water and by oral or nasal discharges from infected birds. Migratory birds have been reported as a major source of fowl cholera (Christensen *et al*., 1998). The incubation period ranges from four to nine days, but acute outbreaks can occur within two days of infection. Most outbreaks of fowl cholera affect chickens, turkeys, ducks and geese (Rimler and Glisson, 1997). The disease is worldwide in distribution and is reported in most avian species; among poultry species, water fowl appears to be more susceptible, followed by turkey and chicken. Older birds are more susceptible than young chickens less than 16 weeks. Heavy losses have been observed in mature chickens of up to 16 weeks of age. This means that mature chickens are more susceptible than young chickens (Rimler and Glisson, 1997). Fowl cholera usually occurs as a fulminating disease with high bacteraemia, high morbidity and mortality (OIE, 2008). Typically, fowl cholera occurs abruptly in healthy turkeys of market age; it causes high mortality over a short period oftime and/or high condemnation rates at processing plants, despite prompt medication efforts. The severity of the disease and its incidence are also influenced by environmental factors such as overcrowding, climatic changes, nutrition and concurrent diseases (Harper *et al*., 2006).

Five capsular serogroups (A, B, D, E, and F) and 16 serotypes have been identified based on capsular antigen, outer membrane protein and lipopolysaccharhide (Harper *et al*., 2006). *Pasteurella multocida* is not host specific (Rimler and Glisson, 1997; Arashima and Kumasaka, 2005). The *P. multocida* type A strains are responsible for fowl cholera in birds, snuffles in rabbit and bovine pneumonia, while atrophic rhinitis in swine is caused by serogroup D. Haemorhagic septicaemia in cattle and buffalo is caused by serogroup B or E. Type F strains have been recovered primarily from turkey, but their role in disease is unclear. Serotypes 1, 3 and 4 are the most common isolates in poultry. Serotype 1 is the most frequent cause of disease in wild water birds (Botzler, 1991). Variable mortality rates have been reported in different poultry types in many parts of the world; 0-20% in chickens, 35-45% pullets, 50% in ducks, and 17-68% in turkeys (Rimler and Glisson, 1997). The disease often results in reduced egg production, localized infections and death. Chronically infected birds are major reservoirs of infections (Muhairwa *et al*., 2001; Glisson *et al*., 2003). Although cats and dogs are asymptomatic carriers, their roles in the epidemiology of fowl cholera are becoming more significant. A case of pasteurellosis in a lioness was reported by Rama and Raja, (2000).

Dogs have been reported to transmit *P. multocida* to poultry. Fomites such as contaminated crates, feeders, or waterers may introduce *P. multocida* to new intensively managed birds. Excretions from nostrils, beaks, conjunctiva, and vent of infected birds may spread the disease within a flock either directly or indirectly (Rama and Raja, 2000). In Nigeria, documented reports on pasteurellosis associated with *Pasteurella multocida* in poultry indicated that fowl cholera is a major bacterial disease in poultry affecting chickens, turkeys,

ducks, geese and quails; outbreaks could cause high mortality of up to 80% (Abdu *et al*., 1985; Ambali *et al*., 2003; Odugbo *et al*., 2004; Yunusa, 2008; Mwankon *et al*., 2009; Raji *et al*., 2010). Different prevalence rates of fowl cholera in Nigeria were reported by many researchers, Abdu *et al*. (1985) documented a prevalence rate of 3.2%, Molokwu *et al*. (1990) 3.5%, Oladele *et al*. (1999); Ambali *et al*. (2003) 0.1-33%, Masdooq *et al*. (2008) 6.4% and Raji *et al*. (2010) (2.3% in 2001 and 7.4% in 2005).

1.3 STATEMENT OF RESEARCH PROBLEM

Fowl cholera is widely identified as one of the poultry diseases of economic importance wherever intensive poultry production occurs; particularly in the United States of America (Rimler *et al.*, 1998; Gueye, 1999). Fowl cholera is endemic in regions of Asia and Africa where it causes immense economic losses to the poultry industry (Townsend *et al*., 1997). Fowl cholera has often occurred as a sporadic disease in many parts of the world including Nigeria with grave economic consequences on both poultry and humans.

In Nigeria and most of the developing countries in Africa, a great loss in poultry production as a result of this disease still remains one of the major constraints hindering the success and growth of the poultry industry, where disease prevention and control measures are rare and high mortality are common even in vaccinated flocks in some cases (Pandey, 1992). Fowl choler was identified as one of poultry diseases diagnosed in Zaria from 1981-1984 with a prevalence rate of 3.2% (Abdu *et al*., 1985). Fowl cholera and infectious coryza have been identified as two bacterial diseases that contribute to the respiratory disease complex and

both of these diseases present considerable challenges for diagnostic laboratories (Blackall and Marrow, 1990).

Despite the application of advanced investigation and diagnostic techniques on both the organism and the affected animal species, *Pasteurella* infections have continued to contribute to heavy losses in animal production as well as hazardous threat to human health worldwide (Patrick *et al*., 2000; Ragy, 2006).

1.4 JUSTIFICATION OF THE RESEARCH

Poultry contributes a substantial proportion of Nigeria quality animal protein and has the potential to diversify and increase our foreign exchange. It is a quick source of income for Nigerians and it requires less space as compared to cattle (Oladele and Raji, 1997).Sporadic outbreaks of fowl cholera associated with massive losses in chickens have been experienced in Jos despite vaccination; and sources of infections could not be traced (Unpublished observations).

The current importance of fowl cholera in the country's poultry industry is reflected by a recent estimate that *P. multocida* accounts for a prevalence rate of 1.2% and 4.9% in apparently healthy and clinically sick birds respectively in an investigative study conducted on the disease in Sokoto State (Yunusa, 2008). Infections due to *Pasteurella multocida* have resulted in immense economic losses to the poultry industry due to death of birds, weight loss, unthriftness, cost of treatment and short fall in supply of protein (Pandey, 1992).

The significance of *P. multocida* as a pathogen of poultry and other animals in Nigeria has been established and sources of outbreak of fowl cholera are not traced in most cases; therefore; molecular and phenotypic identification of *P. multocida* will be useful for the determination of reservoirs of infection, the epidemiology of the disease and appearance of new serotypes (Abdu *et al*., 1985; Oladele *et al*., 1999; Muhairwa *et al*., 2001; Raji *et al*., 2010).

There is paucity of information on the capsular and somatic groups of this organism in Nigeria. Strains of *P. multocida* causing avian pasteurellosis represent at least 16 immunotypes and 5 capsular serogroups A-F, which makes the prevention of the disease seemingly difficult using vaccines, since protection is serotype specific (Heddleston *et al*., 1972; Glisson *et al*., 2003).

Antimicrobial therapy is the most widely available conventional tool for the prevention and control of fowl cholera (Kehrenberg *et al*., 2001; Bronzwaer *et al*., 2002; Lion *et al*., 2006). Antibiotic resistant pathogenic bacteria in food-producing animals and environment are recognized as global problem for public health (Bronzwaer *et al*., 2002; White *et al*., 2002). *P. multocida* with multi-drug resistan to more than 5 classes of antimicrobials has been reported in China. The prevalence of multiresistance in China has increased from 47.8% in 2003, 54.1% in 2004 and 57.6% in 2005 to 81.6% in 2006 and 97.1% in 2007. Also, the proportion of *Pasteurella multocida* isolates with multiresistance to more than 7 antimicrobials increased to approximately fourfold between 2003 and 2007, from 16.2% to 62.8% (Xibiao *et al*., 2009). Strains of *P. multocida* vary in susceptibility to

chemotherapeutic agents and resistance to treatment may develop, especially during prolonged use of these agents (Donahue and Olson, 1972; Walser and Davis, 1975; Arora *et al*., 2004).

1.5 AIM OF THE STUDY

The aim of the study was to isolate and study *P. multocida* from chickens in Jos metropolis using phenotypic and genotypic methods.

1.7 OBJECTIVES OF THE STUDY

The specific objectives of the study were to:

1. Isolate *P. multocida* from apparently healthy and clinically sick chickens in Jos metropolis.
2. To characterize *P. multocida* isolated using conventional biochemical methods, and rapid kit system.
3. Determine the serotypes of *P. multocida* isolated using commercial antisera.
4. Assess the sensitivity profiles of *P. multocida* isolated from healthy and clinically sick chickens to a panel of conventional antimicrobial agents.
5. Confirm isolated *P. multocida* using polymerase chain reaction (PCR).
6. Determine capsular serogroups using multiplex polymerase chain reaction.
7. Test for pathogenicity of isolated *P. multocida* in chickens, mice and Japanese quails.

1.8 RESEARCH HYPOTHESES (H_0)

(a) There is no significant difference between the prevalence of *P. multocida* isolated from apparently healthy chickens and clinically sick ones in Jos metropolis.

(b) There is no genotypic relationship between capsular and somatic types of *P. multocida* isolated from apparently healthy birds and clinically sick ones in Jos metropolis.

(c) There is no significant difference between antibiotic susceptibility pattern of *P. multocida* isolated from clinically sick and those recovered from apparently healthy chickens.

(d) The isolates *P. multocida* are not pathogenic to chickens, quails and mice.

CHAPTER 2

LITERATURE REVIEW

2.1 NOMENCLATURE OF FOWL CHOLERA

Several names such as avian cholera, fowl cholera, avian pasteurellosis and avian haemorrhagic septicemia have been ascribed to the fowl cholera in the history of its discovery in birds. The name *fowl cholera* was first used by Mailet of France in 1836 who studied a disease that caused several epornitics among fowls in Europe during the half of the 18th century. A researcher named Huppe in 1886 referred to the disease as *haemorrhagic septicemia*, while Lignieres used the name *avian pasteurellosis* (Glisson *et al*., 2003). In 1851, Benjamin demonstrated that the disease had the capacity to spread by cohabitation; hence he formulated procedure for its prevention. In 1877 and 1878, Perroncito of Italy and Semmer of Russia observed in tissues of affected birds a bacterium that had a rounded form and occurred singly or in pairs. In 1879, Toussaint isolated the bacterium and proved it was the sole cause of the disease. Louis Pasteur isolated the causative agent of fowl cholera and grew pure cultures in chicken broth. He used the FC organism to perform his classic experiment in attenuation of bacteria for use in producing immunity. The aetiologic agent was named after Pasteur "*Pasteurella multocida"* (Glisson *et al*., 2003).

2.2 AETIOLOGIC AGENT OF FOWL CHOLERA

Fowl cholera is caused by *P. multocida* which is a gram negative, bipolar, non-motile, non-spore forming rod-shaped bacterium. The bacterium occurs singly, in pairs, and occasionally as chains or filaments. It measures 0.2-0.4 x 0.6- 2.5 μm, but tends to be pleomorphic after repeated cultures. The organism can occur as a commensal in the nasal cleft, naso and oro-

pharyngeal region of apparently healthy birds, normally difficult to isolate in non clinical cases (Carpenter *et al*., 1989). The organism can be isolated readily from the liver, bone marrow, and heart blood of birds that die of acute fowl cholera and usually from localized lesions of chronic cholera. *P. multocida* grows aerobically or anaerobically, at 37^0C, with optimal PH range from 6.2- 9.0. The bacterium will grow on meat infusion media; growth is enhanced when the medium is enriched with peptone, casein hydrolysate, or avian serum. Dextrose starch agar with 5% avian serum and blood agar are also good media for isolation and growth of *P. multocida.* On primary isolation from birds with fowl cholera, colonies may be iridescent (due to capsules) and sectored (Rimler and Glisson, 1997). Capsular typing and somatic serotyping are based on immunospecificity of the capsular antigens, lipopolysaccharides and major membrane protein *OMPH* (Heddleston *et al*., 1972; Marandi *et al*., 1997). Sixteen somatic serotypes and five capsular serogroups (*CAP A, CAP B, CAP D, CAP E* and *CAP F*) have been identified (Heddleston *et al*., 1972).

2.2.1 Genomics of *Pasteurella multocida*

The genomic sequence of an avian clone of *Pasteurella multocida*, *Pm70* has helped in elucidating the virulent factors used by this organism to cause clinical disease in poultry. The genome of *Pm70* consists of a single chromosome 2,257,487 base pairs in length, 2,014 predicted coding regions, 6 ribosomal RNA operons and 57 tRNAs (May *et al*., 2001). The *P. multocida* genome shows 129 lipo-proteins that are secreted and located in the outer membrane. Protein H has been found to be the major polypepetide in the outer membrane of *P. multocida*. Microarrays analyses showed that more than fifty *Pm70* genes have the role of acquiring iron and metabolism. Gene expression for energy metabolism and electron

transport decreases by 2-6 times, and gene for iron binding and transport increases 2-7 times during growth for the first two hours (May *et al*., 2001). *Pasteurella multocida* produces a 140 kDa protein toxin that activates signal transduction pathways, which activate phospholpase C beta, Rho A, Jun kinase and ERK. DNA microarray and proteomic techniques used in testing the capability of *P. multocida* to produce disease showed that the bacteria are in host niches that force them to change their gene expression for central energy metabolism and for uptake of iron, amino acids and other nutrient. *Pasteurella multocida* genes that are up regulated in times of infection are usually involved in nutrient uptake and metabolism. The true virulence genes may only be expressed and upregulated during the first stages of the infection.

2.2.2 Phenotypic Characterization *Pasteurella multocida*

Identification and characterization of *P. multocida* has relied on the ability to purify the organism in the laboratory. A comprehensive phenotypic profile of *P. multocida* can only be achieved through detailed carbohydrate fermentation, colonial, serological and biochemical studies. However, significant variations in phenotypic properties of *P. multocida* have been reported by several authors (Heddleston, 1976; Christensen *et al*., 2004). Culture conditions can influence the expression of phenotypic attributes thus diminishing the stability and reliability of the phenotypic methods for strain identification (Matsumoto and Strain, 1993; Jacques *et al*., 1994).

2.2.2.1 Microscopic appearance

P. multocida is a Gram negative, bipolar, non-motile, non-spore forming rod-shaped bacterium. The bacterium occurs singly, in pairs, and occasionally as chains or filaments. It measures 0.2-0.4 x 0.6- 2.5 µm, but tends to be pleomorphic after repeated cultures (Morishita *et al*., 1996).

Colonial morphology: Primary culture of *P. multocida* on blood agar shows circular (2-3 mm), smooth, convex, translucent, mucoid, glistening, and butyrous appearance without haemolysis. The colonies are dew-drop like and have the tendency to coalesce. The organism does not grow on MacConkey agar (Morishita *et al*., 1996).

2.2.2.2 Carbohydrate Fermentation

Identification of *P. multocida* on the basis of sugar fermentation reaction indicates that the organism ferments sugars, such as fructose, galactose, mannitol, mannose, sorbitol, glucose, and sucrose without gas production. Maltose, lactose, inositol, inulin, salicin, and rhamnose are not fermented by the organism (Morishita *et al*., 1996).

2.2.2.3 Biochemical Characterization

The biochemical activities of *P. multocida* have shown that the bacterium is positive for some specific tests like indole, catalase, oxidase, voges proskauer, ornithine decarboxylase and it reduces nitrate to nitrite. On the other hand the organism is negative for urease, citrate, motility and methyl red (CLSI, 2009).

2.2.2.4 Serological Identification

Serologic tests are rarely used for identification of *P. multocida*. However, they are commonly used for antigenic characterization of strains of *P. multocida*. A gel-diffusion precipitin test (GDPT) is used for serotyping based on differences in somatic antigens (somatic serotyping) with appropriate antisera (Heddleston *et al*., 1972). Sixteen serotypes (1-16) have been reported; strains representing each 16 serotypes have been isolated from avian host (Glisson *et al*., 2003).

An indirect (passive) haemagglutination test is used for serogrouping based on differences in capsular antigens (capsular serogrouping). Five capsular serogroups (A, B, D, E and F) have been reported (Heddleston *et al*., 1972). Serogroup A, D and F strains produce capsules containing mucopolysaccharides.

2.2.3 Molecular Identification

The advent of molecular analysis has provided endless and countless opportunities for scientific researchers to explore the most fundamental properties of an organism such as its genetic information during identifying and characterization. Genomic characterization possesses versatility surpassing traditional phenotypic methods, as nucleic acid analyses facilitate identification and rapid detection of an organism, determination of its taxonomic position, and investigation of intra-species genetic relationships (Owen, 1989). Molecular approaches such as DNA hybridization and nucleic acid amplification have allowed bacterial detection directly from clinical samples, dramatically reducing the time required for identification of organisms that are generally difficult or slow to cultivate in the laboratory.

Molecular technology has also significantly influenced the identification and characterization of *P. multocida* (Owen, 1989). Restriction endonuclease analyses of chromosomal DNA and ribotyping have provided considerable information about genomic characteristics of *P. multocida* (Townsend *et al*., 1997; Jabbari *et al*., 2002).

2.2.3.1 Pasteurella multocida Specific PCR assay

The species-specific PCR was developed only recently for the detection of *P. multocida* in mixed cultures or clinical samples (Kasten *et al*., 1997; Townsend *et al*., 1998). Kasten *et al*. (1997) described the use of oligonucleotide primers constructed to amplify the *psl* gene encoding the P6-like protein of *P. multocida*. The amplified products of *P. multocida* serotype will show bands that are expected to correspond to amplicon size of 460 base pairs (Townsend *et al*., 2001).

It has been shown that neither mouse inoculation nor the PCR assay accomplished total detection among infected flocks. However, increased detection by PCR may be possible with further optimization of the sample preparation procedure. Another advantage of this technique is that, to achieve the maximum sensitivity of 10 organisms, additional hybridization with *psl* was required. While PCR has been increasingly incorporated in laboratories around the world, hybridization is still usually possible only in specialized laboratories (Lee *et al*., 2000).

The PM-PCR developed by Townsend *et al*. (1998) demonstrated a sensitivity of less than 10 organisms without the need of additional hybridization (Lee *et al*., 2000). This assay is based

on the amplication of DNA sequence unique to *P. multocida* (KMT1) that was isolated by subtractive hybridization (Townsend *et al*., 1998; Hunt *et al*., 2000). While the sensitivity of this assay is high, the specificity is decreased slightly by the amplification of *Pasteurella canis* biovar 2, once recognized as an atypical *P. multocida*. Indeed, 16S rRNA sequencing indicates that the two species are closely related (Dewhirst *et al*., 1993). As species within *Pasteurella sensu stricto* other than *P. multocida* and *P. avium* were not examined by Kasten *et al.* (1997), it is not known whether *P. canis* biovar 2 will remain positive with the PCR-H assay. DNA: DNA hybridization studies support the recognition of *P. canis* biovar 2 as a distinct species (Mutters *et al*., 1985).

While false positives may occur with some isolates from pneumonic cattle and pigs with the PM-PCR, *P. multocida* subspecies and *P. canis* biovar 2 can be distinguished on the basis of indole and mannitol fermentation. Optimization of the PM-PCR using regions surrounding the KMT1 sequence may improve the specificity of the assay, although this requires further examination. Although some disadvantages in specificity are evident in both assays, the assay derived by Townsend *et al*. (1998) is more versatile as additional hybridization is not required for optimal sensitivity.

2.2.3.2 Molecular Characterization of Pasteurella multocida

Several efforts have been made to classify the *P. multocida* species according to phenotypic attributes such as serological antigen presentation (capsular and somatic) and biochemical fermentation profiles. However, these techniques offer limited characterization and insufficient information for epidemiologic studies of *P. multocida* (Wilson *et al*., 1992). The

ability to differentiate phenotypically similar isolates is critically important in epidemiology, particularly when establishing the identity of bacterial vaccine strains (Stull *et al.,* 1988). The development of DNA-based techniques has provided alternative methods of characterization that overcome the limitation of phenotyping, while identifying precisely individual strains of closely related bacteria (Owen, 1989). Genomic characterization techniques have supplemented or replaced the traditional typing methods for the discrimination of isolates from a wide range of bacterial pathogens. DNA fingerprinting encompasses a large range of methods with variable specificity and discriminatory power, most of which have been used to differentiate phenotypically similar *P. multocida* isolates (Tenover *et al.,* 1995).

Restriction endonuclease analysis:

So many restriction enzymes, such as *Hha1*, *Hha11*, *BamH1*, *Hpa11* and *EcoR 1* among others have been used to digest genomic DNA of *P. multocida* isolates. The characteristics will be based on polymorphism of the sequence obtained after agarose gel separation (Wilson *et al*., 1992; Glazer and Nikaido, 1995). Restriction endonuclease analysis (REA) has proved to be a valuable component of bacterial epidemiologic studies, particularly in the investigation of outbreaks of pasteurellosis. This method is a highly reproducible technique that is not influenced by the inconsistency expression of phenotypic traits that limit the sensitivity and specificity of conventional typing methods (Snipes *et al*., 1989). The use of REA, either solely or in conjunction with ribotyping can provide sensitive, distinctive banding profiles capable of differentiating *P. multocida* isolates of similar serotype. Of all the restriction enzymes used in DNA fingerprinting of *P. multocida* isolates, *Hha1* and *Hpa11* have been found to yield the most informative and easily distinguishing profiles from

a wide range of serotypes (Wilson *et al*., 1992, 1993; Blackall *et al*., 1995, 1996; Diallo *et al*., 1995).

Ribotyping of *Pasteurella multocida* genomic DNA:

The banding patterns produced by REA are often complex, making visual interpretation of results difficult. Ribotyping like REA utilizes restriction enzyme digestion of genomic DNA and agarose gel electrophoresis for DNA fragment separation. The additional use of Southern blotting and hybridization with a labeled DNA probe reduces the complexity of the restriction patterns, and highlights restriction fragment length polymorphisms (RFLPs) within the bacterial genome without the computer analysis that is sometime required (Snipes *et al*., 1989).

Ribosomal RNA (rRNA) molecules are highly conserved, ubiquitous molecules that constitute the major proportion of RNA in the bacterial cell (Grimont and Grimont, 1986). As (rRNA) operon vary in copy number genomic location between strains and species, DNA probes specific for rRNA gene sequence can be used to identify RFLPs within and/or around the ribosomal operon, thus providing the basis for bacterial strain differentiation. Ribotyping, in conjunction with REA, has been used to characterize successfully and differentiate avian species and porcine isolates of *P. multocida* (Snipes *et al*., 1989; Gardner *et al*., 1994; Blackall *et al*., 1995).

Pulsed-field gel electrophoresis:

Field alternation electrophoretic methods, also known as pulsed-field gel electrophoresis (PFGE), remain the 'gold standard' fingerprinting method for molecular epidemiology, as polymorphism throughout the chromosome are examined without the complexity of REA patterns and the restriction view of genetic variation produced by ribotyping (Goering, 1993). An extensive study by Gunarwardana *et al*. (2000) confirmed the standing of PFGE as the 'gold standard' technique for molecular epidemiology, as it was shown that PFGE was more discriminatory than repetitive extragenic palindromic PCR (REP-PCR) for the characterization of avian *P. multocida.* In spite of the importance of PFGE analysis as an integral component of bacterial genetics and molecular epidemiology in the last decade, it has had limited application in the comparative typing of *P. multocida* isolates (Blackwood *et al.*,1996; Townsend *et al*.,1997; Donnio *et al*.,1999).

Polymerase Chain Reaction fingerprinting:

Polymerase chain reaction fingerprinting has been shown to be useful for the differentiation of *P. multocida* **(**Townsend *et al*., 1997; Hopkins *et al*., 1998; Gunarwardana *et al*., 2000). Arbitrarily primed PCR (AP-PCR) was effective in discriminating *P. multocida* isolates from the respiratory tract of pigs and also post-vaccination isolates from turkey (Zucker and Horsch, 1996; Hopkins *et al*., 1998). However, the use of a radioactive label by Hopkins *et al*. (1998) to increase sensitivity may limit the feasibility of these primers in some laboratories.

2.3 TRANSMISSION OF FOWL CHOLERA

The ways in which the causative agent of fowl cholera is introduced into a flock is often impossible to determine. Chronically infected birds are considered to be a major source of infection. Free flying birds having contact with poultry may be a source of the aetiologic agent of fowl cholera. Contaminated crates, feed bags or any equipment used previously for poultry may serve in introducing fowl cholera into a flock (Rimler and Glisson, 1997). Dissemination of *P. multocida* within a flock is primarily by secretions from the mouth, nose, and conjunctiva of diseased birds that contaminate their environment, particularly feed and water. Chronically infected birds may succumb, remain infected for long period (carriers) or recover (Rimler and Glisson, 1997).

2.3.1 Risk Factors

A thorough knowledge as well as proper understanding of the risk factors and dynamics involved in the introduction and dissemination of fowl cholera in an area will help as a counter measure to adequately contain and control the disease. Some factors have higher propensity of introducing of infection into an area, while others are linked to transmission within flocks (Botzler, 1991). Some of the risk factors that contribute as epidemiological propellant of fowl cholera include:

2.3.1.1 Migratory Birds

It is clear that migratory waterfowls, seabirds and shorebirds are the natural reservoirs of fowl cholera (Waller and Underhill, 2007). *P. multocida* is circulating within at least some species of wild birds including migratory species and are considered to represent a major

source of fowl cholera infection (Botzler, 1991). Globally, the frequency of avian cholera outbreak is on the increase because the disease is perpetuated by avifauna (Waller and Underhill, 2007).

In Nigeria, over 910 wild bird species have so far been recorded (Ezealor, 2006). Three types of bird migrations occur in Nigeria: Intra-national migrant by Green-backed heron (*Botorides striatus*) and cattle egret (*Bubulcus ibis*), intra-African migrants April/May by Abdim's stork (*Ciconia abdimii*) and intercontinental migrants October/November by Pin-tauleo-duck (*Anas acuta)* (Ezealor, 2006). Three flyways enter Nigeria through Northwestern, Northeastern and Central north boundaries bringing birds into the Sokoto–Rima Basin, Kano-Kazaure Wetlands and the Chad Basin Wetland including Hadeja-Nguru Wetlands. From these sites, migratory birds spread into other suitable wintering grounds in the hinterland such as middle Niger/lower Kaduna river floodplain, Niger-Benue confluence, lower Niger/Anambra river flood plain, upper Benue flood plain and Itu wetland. Other routes are mudflats, lagoons beaches and creeks of the Lagos, Akassa, Brass and Bonny area (Ezealor *et al*., 1996).

2.3.1.2 Poor Biosecurity

Low biosecurity standard presented in unrestricted movement of people, animals, materials as well as lack of foot dip on poultry farm contribute to high risk of fowl cholera outbreaks. Equipment, clothing, shoes, vehicles and eggs are good means of spreading *P. multocida* infection. If movement of farm workers and personnel from infected to non-infected areas are

not restricted, there will be high risk of introduction of the bacterium to new areas. Basic concepts of biosecurity are observed to be the most critical way to eliminate fowl cholera (Glisson *et al*., 2003).

2.3.1.3 Predators

Vermin such as rats have constituted a significant source of introducing P. multocida to uninfected flocks. Brown rat (*Rattus norvegicus*) found notably in Europe and North America and black rat (*Rattus rattus*) found in the tropics such as Nigeria and Africa are known to be significant reservoirs of *P. multocida* (Rhoades and Rimler, 1989). In a study conducted by Mwankon *et al*. (2009) in Vom, Nigeria, they isolated *P. multocida* from rats cought around Japanese quail farm experiencing recurrent outbreaks of fowl cholera and they was concluded that rats could be responsible for the transmission of the causative agent of fowl cholera in the farm. If poultry houses are not well secured, some of these predators may gain access thereby introducing pathogens. Dogs and cats having free access to poultry house have been reported to introduce *P. multocida*, a causative agent of fowl cholera to free flocks; therefore, their movements should be restricted as much as possible (Muhairwa, 2000).

2.3.1.4 Poultry Faeces as Manure and Livestock Feed

The widespread practice of using poultry manure in agriculture and aquaculture as fertilizer, or in untreated form as food for pigs and fish is very alarming, and needing closer investigation. Birds infected with the *P. multocida* excrete the bacterium in their faeces. Introducing untreated faeces from infected birds into fish ponds and fields provides a potential new source of infection (Birdlife International, 2006).

2.4 PATHOGENESIS OF *PASTEURELLA MULTOCIDA*

The mechanisms of pathogenesis and determinants for most species other than *P. multocida* have been studied both phenotypic and genotypically. The genus is complex and diverse with respect to host predilection and antigenic variation. Severity may vary depending on species of birds, age, genetic line of host, bacterial strain, concurrent diseases and environmental factor (crowding, climate). *P. multocida* strains associated with fowl cholera usually enter avian tissue through the mucous membranes of the pharynx, upper respiratory passage, conjunctivae, or through cutaneous wounds. The attachment and replication of *Pasteurella multocida* in susceptible host is crucial to its pathogenicity (Lee *et al*., 1994). The virulence in this organism has been attributed to a number virulence factors such as capsule, endotoxin, exotoxin, adhesins, neuraminidase, iron binding system, antibody cleaving enzymes, haemagglutinins, multocidin and proteases. These virulence factors facilitate colonization and invasion of the host, avoidance or disruption of the host defense mechanisms, injury to the host tissue, and stimulation of noxious host inflammatory responses (Hunt *et al*., 2000; Harper *et al*., 2006). Neuraminidase could cause degeneration of the acid, expose other carbohydrate residue which could act as receptors for strains of *P. multocida*, thereby

facilitating invasion. *Pasteurella multocida* adheres to the mucosal lining of susceptible host by using fimbriae which attaches to the N-acetyl-D-glucosamine receptor (Lee *et al*., 1994). Capsule has a significant role in determining access of certain molecules into the cell membrane, mediating adherence to surface and increasing tolerance to desiccation. Capsulated forms of the organism multiply to a 1000,000 folds higher concerntration than non capsular variant in the liver and spleen and are subsequently released into the blood stream. The cause of death is assumed to be due to endotoxic shock. Furthermore, the capsule of *P. multocida* impairs phagocytosis and may reduce the action of complement mediated killing (Finlay and Falkow, 1989). Capsule has been implicated in virulence in *P. multocida* as encapsulated strains have been shown to be more virulent and able to resist complement mediated killing (Hansen and Hjrsh, 1989; Jacques, 1994; Ragy, 2006). *P. multocida* expresses a siderophore called multocidin, which scavenges iron from host iron-binding glycoproteins. *Pasteurella trehalosi* produces collagenase, which destroys collagen from the lung parenchyma, allowing for deeper penetration of the bacterium and further pulmonary inflammations (Lax and Grigoriadis, 2001).

2.5 RESISTANCE OF *PASTEURELLA MULTOCIDA* TO ANTIBIOTICS

Antibiotic therapy is an effective tool for the treatment and prevention of infectious diseases in food animals. Generally, *P. multocida* isolates are susceptible to most of the widely used commercial antibiotics, but their excessive and unjustified use accelerates the emergence of resistant strains (Arora *et al*., 2005; Sellyei *et al*., 2009). In Nigeria, like in most developing countries, antibiotics are widely used without prescription and the withdrawal period are not usually observed (Kabir *et al*., 2004; Ezenduka *et al*., 2011). WHO (2006) estimated that

about 30% of medicine sold in Africa are counterfeit of which antimicrobial agents being the most popular target. Sub-standard products lower-than-stated doses promote resistance, and those containing no antimicrobial drug at all promote microbial dessimination (Okeke, 2007). The resistant strain of *P. multocida* can result from widespread use of antibiotics in feed, both for prophylaxis, metaphylaxis and for growth promotion (Shivachandra *et al*., 2004). Bacterial agents over a period of time change their antibiogram pattern and develop resistance against commonly used chemotherapeutic agents.The administration of sub-therapeutic doses livestock will no doubt increase the incidences of disease and elevate the virulence of microorganisms that evolved after such exposure (Okeke *et al*., 2007). The resistance of *P. multocida* to common antibiotics and the emergence of multidrug resistant phenotypes have become of increasing concern globally (Caprioli *et al*., 2000; White et al., 2002; Walsh and Fanning, 2008). In Nigeria, there is paucity of information pertaining to resistance of *P. multocida* to antibiotics as the disease caused by this bacterium is treated with oxytetracycline, enrofloxacin, ciprofloxacin, gentamicin, streptomycin among others. Although there is inadequate information on the resistance profiles of *P. multocida* in Nigeria, there have been reported cases of drug resistance due to the organism; in China, resistance of *P. multocida* to a wide range of antibiotics such as amoxicillin, tetracycline, chlortetracycline, lincomycin and sulfadimidine have been reported (Xibiao *et al*., 2009). Previous studies have shown that injurdicious use of antimicrobials have contributed in the promotion of the spread of resistance genes located on plasmids, integrons and transposons (Hunt *et al*., 2000; Kehrenberg *et al*., 2001). This has resulted in the reduction in the efficacy of the antimicrobial agents that are currently available for the treatment of food-producing animals (Kehrenberg *et al*., 2001). Many of these antimicrobials used in animals are identical

or closely resemble drugs used in humans; hence the effect could potentially affect public health (Caprioli *et al*., 2000).

2.6 CLINICAL SIGNS OF FOWL CHOLERA

Clinical manifestations of fowl cholera vary according to strains of the organism involved. The disease occurs in several forms such as per acute, acute and chronic forms (Glisson *et al*., 2003).

The per acute form is probably the most virulent and highly infectious form where there are no pre monitory signs, birds in good body condition are found dead in the nest (Glisson *et al*., 2003).

In the acute form of the disease in chickens, death may occur suddenly and increase rapidly; these could be proceeding a few hours of depression, anorexia, high fever, mucous discharge from the beaks; green foetid diarrhoea and cyanosis of the comb (Einum *et al*., 2003). The chronic form of the disease in chicken is characterized by respiratory infection, conjunctivitis, tracheitis, lameness and dyspnoe. These may be accompanied by swellings in the wattles (Plate I), foot pads, sterna bursa, tendon sheath, joint lesions and infection of the middle ear leading to torticolis and incordination (Heddleston *et al*., 1972).

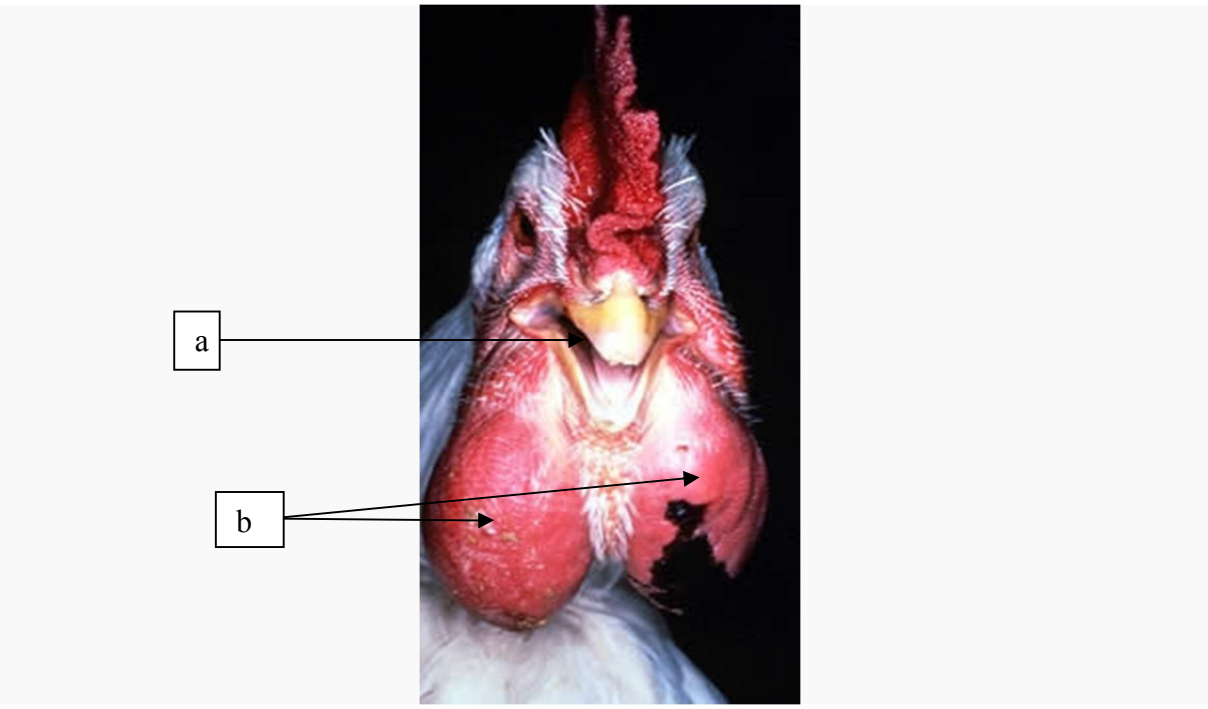


Plate I: Head of a chicken: (a) - opened beaks indicating dyspnea. (b) - swollen wattles in chronic form of fowl cholera. Source: Aiello, 2008.

2.7 DIAGNOSIS OF FOWL CHOLERA

Tentative diagnosis of fowl cholera is based on flock history, clinical manifestation and necropsy findings, while definitive diagnosis is based on animal inoculation, isolation and identification of *P. multocida* (Einum *et al*., 2003). *Pasteurella multocida* can be isolated readily from visceral organs of birds that die of acute fowl cholera and usually from lesions of chronic cases. Bone marrow, heart blood, liver, meninges or localized lesions are preferred for culturing. Bipolar staining round ended rods can be seen in liver imprint and heart blood smear stained by Giemsa (Heddleston *et al*., 1972; Einum *et al*., 2003; Glisson *et al*., 2003). In the per acute form of fowl cholera, there are multiple petechial haemorrhages in the coronary groove, on the proventriculus and serous surface of the sternum of affected bird.

In the chronic form, catarrhal nasal discharges, congested and necrotic lungs may be evident. Purulent lesions may be seen in the bursa and wattles of affected birds (Einum *et al*., 2003).

2.7.1 Gross Pathology of Fowl Cholera

Lesions of fowl cholera vary in type and severity. The greatest variation is related to the course of the disease, whether acute or chronic. Sometimes it is difficult to categorize the disease into these two forms. Clinical signs of infection and lesions may be intermediate to those described for acute and chronic forms (Glisson *et al*., 2003).

2.7.2 Gross Lesions of Acute Form of Fowl Cholera

Gross lesions observed are associated with vascular disturbance such as general hyperemia; petechial and ecchymotic haemorrhages found and may be widely distributed in visceral

organs. Haemorrhages are also observed in subepicardial, myocardium (Plate II), subserosal, lungs, abdominal fat, and intestinal mucosa of affected birds. Increased amounts of pericardial and peritoneal fluid frequently occur. Liver may be swollen, and usually contain multiple small focal areas of coagulative necrosis. Large amounts of viscid mucus may be observed in the digestive tract, particularly in the pharynx, crop, and intestine. Mature follicles often appear flaccid; yolk material from ruptured follicles may be found in the peritoneal cavity (Gustafson *et al*., 1998).

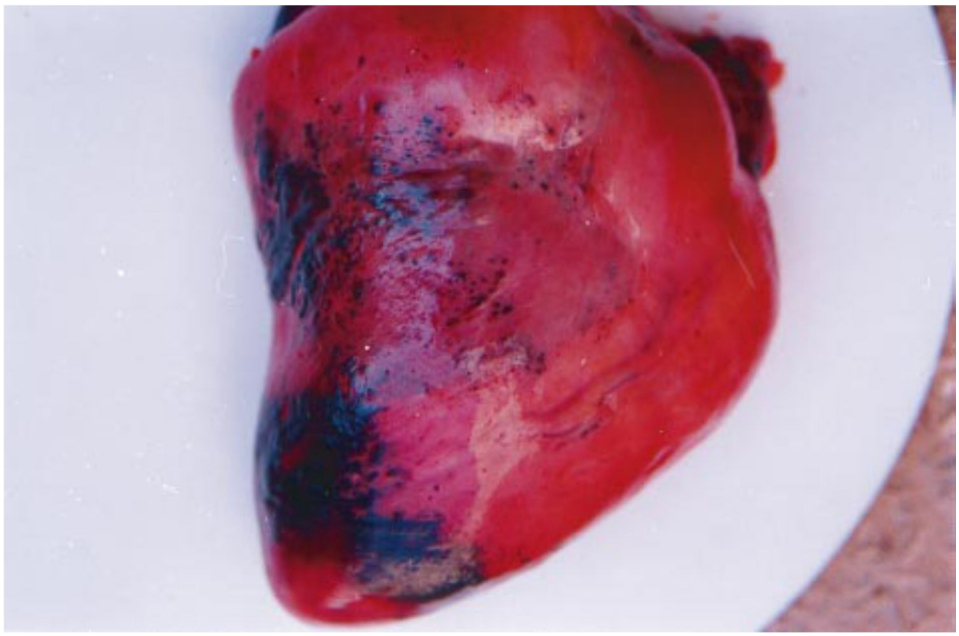


Plate II: Heart of an ostrich suffering from fowl cholera.* Note: (a) - ecchymotic haemorrhages, (b) - petechial haemorrhages on the myocardium. Source: Elfak *et al*. (2002).

2.7.3 Gross Lesions of Chronic Form of Fowl Cholera

Chronic form of fowl cholera is usually characterized by localized infections which become suppurative and may be widely distributed in the body of affected birds. Yellowish caseous exudates in air spaces of the calvarial bones, foot pads, hock joints and peritoneal cavity is usually a consistent gross lesion (Gustafson *et al*., 1998).

2.7.4 Microscopic Lesions of Acute Form of Fowl Cholera

Coagulase necrosis and heterophilic infiltration in the lungs and other parenchymatous organs are usually observed in tissues of affected birds

2.7.5 Microscopic Lesions of Chronic Form of Fowl Cholera

Fibrin and heterophilic infiltration are consistently observed in the air spaces, middle ear, and meninges. Multinuclear giant cells are often associated with necrotic masses of heterophils in air spaces of affected birds (Gustafson *et al*., 1998).

2.8 DIFFERENTIAL DIAGNOSIS OF FOWL CHOLERA

Fowl cholera has to be differentiated from closely related diseases caused by *Riemerella anatipestifer* and *Yersinia pseudotuberculosis.* Several other diseases that are not caused by closely related agents, such as infectious coryza, fowl typhoid, pullorum disease, colibacillosis and staphyloccosis may have histories, signs, or lesions similar to those of fowl

cholera. Differentiation of fowl cholera from these diseases also depends upon isolation and identification of the causative organisms (Rimler and Glisson, 1997).

2.8.1 *Riemerella anatipestifer* Infections

Riemerella anatipestifer infection also known as infectious serositis, duck septicaemia, new duck disease is a septicaemic disease which affects a wide range of hosts such as ducks, geese, turkeys, chickens and quails among others. The disease is worldwide in distribution and it causes significant economic loss due to high mortality, weight loss and condemnations of carcasses. Some of the notable clinical manifestations that can be used to distinguish *Riemerella anatipestifer* infections from fowl cholera are:

a) The acute form of *Riemerella anatipestifer* infection occurs in ducklings 1-8 wk of age, while fowl cholera rarely occurs in birds less than 16 wk of age.

b) Tremors of the head, neck and leg, fibrinous pericarditis, air sacculitis and meningitis are frequent features manifested by *Riemerella anatipestifer* infections in birds (Smith *et al*., 1987). Confirmed diagnosis of fowl cholera is based on isolation and identification of *P. multocida.*

2.8.2 *Yersinia pseudotuberculosis* infection

This organism can affect domesticated, feral, caged birds and variety of mammals, including humans (Rimler and Glisson, 1997). *Yersinia pseudotuberculosis* usually occurs as a septicemia of short duration. In very acute cases, birds may die before other signs of the disease are observed. Common lesions seen in affected birds are emaciation, paralysis,

osteomylitis, enteritis, necrotic foci in visceral organs and muscles (Rimler and Glisson, 1997). Definitive diagnosis depends on isolation and identification of the causative agent of the disease.

2.8.3 Infectious Coryza

Infectious coryza is an acute respiratory disease of chickens caused by *Avibacterium paragallinarum* . The disease occurs worldwide and causes economic losses due to increased culling rate and a marked reduction in egg production. The clinical signs of this disease such as nasal discharge, dypsnea, anoxia, lacrimation and diarrhoea are quite similar to those of fowl cholera. Infectious coryza is often complicated by *P. multocida* (Blackall and Morrow, 1990; Chen *et al*., 1998). Differentiation of infectious coryza from fowl cholera is based on the use of Polymerase Chain Reaction, isolation and identification of causative organisms.

2.8.4 Fowl Typhoid

Fowl typhoid is caused by *Salmonella* Gallinarum. The disease is similar to fowl cholera in terms of clinical manifestation and post mortem features. Birds suffering from fowl cholera normally exhibit signs of depression, weakness, inappetance and ruffled features. There may be congested liver, lungs, spleen and kidneys. All the clinical manifestations are found in birds affected by fowl cholera. However, fowl cholera can be differentiated from fowl typhoid based on: swollen wattles in chronic form of fowl cholera, petechial and ecchymotic haemorrhages on the heart and sub serosal haemorrhages of sterna bones of affected birds. In fowl typhoid, the whole carcass at postmortem shows a marked jaundiced appearance with

dirty brown discolouration of serous membrane. The surface of the liver after exposure to the air for a short time usually shows distinctive green-brooze sheen. This is almost pathognomonic of fowl typhoid. Confirmed diagnosis of fowl cholera is based on isolation and identification of *P. multocida* which is Gram negative rod and coccobacillus; indole and oxidase positive from the organ of affected birds (Glisson *et al*., 2003).

2.8.5 Pullorum Disease

Pullorum disease is caused by *Salmonella* Pullorum. The clinical disease is commonly seen in birds during the first few weeks of life; however, adult chickens, turkeys, and other fowls are also affected. Clinical manifestations of this disease include weakness, inappetance, ruffled feathers, greenish diarrhoea in adult birds, and chalky white diarrhoea in chicks and drop in egg production. Most of these clinical signs are also observed in birds affected with fowl cholera, except that fowl cholera is commonly seen in adult birds. Some of the notably histopathologic lesions associated with pullorum disease are fibrinopurulent and pericarditis; purulent synovitis, focal fibrinoid necrosis and small granulomae in various visceral organs (Gast, 2003). Differentiation of pullorum disease from fowl cholera is based on the isolation and identification of causative organisms.

2.8.6 Colisepticaemia

Clinical manifestation and postmortem features of fowl cholera and colisepticaemia are quite similar. The main frequent lesions in colisepticaemia are pericarditis, epicarditis, generalized visceral serositis and darkening of the liver. Confirmed diagnosis of fowl cholera is based on isolation and identification of *P. multocida* (Glisson *et al*., 2003).

2.8.7 Staphylococccosis

Clinical manifestation of staphylococcal infections such as weakness, inappetance, paralysis, nasal and ocular discharges are similar to those of fowl cholera. However, osteomylitis, arthritis, peri arthritis and synovitis are frequently observed in staphylococcal infections (Andreason, 2003). Differentiation of staphylococccosis from fowl cholera is based on the isolation and identification of causative organisms.

2.8.8 Pneumotropic Form of Newcastle Disease

Dypsnea, coughing, cyanotic comb, haemorrhages and sloughing of the mucosal lining of the respiratory tract seen in the pneumonic form of Newcastle disease is similar to fowl cholera. However, marked lymphocytic infiltration, cuffing with degenerative changes in neurons and ganglia are seen in the pneumotropic form of Newcastle disease.

2.9 TREATMENT OF FOWL CHOLERA

Antibacterial chemotherapy has been used extensively in the treatment of fowl cholera with varying success, depending to a large extent on the promptness of treatment and drug used. Drug sensitivity testing is often advantageous, because strains of *P. multocida* vary in susceptibility to chemotherapeutic agents and resistance to treatment may develop, especially during prolonged use of these agents (Donahue and Oslon, 1972; Arora *et al*., 2004). Some of the chemotherapeutic agents used for the treatment of fowl cholera include: enrofloxacin at 10 mg/kg in drinking water for 5-7 days is very effective against fowl cholera; chloramphenicol administered at 20 mg/kg body weight is also very effective, tetracycline 25-40 mg/kg in drinking for 5-7 days.

2.10 PUBLIC HEALTH IMPLICATIONS OF *PASTEURELLA MULTOCIDA* INFECTIONS

Many species of birds and mammals including human beings are susceptible to *P. multocida*. Infections of *P. multocida* cause live-threaning pneumonia and septicaemia. In humans (especially in immunocompromized) who come in contact with animals with *P. multocida* infection, septicaemia and chronic abcesses characterized by extensive oedema and fibrosis may result (Rhoades and Rimler, 1989; Bisgaard, 1993). A severe cellulitis may develop which may progress to osteomyelitis and subsequently to septicaemia (Francis, 1975).

P. multocida has been added to the list of infective agents that are potentially transmitted after an animal contact. Although statistics on the zoonotic diseases caused by P. *multocida* in Nigeria is lacking, it has been documented that *P. multocida* is estimated to infect 20% - 50% of the 1-2 million Americans (primarily children) who are bitten or scratched each year by a variety of animals including dogs, cats, pigs, rats, lions, opossums and rabbits (Ilaria and Cascio, 2007). A new species of *P. multocida* called *P. tigris* has been associated with tiger bite (Harper, 2006). Bite wound infections caused by pigs have also been reported (Frederiksen, 1989; Frederiksen, 1993).

2.11 PREVENTION AND CONTROL OF FOWL CHOLERA

Prevention of fowl cholera can be effected by eliminating reservoirs of *P. multocida* or by preventing their access to poultry flock. Good management practices, with emphasis on sanitation should be strictly observed. Strict sanitation and biosecurity are assential for successful poultry production (Glisson *et al*., 2003). There is a documented report suggesting that rat (*Rattus rattus*) around poultry houses are involved in the transmission *P. multocida* to

poultry (Mwankon *et al*., 2009). Good biosecurity should be instituted by preventing rodents from getting access to poultry farms. Movement of people and animals should be restricted in order to prevent the transmission of *P. multocida* to uninfected flock. If movement of farm workers and personnel from infected to non-infected areas are not restricted, there will be high risk of introducing the bacterium to new areas. Construction foot dips on poultry farms should be encouraged so the high risk of fowl cholera outbreaks would be reduced. Equipment, clothing, shoes, vehicles and eggs from infected farm should not be taken to uninfected flocks, since they are good sources of spreading *P. multocida* infection. Different species of birds should not be raised on the same premises. Adequate measures should be taken in order to prevent flying birds, pigs, dogs and cats from having access to poultry area (Glisson *et al*., 2003). All housing and equipment should be cleaned and disinfected before restocking.

2.12 VACCINATION AGAINST FOWL CHOLERA

Vaccination is an effective means to prevent or reduce the adverse effects of specific disease. Vaccination programmes should be designed in an area where fowl cholera is prevalent. It is advisable to incorporate the prevalent serotypes of *P. multocida* in fowl cholera vaccine intended to be used in a given area for the control of the disease (Glisson *et al*., 2003). Commercially produced live vaccines are available. In Nigeria, the fowl cholera vaccine for the control of the disease is produced by National Veterinary Research Institute, Vom. The antigen of the killed fowl cholera is *P. multocida* serotype A: 1 (NVRI Reports, 2002). The use of live fowl cholera vaccine stimulates an effective immune response, but has the disadvantage of potentially resulting in mortality in vaccinated birds (Bierer and Derieux, 1972). Vaccination is not a substitute for effective management, because vaccines may be

effective in reducing the risk of flock coming down with fowl cholera, but exposed birds in most cases become infected and shed disease organism (Glisson *et al*., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY AREA

Jos, the capital of Plateau State was purposively selected for this study because of the high concentration of poultry farms and the availability Veterinary Hospitals and Clinics. Jos North and South Local Government Areas of Plateau State are located between Latitudes 8.50^0 -100.46^0 N and Longitudes 8.20^0-10.36^0E in the North-Central geopolitical zone of Nigeria**.** The altitude ranges from 1,200 metres (about 400 feet) to a peak of 1,829 metres above sea level in the Shere hills. The land area of Plateau State is estimated to be 30,913 km^2 which is 2.8% of the land area of Nigeria (923,000 km^2) (Nigeria location map.svg., 2010). Mean annual rainfall varies between 1317 mm in the southern part of the State and 1,460 mm on the high Plateau. There are four types of vegetation zones: Northern Guinea Savannah, Mid altitude or Montane, Southern Guinea Savannah and Sub- Sudan zones (NPC, 2006; NFDP, 2009). The State has an estimated population of 808,298 commercial poultry; of this figure, Jos North has 248,934, Jos South 371,606, while the remaining 15 LGA accounted for 187,758 (Poultry Farm Register of Plateau State Govt., 2009). The human population was estimated at 3.5 million people with over 30 ethnic groups. Plateau State shares common borders with four of the 36 states of the country. To the South-West is Nassarawa, while to the North-West and North-East are Kaduna and Bauchi respectively and to the South-East is Taraba state (Figure 1). There are two seasons: rainy season which starts from April to October and dry season from November to March (NFDP, 2009).

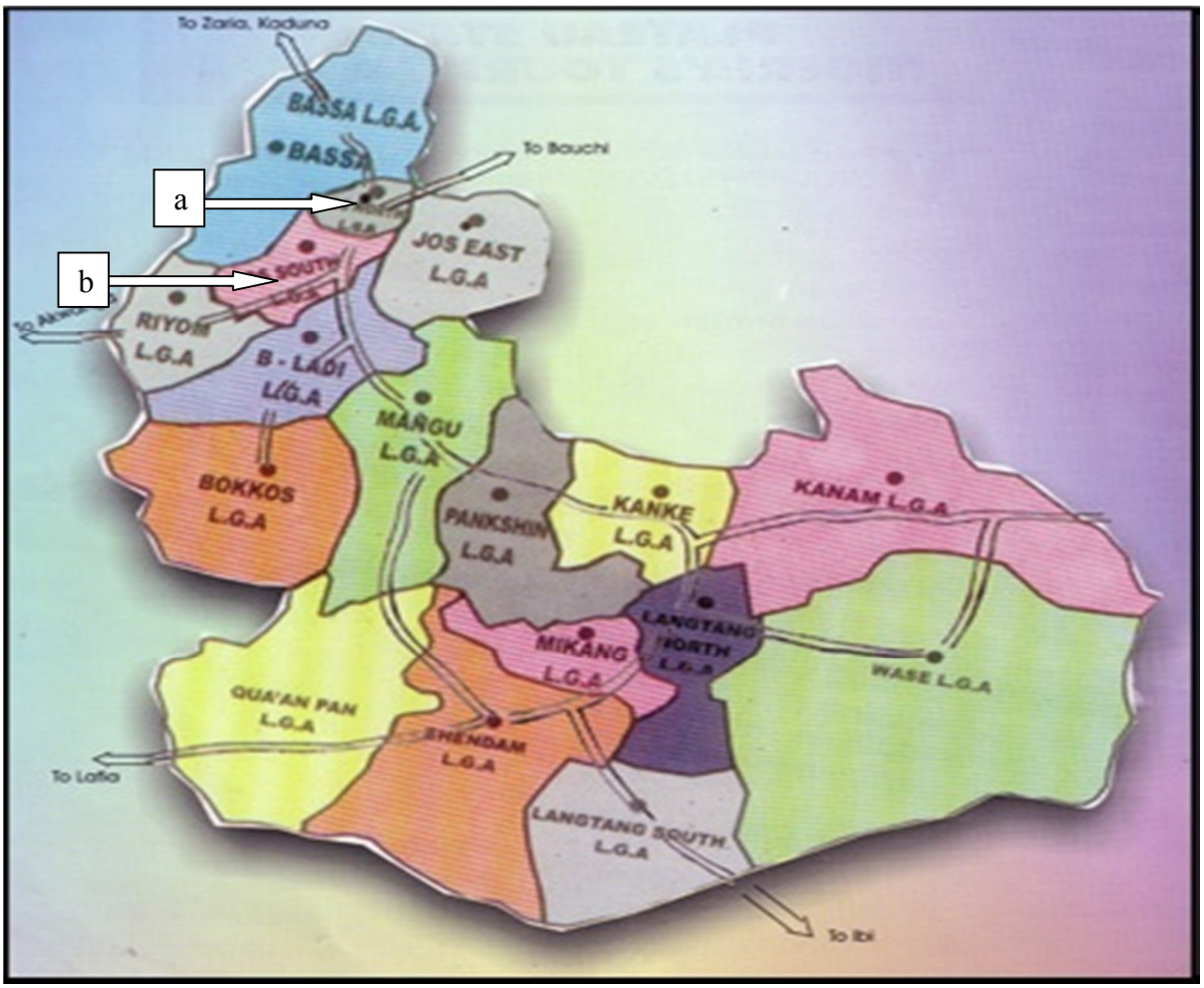


Figure 1: Map of Plateau State showing the various Local Government Areas. A - representing Jos North , b – representing Jos South . Source of Map: Nigeria location map.svg. (2010).

3.2 SAMPLE COLLECTION

3.2.1 Sample size determination.

Sample size was determined using the formula given by Thrusfield (1997).

$n = Z^2PQ/ L^2$

Where n= sample size

Z= appropriate value for the standard normal deviation for the desired confidence interval = 1.96

P= prevalence (38% estimated prevalence used by Yunusa in Sokoto in 2008)

Q = 1-p

L= allowable error - 0.05%

Therefore:

$N = 1.9^2 \times 0.35(1-0.35)/(0.05)^2$

This gave an estimated sample size of 400 chickens based on these parameters. Base on this estimate, we decided to sample 400 clinically sick chickens and 400 apparently healthy ones).

3.2.2 Sampling Method

Systematic random sampling method (one in five; every 5^{th} bird on each visit) was applied for the selection of 400 apparently healthy and 400 clinically sick chickens between November, 2010 and October, 2011 (8 chickens/week for apparently healthy chickens and 8/week for clinically sick). The breed, sex and age of each chicken were recorded.

3.2.3 Inclusion Criteria

Inclusion criteria used were chickens with history of sudden dead as well as those showing any of the following clinical signs such as swollen combs and wattles, cyanotic combs, diarrhoea, emaciation, torticolis, ocular and nasal discharges were selected as presumptive cases of fowl cholera. Chickens from 0-10 weeks were classified as chicks, 11-15 growers and 16 weeks above as adults.

3.2.4 Exclusion Criteria

Chickens without any of these clinical signs swollen combs and wattles, cyanotic combs, diarrhoea, emaciation, torticolis, ocular and nasal discharges were not selected.

3.3 SAMPLING LOCATIONS

3.3.1 Sampling of clinically sick chickens

Purposive sampling technique according to Porney and Watkins (2007) was used in selecting three sampling points which were Central Diagnostic Laboratory of the National Veterinary Research Institute, located in Vom, Plateau State Veterinary Hospital, Jos and ECWA Veterinary Clinic, Bukuru were used for the collection of tissue samples from sick chickens submitted for diagnosis in these three sampling points. Breed of chickens sampled at these points were Isa brown, Hubbard, Marshall, Ross, Dorminant black and others. One hundred and thirty three clinically sick chickens were collected at each sampling point to give a total of three hundred and ninety nine. One chicken was added at Central Diagnostic Laboratory of the National Veterinary Research Institute, Vom, to make up four hundred samples.Tissue samples collected were heart blood, femur, lungs, spleen and liver (400 each from clinically

sick chickens, giving a total of 2000 tissue samples). One hundred and thirty three clinically sick chickens were sampled at each sampling point.

3.3.2 Sampling of apparently healthy chickens

National Veterinary Research Institute Canteen and Poultry Division Vom, Kugiya in Bukuru, Railway-terminus and Yan Kaji market in Jos North were used for the collection of four hundred oro-pharyngeal swabs from apparently healthy chickens. Eighty oro-pharyngeal swabs were collected at each of the five locations. Breed of chickens sampled were same as in 3.3.1.

3.3.4 Transportation of Samples

The samples collected were transported on ice to the Bacteriology Unit of the Central Diagnostic Laboratory, NVRI, Vom for culture and microbiological examination as described by Cowan and Steel (2004).

3.4 CULTURE AND ISOLATION OF *PASTEURELLA MULTOCIDA*

The surface of each organ was seared with spatula and incised with a small sterile scapel blade. Swabs from these organs were inoculated directly onto selective medium which were Casein Sucrose Yeast (CSY) agar and general purpose medium i.e. Blood agar and incubated aerobically at 37^0C for 24 h. Oro-pharyngeal swabs were cultured indirectly by inoculating into 5 ml of brain heart infusion broth (BHI), incubated at 37^0C for 24 h and then streaked unto Casein Sucrose Yeast (CSY) agar and Blood agar. Cultural and morphological examinations were conducted as described by Cowan and Steel (2004). Presumptive *P. multocida* based on colonial morphology were subjected to Gram and methylene blue

staining for cellular morphology. All cultures showing Gram negative, with bipolar coccobacilli characteristics were cultured on MacConkey agar and incubated at 37^0C for 24 h. Isolates that did not grow on MacConkey after 48 h of incubation were subjected to further analysis such as biochemical and molecular characterization. Coccobacilli and bipolar organisms were further confirmed as *P. multocida* by biochemical tests according to Cowan and Steel (2004).

3.4.1 Phenotypic Characterization of *Pasteurella multocida*

Pasteurella multocida obtained from various samples were sub-cultured on blood agar and subjected to comprehensive phenotypic characterization. Presumptive isolates of *P. multocida* were futher subjected to Gram reaction, oxidase, catalase, indole, citrate ultilization and nitrate reduction test according to CLSI (2009).

3.4.1.1 Oxidase test

Standard oxidase discs were used to perform the test. The loopful culture from single colony was applied on the disc. Immediate development of blue colour was considered as positive and those that didn't show blue colour were considered negative.

3.4.1.2 Catalase test

Catalase test was performed by taking 2-3 drops of three percent H_2O_2 on clean grease free glass slide and single colony was mixed using wire loop. Immediate formation of gas bubbles were considered as a positive test.

3.4.1.3 Indole test

Few drops (2-3) of Kovac's reagent was added into the overnight peptone water culture containing *P. multocida* organism and mixed gently to dissolve indole. Pink layer above peptone water was considered as a positive reaction (Appendix VI).

3.4.1.4 Citrate utilization

Slant of Simmon's citrate agar was inoculated with *P. multocida* isolate and incubated at 37^0C for 24 h. Growth with a development of intense blue colour of the medium was considered as a positive reaction (Cowan and Steel, 2004).

3.4.1.5 Nitrate reduction

Few drops (2-3) of nitrate were added to two days old broth culture of *P. multocida* in peptone water containing 0.1 per cent Potassium nitrate and then incubated at 37^0C for 24 h. The 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 a positive test.

3.4.1.6 Carbohydrate fermentation test

Fermentation reactions of *Pasteurella multocida* on sugars such as glucose, mannitol, sucrose, mannose, maltose, arabinose, lactose, dulcitol, inositol, lactose and trehalose were conducted. One per cent of each sugar in peptone water base with one per cent Andrade's indicator was used. Few drops (2-3) of one-day growth of the isolate in peptone water were inoculated into each sugar. Tubes were incubated at 37^0C and readings were recorded after

every 24 hours. Sugars that showed colour change were considered as positive reaction. Proper controls were always kept for each of the biochemical tests performed.

3.4.3 Microbact Test

All *P. multocida* isolates that were identified by biochemical tests were further subjected to analytical profile test using commercially available kit (Macrobact GNB 24E kit) Oxoid® ,United Kingdom, ccording to the manufacturer's instruction. The kit consists of 24 tests (12 biochemical and 12 sugar tests) (Appendix II). Presumptive *P. multocida* organisms with a cut off point of 75% and above with a probability index of 1/100,000, 000 were considered as confirmed *P. multocida* isolates.

3.4.4 Preservation of *P. multocida* Isolates

Twenty five isolates identified as *P. multocida* by Microbact test were preserved in nutrient agar slant and in cryo vials containing 10% glycerol in tryptic soya broth transferred in a screw capped test tubes and kept under refrigeration temperature of -80^0C (in lyophilized state) for further molecular studies.

3.5 ANTIBIOTIC SUSCEPTIBILITY TEST

Twelve isolates of *Pasteurella multocida* identified by Polymerase Chain Reaction were tested for their susceptibility against 15 most commonly used antibiotics in Veterinary Medicine in Nigeria. Antimicrobial agents that were tested include chloramphenicol (30 μg), enrofloxacin (10 μg), ampicillin (10 μg), amoxicillin and clavulanate acid (20 μg/10 μg), gentamycin (10 μg), oxytetracycline (10 μg), erythromycin (10 μg), streptomycin (10

μg), trimetoprim/sulfamethoxazole (1.25μg/23.75μg), ciprofloxacin (10 μg), pefloxacin (10 μg), rocephin (25 μg), furasol (10 μg), tylosin (10 μg) and anicillin (10 μg). The antibiogram of all the isolates was determined on Muller Hinton medium supplemented with 5% defibrinated sheep blood according to the disc diffusion method by Bauer *et al*. (1966). Briefly, 3 colonies of *P. multocida* were made into homogenous suspension in 5 ml of sterile Muller Hinton broth and incubated for 5 min. The turbidity of each isolate in the homogenous suspension was measured in a Nephelometer in order to standardize to a turbidity equivalent of 0.5 Mc Farland standard which correspond to $1x10^7$ colony forming units. Each homogenous suspension consisting of a 24 hour old culture of *P. multocida* was spread evenly on plates. The culture was allowed to dry for about 10 min. Subsequently, each antimicrobial disc was picked with a sterile forcep and placed on the plate at an appropriate distance from each other. The plates were later incubated at 37^0C for 24 h. The resistance profile of *P. multocida* was assessed as described by Shivachandra *et al.* (2004). Isolates resistant to at least three different antibiotic classes was classified as multidrug resistant. The diameter of the zone of inhibition of each antibiotic was measured and matched with respective standard zone diameter to interpret the test culture as resistant, intermediate or sensitive according to the procedure of Bauer *et al*. (1966).

3.6 SOMATIC SEROTYPING OF *PASTEURELLA MULTOCIDA* ISOLATES

Pasteurella multocida isolates were shipped to the National Veterinary Services Laboratory Ames, Iowa, United State Department for Agriculture (USDA) for somatic serotyping. Somatic serotyping based on lipopolysaccharide was carried out using agar gel diffusion precipitation test (AGDPT) as described by Heddleston *et al.* (1972).

3.7 CULTURE LYSATE METHOD OF DNA EXTRACTION

Two mililitre of 18 h broth culture was pelleted by centrifugation at 7826 g for 10 minutes (10,000 rpm, rotor radius of 7 cm). The pellet was washed with sterile PBS twice and re-suspended in 30 µl sterile nuclease free water and boiled for 10 minutes. After boiling, the sample was chilled over ice. The samples were centrifuged at 704 g for 10 minutes to sediment the cell debris and supernatant stored at -20^0C until used (3,000 rpm, rotor radius of 7 cm).

3.8 *PASTEURELLA MULTOCIDA* ASSAY BY POLYMERASE CHAIN REACTION METHOD.

Presumptive isolates of *P. multocida* were confirmed by a PCR assay using *P. multocida*-specific primer (KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'- forward; KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'- reverse) for amplification of KMT 1 gene. The amplified products of *P. multocida* serotype showed bands that are expected to correspond to amplicon size of 460 base pairs (Townsend *et al*., 2001). Bacterial culture lysate was used as template DNA (50 ng), was added to the PCR mixture (total volume of 25 µl) containing 1 x PCR buffer, 200 µM of each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 20 pmol of each primer and 1 unit *Taq* DNA polymerase. Amplification programme using automated thermal cycler were as follows:

The initial DNA denaturation was achieved at 95^0C for 10 min. Denaturation during cycling was done at 95^0C for 2 min. Annealing was carried out at 55^0C for 30 sec. Extension was done at 72^0C for 1 min, while the final extension was achieved at 72^0C for 10 min. Cycle was repeated 35 times to produce millions of copies. The reaction was held at 4^0C until it was

required for electrophoresis; 5 µl of each sample was electrophoresed on a 2% agarose gel in 1 x Tris-acetate running buffer (TAE) at 4 V/cm for 1 h. The gel was stained with ethidium bromide at 5 µl of 10 mg/ml. The amplified DNA fragments were viewed on a transilluminator and photographed.

3.9 MULTIPLEX CAPSULAR POLYMERASE CHAIN REACTION TYPING

The capsular types were determined by multiplex PCR typing and capsular specific primers (CAP A, CAP B, CAP D, CAP E and CAP F) listed below were used as previously reported by Townsend *et al.* (2001).

Multiplex capsular PCR primers	**Amplicon size**
CAPA-FWD 5'-TGC-CAA-AAT-CGC-AGT-GAG-3'	1,044 bp
CAPA-REV 5'-TTG-CCA-TCA-TTG-TCA-GTG-3'	
CAPB-FWD 5'-CAT-TTA-TCC-AAG-CTC-CAC-C-3'	760 bp
CAPB-REV 5'-GCC-CGA-GAG-TTT-CAA-TCC-3'	
CAPD-FWD 5'-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC-3'	657 bp
CAPD-REV 5'-CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG-3'	
CAPE-FWD 5'-TCC-GCA-GAA-AAT-TAT-TGA-CTC-3'	511 bp
CAPE-REV 5'-GCT-TGC-TGC-TTG-ATT-TTG-TC-3'	
CAPF-FWD 5'-AAT-CGG-AGA-ACG-CAG-AAA-TCA-G-3'	851 bp
CAPF-REV 5'-TTC-CGC-CGT-CAA-TTA-CTC-TG-3'	

3.10 PATHOGENICITY TEST OF *PASTEURELLA MULTOCIDA*

Pasteurella multocida isolated from apparently healthy and clinically sick chickens were used for pathogenicity studies using established models like chickens, quails and mice as described below (Rimler and Glisson, 1997):

3.10.1 Chickens Inoculated with *Pasteurella multocida*

Commercial chickens of eighteen weeks old were provided by the National Veterinary Research institute, Vom, for this experiment. One week before the onset of the study, all chickens to be used for the study were screened for the presence of *P. multocida* by taking oro-pharyngeal swabs. All chickens were fed with commercial pelleted layer feed and water *ad libitum* before and during the experiment. The floor space of the pen was 4 x 5 m^2 measurement in size. Chickens were allowed to acclimatize for two weeks prior to the commencement of the pathogenicity study. Five groups of six chickens were used for the pathogenicity test. The first group were inoculated with a concentration of 10^8, 10^7 and 10^6 CFU of *P. multocida* serotype A: 1 (contained in 0.1ml of Typtose Soya broth) intramuscularly at the breast muscle. The same concentration of 10^8, 10^7 and 10^6 CFU A:3 was administered to the second group, while serotype A:4 was given to the third group at the same concentration as stated above and the 4^{th} were given vaccine strain. Appropriate control, i.e., the 5^{th} group was inoculated with 0.1 ml normal saline. Death occurring within the period of twenty one days of the experiment was recorded. Findings from postmortem examination of carcasses and histopathological features were recorded.

3.10.2 Japanese quails inoculated with *Pasteurella multocida*

Thirty adult Japanese quails (*Coturnix coturnix japonica*) were also divided into five groups. All the Japanese quails were fed with commercial pelleted layer feed and water was given *ad libitum*. The floor space was 35 cm^2 measurement in size. Pathogenicity test was conducted as described in 3.10.1.

3.10.3 Mice Inoculated with *Pasteurella multocida*

Five groups of six mice were used for the pathogenicity test. In the first three groups consisting of eighteen apparently healthy 4 week old Swiss-Webster mice were challenged by inoculating intra-peritoneally with 10^8, $10^7$and 10^6 CFU of *P. multocida* serotypes A: 1, A: 3 and A: 4 in 0.1 ml Tryptic Soya broth and returned to their cages (floor space of 30 cm^2). Mice were fed with pelleted feed and water *ad libitum* before and during the experiment. The mice were observed for death over 24 h. The fourth group consisting of six mice were inoculated with *P. multocida* vaccine strain obtained from National Veterinary Reseach Institute, Vom, at the same concentrations above. The fifth group which was the control also consisted of six apparently healthy mice, which received 0.1 ml normal saline. All experimental mice were kept under the same environment. Clinical and postmortem examination was conducted on any mouse that died and the lungs, heart blood, spleen and liver were collected for histopathology and re-isolation of *P. multocida.*

3.11. CLINICAL AND PATHOLOGICAL EXAMINATION

3.11.1 Clinical examination of chickens, Japanese quails and mice.

All birds and mice in the infected and control groups were examined for clinical manifestions post inoculation with *P. multocida* serotypes A: 1, A: 3, A: 4; vaccine stain and normal saline.

3.11.2 Postmortem examination of chickens, Japanese quails and mice

Postmortem examination was carried out on carcasses of chickens, Japanese quails and mice. Birds and mice used as controls were euthanized at the end of the pathogenicity study and

necropsy was also carried out on the carcasses. Grossly affected organs such as heart, liver, lungs and spleen were taken and preserved in 10% buffered formalin for histopathology.

3.11.3 Preparation of tissues for histopathology

After the gross pathological examination and diagnosis, tissue specimens of grossly affected organs were collected and fixed in 10% buffered formalin; paraffin embedded tissues, were sectioned at 5 microns, stained with haematoxylin and eosin (H & E), mounted on glass slides and examined under light microscope at X200 and X400 respectively.

3.12 STATISTICAL ANALYSIS

Data were used to generate tables and figures in order to determine prevalence. Chi-square test was used to test for association between *P. multocida* isolated in clinically sick and apparently healthy chickens with respect to breed, age, and serotypes (Thrusfield, 1997). Values of $P \leq 0.05$ were considered significant. Analytical profile of all positive isolates was carried out using Microbact GNB 24E supplied software version Microbact ^{Tm} 200 identification package V2.03 (Windows ^{TM}). The percentage probabilities of isolates above 75% were considered positive, while those with less than 75% were regarded as negative.

CHAPTER 4

RESULTS

4.1 BACTERIOLOGY

Table 4.1 shows the distribution *Pasteurella multocida* isolated from different tissues of clinically sick chickens in Jos, Nigeria.The result indicates that a total of 20 *Pasteurella multocida* were isolated from tissues of clinically sick chickens. All the 20 *Pasteurella multocida* isolates were recovered from adult chickens above 16 weeks of age and the presence of the bacterium was highest in the heart blood (8 out of the total of 20 isolates) and lowest in the spleen (1 out of the total of 20 isolates). The isolation rates of *P. multocida* in different breeds of chickens showed that 20% of the organisms were isolated from Isa brown, Ross and Dominant black. Sixteen percent (16%) were recovered from Hubbard and Marshall, while 8% were isolated from other chickens whose breeds were not determined (Table 4.3). There was no significant difference ($P>0.05$; $X^2=2.19$, df=3) in the isolation rate of *P. multocida* and breed of chickens sampled (Appendix III). A significant association ($P<0.05$; $X^2=6.01$, df=2) was found between the isolation rate of *P. multocida* and age of sampled chickens as all the *P. multocida* isolates were recovered from chickens above 16 weeks (Appendix IV). Biochemical profiles of all the 25 *P. multocida* isolates indicated that 100% were Gram negative rods, indole positive and all reduced nitrate to nitrite. Variable reactions were observed for hydrogen sulphide production (88.9%), lactose fermentation (83.7%), ornithine decarboxylase positive (63.9%), urease production (91.7%) and oxidase production (69.4%) (Appendices V and VI). The results indicated that the frequency of isolation of *P. multocida* was highest between July and October (Table 4.2).

Five (5) *Pasteurella multocida* isolates were isolated from the oro-pharyngeal swabs of apparently healthy chickens. All the isolates of *Pasteurella multocida* were recovered from adult chickens above 16 weeks of age.

Table 4.1: Distribution of *P. multocida* in tissues of clinically sick chickens in Jos metropolis, Nigeria.

Tissue	Number of tissues examined	Number of *P. multocida*	Percentage
Bone marrow	400	4	1
Heart blood	400	8	2
Liver	400	4	1
Lung	400	3	0.75
Spleen	400	1	0.25
Total	2000	20	1

Table 4.2: Distribution of *Pasteurella multocida* isolates by month of isolation (between November, 2010 and December, 2011) in Jos metropolis, Nigeria.

Month	Number of *P. multocida* isolates	Percentage
January	2	8
February	1	4
March	2	8
April	2	8
May	-	-
June	1	4
July	5	20
August	4	16
September	3	12
October	3	12
November	2	8
December	-	-
Total	25	100

Table 4.3: The isolation rates of *Pasteurella multocida* in different breeds of apparently healthy and clinically sick chickens in Jos.

Breed of chickens	No. *P. multocida* Isolated	Percentage (%)
Isa brown	5	20
Hubbard	4	16
Marshall	4	16
Ross	5	20
Dominant black	5	20
Others	2	8
Total	25	100

* Out of the 5 isolates from apparently healthy chickens, 2 each were from Isa brown and Marshall and 1 from Ross.

4.2 MICROBACT

Of the 35 *P. multocida* presumptive isolates (25 from clinical cases and 10 from apparently healthy chickens) identified by biochemical tests, twenty of the isolates from clinically sick and five from apparently healthy chickens were identified as *P. multocida* by Microbact kit (Table 4.4 and Appendix II). Microbact GNB 24E test identified 25 *P. multocida* isolates with cut-off point of 75% above and probability index of <1/100,000,000. All isolates were found to ferment glucose, mannitol, ONGP and sorbitol, while 96% were indole positive, 64% lactose negative, 68% produces H_2S and 80% were ornithine positive.

Table 4.4: Identification of *Pasteurella multocida* isolated from chickens using Microbact GNB 24E kit.

Serial number	*Pasteurella Multocida* isolate	Octal number	Percentage Identification	Probability index
1	1	537736600	99.19	<1/100,000,000
2	12	576611410	99.75	<1/100,000,000
3	57	577611421	99.97	<1/100,000,000
4	72	567610666	99.70	<1/100,000,000
5	122	546600400	98.76	<1/100,000,000
6	150	577623550	98.77	<1/100,000,000
7	200	577623600	99.99	<1/100,000,000
8	207	577721421	96.03	<1/100,000,000
9	231	566723400	99.79	<1/100,000,000
10	236	577711421	99.99	<1/100,000,000
11	258	576723400	99.79	<1/100,000,000
12	354	576623420	83.26	<1/100,000,000
13	30	576611421	89.48	<1/100,000,000
14	46	576600410	99.38	<1/100,000,000
15	40	576623420	83.26	<1/100,000,000
16	4	576611410	99.75	<1/100,000,000
17	111	577623600	99.99	<1/100,000,000
18	63	567600665	99.29	<1/100,000,000
19	182	547600460	99.75	<1/100,000,000
20	180	576623420	83.26	<1/100,000,000
21	382	576623421	83.26	<1/100,000,000
22	392	577623610	99.21	<1/100,000,000
23	293	576723401	93.23	<1/100,000,000
24	398	577720420	79.84	<1/100,000,000
25	251	577723420	99.55	<1/100,000,000

4.3 *PASTEURELLA MULTOCIDA* ISOLATES IDENTIFIED BY POLYMERASE CHAIN REACTION ASSAY

Out of the twenty *P. multocida* isolated from clinical cases and further confirmed by Micobact GNB 24E, only 10 were found to give an amplicon of 460 bp using *P. multocida* - specific primer (KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'- forward; KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'- reverse), whereas no amplicon was observed from the remaining 10. Two *P. multocida* isolates out of five from apparently healthy birds also gave amplified product; the remaining three were negative (Plates III and IV).

4.4 *PASTEURELLA MULTOCIDA* ISOLATES IDENTIFIED BY MULTIPLEX POLYMERASE CHAIN REACTION

A total of twelve *P. multocida* isolates (10 from clinical and 2 from apparently healthy chickens) confirmed by PCR gave an amplicon size of 1,044bp, which corresponded to Capsular type A. No amplicon was found to belong to capsular types B, D, E and F (Plates V and IV).

4.5 SOMATIC SEROTYPING OF *P. MULTOCIDA* ISOLATES

Agar gel diffusion precipitation test (AGDPT) carried out on the 12 isolates identified five (41.7%) as belonging to serotype 1, two (16.7%) to serotype 3, one (8.3%) to serotype four and four (33.3%) of the isolates were Untypable (Table 4.5).

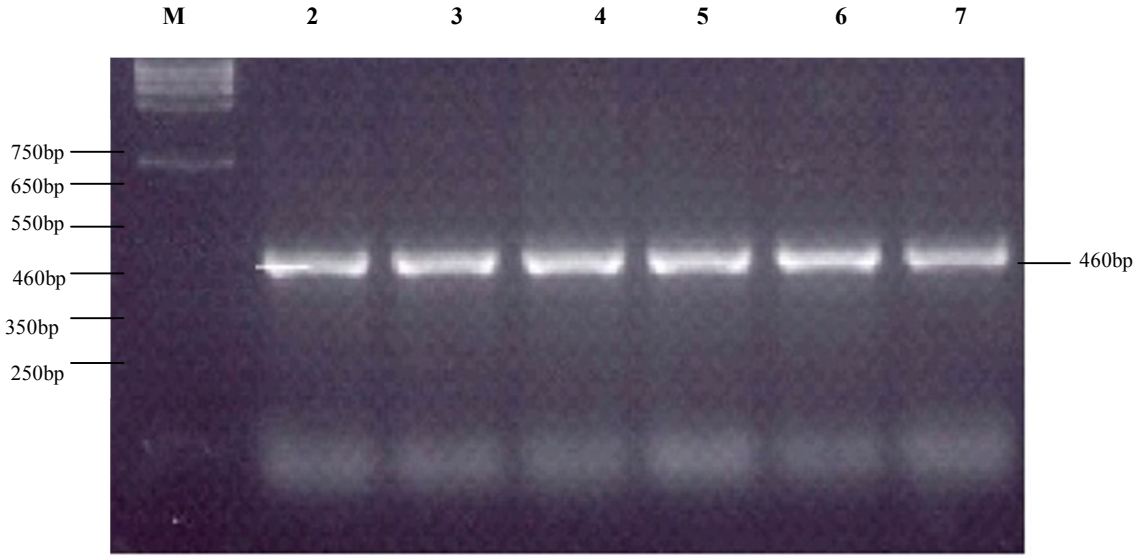


Plate III: *Pasteurella multocida* – Specific PCR. Lane M - 100 bp molecular weight marker; lane 2- positive control, lanes 3-7 are *P. multocida* isolates 1, 12, 57, 72 and 122 respectively which yielded the 460 bp amplicon.

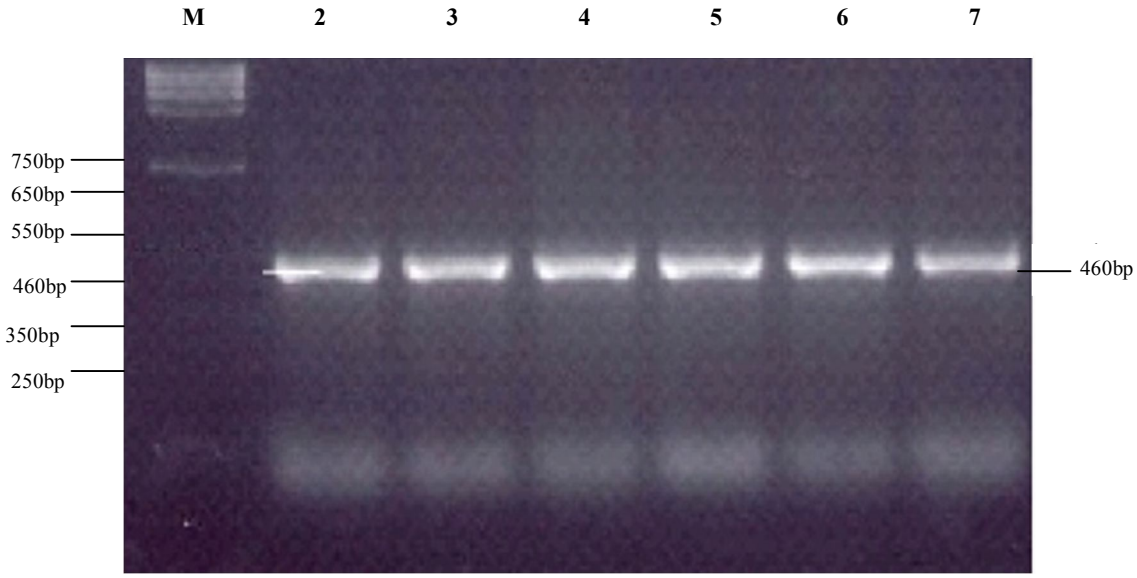


Plate IV: *Pasteurella multocida* – Specific PCR. Lane M - 100 bp molecular weight marker; lane 2- positive control, lanes 3-7 are *P. multocida* isolates 200, 207, 57, 231, 236 and 354 which yielded the 460 bp amplicon**.**

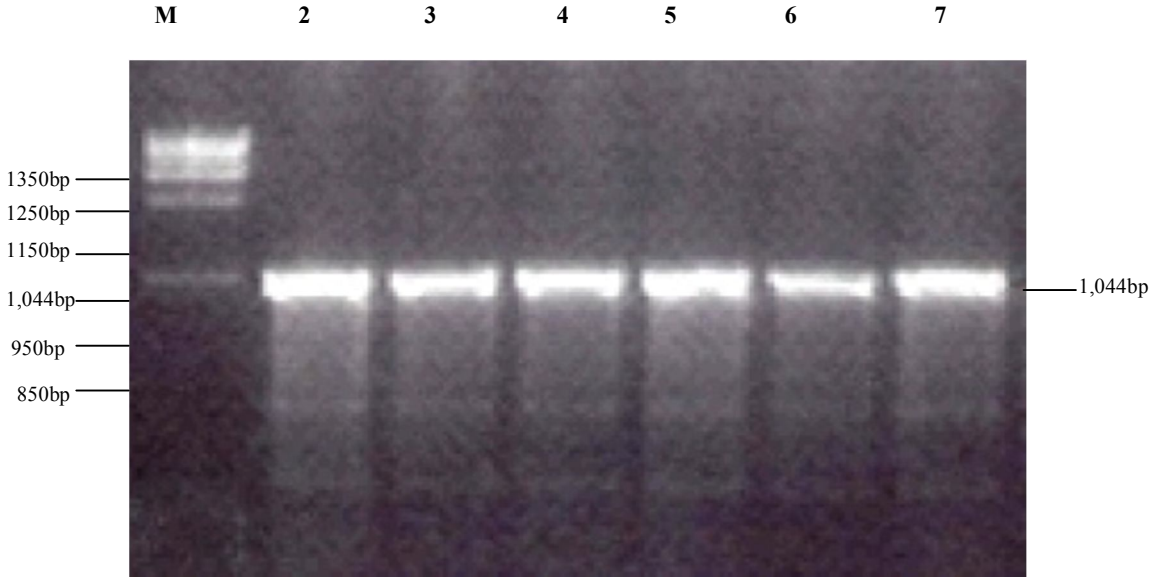


Plate V: Multiplex capsular PCR typing. Lane M - 100 bp molecular weight marker; lane 2- positive control, lanes 3-7 are *P. multocida* isolates 1, 12, 72 and 122 respectively which yielded 1,044 bp amplicon**.**

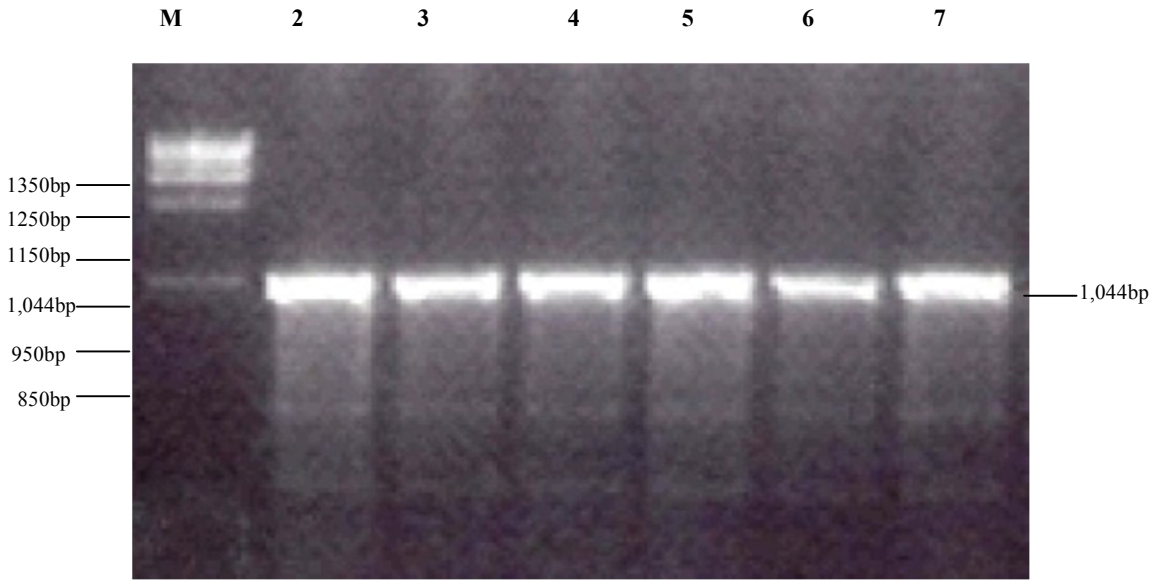


Plate VI: Multiplex capsular PCR typing. Lane M - 100 bp molecular weight marker; lane 2- positive control, lanes 3-7 are *P. multocida* isolates 200, 207, 231, 236, and 258 respectively which yielded 1,044 bp amplicon**.**

Table 4.5: Serotypes of *Pasteurella multocida* isolated from chickens in Jos, Nigeria.

Serotypes	Number of Isolates	Percentage total (%)
1	5	41.7
3	2	16.7
4	1	8.3
Untypeable	4	33.3
Total	12	100

4.6 ANTIMICROBIAL SUSCEPTIBILITY OF *PASTEURELLA MULTOCIDA* ISOLATES

All the *P. multocida* isolates that were tested for sensitivity against a panel of 15 antimicrobial agents exhibited 100% susceptibility to gentamicin, streptomycin and ciprofloxacin; followed by, oxytetracycline and pefloxacin 91.7%; chloramphenicol, enrofloxacin and furasol 83.3% each, recephin75%. High resistance was shown to ampicillin, 91.7% and amoxicillin/clavulanate 83.3%. Five isolates (41.7%) of *P. multocida* were resistant to six or more antibiotics (Tables 4.6, 4.7 and Appendices VII-XIX).

Table 4.6: Antimicrobial susceptibility profile of *Pasteurella multocida* isolated from clinically and apparently healthy chickens in Jos metropolis, Nigeria.

Antibiotic agent	Disc concentration (μg)	No. of strains resistant	No. of strains sensitive	Percentage sensitivity	Percentage resistant
Chloramphenicol	30	2	10	83.3	16.7
Enrofloxacin	10	2	10	83.3	16.7
Ampicillin	10	11	1	8.3	91.7
Amoxicillin/Clavulanic	30	10	2	16.7	83.3
Gentamicin	10	-	12	100	0.0
Oxytetracycline	10	1	11	91.7	8.3
Erythromycin	10	7	5	41.7	58.3
Steptomycin	10	-	12	100	0.0
Trimethoprim/Sulfamethoxazole	30	8	4	33.3	66.7
Ciprofloxacin	10	-	12	100	0.0
Pefloxacin	10	1	11	91.7	8.3
Reocephin	25	3	9	75	25
Furasol	10	2	10	83.3	16.7
Tylosin	10	4	8	66.7	33.3
Anicillin	10	7	5	4.7	58.3

Table 4.7: Antibiotic resistance profile of twelve *Pasteurella multocida* isolates tested against 15 antimicrobial agents.

Pasteurella multocida Isolates	Total number of drugs to which isolate was resistant	Antibiogram	Percentage resistance
1	8	CH,Cx,Am, Au, E, SXT, Ro, Ani	53.3
12	6	Cx, Am, Au, O, E, SXT	40
57	6	CH, Am, Au, Ani, E,SXT	40
72	5	Am, Au, Ani, E,SXT	33.3
122	3	Am, E, Ani	20
150	7	Am, Au, E, SXT, Fur, Tyl, Ani	46.7
200	2	Am, Au	13.3
207	3	Am, Au, SXT	20
231	5	AM, Au, SXT, Tyl, Ani	33.3
236	6	AM, Au, SXT, Pef, Ro, Ani	40
258	1	Tyl	6.7
354	5	Am, Au, E, Ro, Fu	33.3

KEY
CH – chloramphenicol
CX – enrofloxacin
CPX –ciprofloxacin
AM – ampicillin
AU – amoxicillin/clavulanate
CN – gentamicin
O – oxytetracycline
E – erythromycin
SXT –trimethoprim/sulfamethoxazole
Pef – perfloxacin
Ro – rocephin
Fur – furasol
Tyl – Tylosin
Ani - anicillin
S – streptomycin

4.7 PATHOGENICITY TEST

4.7.1 Respones of chickens inoculated with *P. multocida* serotypes A: 1, A: 3 and A: 4

Experimental chickens inoculated with *P. multocida* serotypes A: 1, A: 3 and A: 4 manifested clinical signs such as depression, inappetence, ruffled feathers, dyspnoe, sitting on hock within 24 h post inoculation and signs continued for four days (Table 4.8). No clinical sign was noticed in the group inoculated with vaccine strain and the control chickens. On day five, four carcasses were picked in the group inoculated with *P. multocida* serotypes A: 1 at concentration of 10^8 CFU and three in group inoculated with *P. multocida* serotype A: 3 at concentrations of 10^8 cfu and 10^7 CFU. No death was recorded in vaccine strain, A: 4 and control groups. One bird died in the group inoculated with A: 1 on day nine (Appendix XX). Amelioration of clinical signs in affected chickens was noticed on day ten and chickens were fully recovered and active. Pure isolates of *P. multocida* were recovered from hearts of 4 chickens and livers of two necropsied chicken carcasses on day five. Lesions observed during postmortem examinations in chickens inoculated with *P. multocida* serotypes A1 and A3 were: prominent keel with congested heart, liver, kidneys and lungs (Table 4.9 and Plate VII). Frothy exudates from the lungs, petechial and ecchymotic haemorrhages were seen on the heart, subserosal haemorrhages (Plate VIII), fatty degeneration and egg yolk peritonitis were also observed in some birds.

4.7.2 Respones of Japanese quails inoculated with *P. multocida* serotypes A: 1, A: 3 and A: 4

Japanese quails inoculated with *P. multocida* serotypes A:1, A:3, A:4 and vaccine strain did not exhibit severe clinical signs. On day one of inoculation, weakness and inappetence were observed in some Japanese quails in the groups inoculated with strains of *P. multocida* A: 1,

A: 3 and A: 4 within 24 h (Table 4.10). Five Japanese quails in group A1, four in group A: 3 and two in group A: 4 died without any premonitory signs on day seven post inoculation and *P. multocida* were re-isolated from hearts of two quails and a liver (Appendix XXI). On day 14, Japanese quails were fully recovered. Japanese quails inoculated with vaccine strain and control group did not manifest any clinical sign. Ecchymotic haemorrhages of the heart and breast muscles were also observed. Mild postmortem lesions, such as congested heart, liver, and lungs were seen (Table 4.11) (Plates IX and X).

Table 4.8: Clinical signs in chickens inoculated with *P. multocida* serotypes A: 1, A: 3 and A: 4

Clinical signs	*Pasteurella multocida* serotypes A1	A3	A4
Inappetance	6/6 (100%)	6/6 (100%)	0/6 (0%)
Ruffled Feathers	6/6 (100%)	3/6 (100%)	0/6(0%)
Dyspnoe	3/6 (50%)	3/6 (100%)	0/6 (0%)
Sitting on hock	3/6 (50%)	0/6(0%)	0/6(0%)

Table 4.9: Gross lesions in chickens inoculated with *Pasteurella multocida* serotypes A: 1, A: 3 and A: 4

	Pasteurella multocida serotypes		
Gross Lesions	A1	A3	A4
Congested liver	6/6 (100%)	6/6 (100%)	0/6(0%)
Congested heart	6/6 (100%)	6/6 (100%)	0/6 (0%)
Congested kidneys	6/6 (100%)	6/6 (100%)	0/6(0%)
Congested lungs	6/6 (100%)	6/6 (100%)	0/6(0%)
Frothy exudates from lung	3/6 (50%)	0/6 (0%)	0/6 (0%)
Petechial and Ecchymotic haemorrhages on heart	6/6 (100%)	6/6 (100%)	0/6(0%)
Fatty degeneration	1/6 (16.7%)	0/6(0%)	0/6 (0%)
Egg yolk peritonitis	3/6 (50%)	3/6 (50%)	0/6(0%)
Prominent keel	6/6 (100%)	6/6 (100%)	0/6 (0%)

Table 4.10: Clinical signs in Japanese quails inoculated with *Pasteurella multocida* Serotypes A: 1, A: 3 and A: 4

Clinical sign	*Pasteurella multocida* serotypes A1	A3	A4
Weakness	6/6 (100%)	3/6 (50%)	3/6 (50%)
Inappetance	6/6 (100%)	6/6 (100%)	3/6 (50%)

Table 4.11: Gross lesions in Japanese quail inoculated with *Pasteurella multocida* Serotypes A: 1, A: 3 and A: 4

	Pasteurella multocida serotypes		
Gross Lesions	A1	A3	A4
Congested heart	6/6 (100%)	6/6 (100%)	3/6 (50%)
Congested liver	6/6 (100%)	6/6 (100%)	6/6 (100%)
Congested lungs	3/6 (50%)	3/6 (50%)	3/6 (50%)
Petechial & ecchymotic hemorrhages of the heart	6/6 (100%)	6/6 (100%)	0/6 (0%)
Hemorrhages of breast muscle	3/6 (50%)	3/6 (50%)	0/6(0%)



PlateVII: A - Congested liver in chicken inoculated with *Pasteurella multocida* serotypes A: 1.

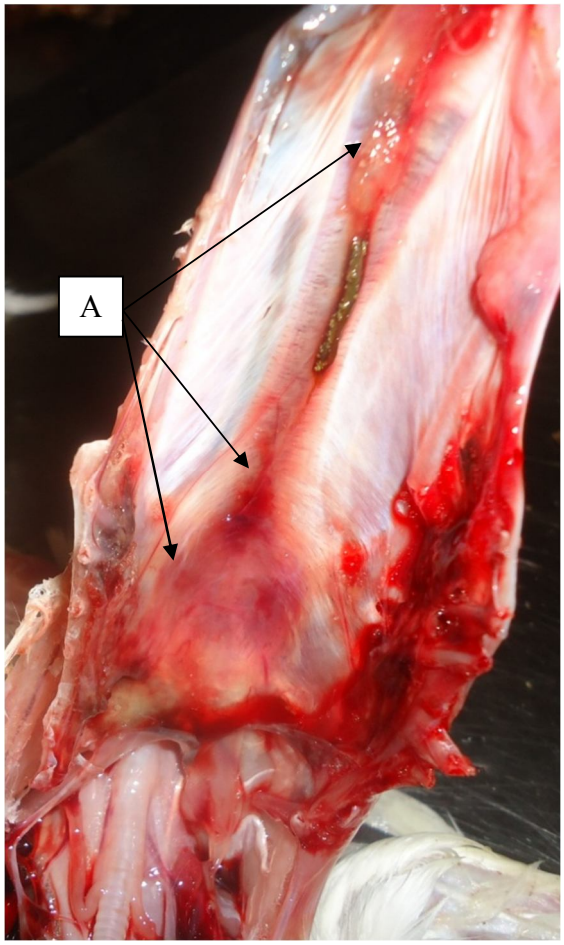


Plate VIII: A - Haemorrhages in subserosal layer of sternal bone of chicken inoculated with *Pasteurella multocida* serotypes A: 1.

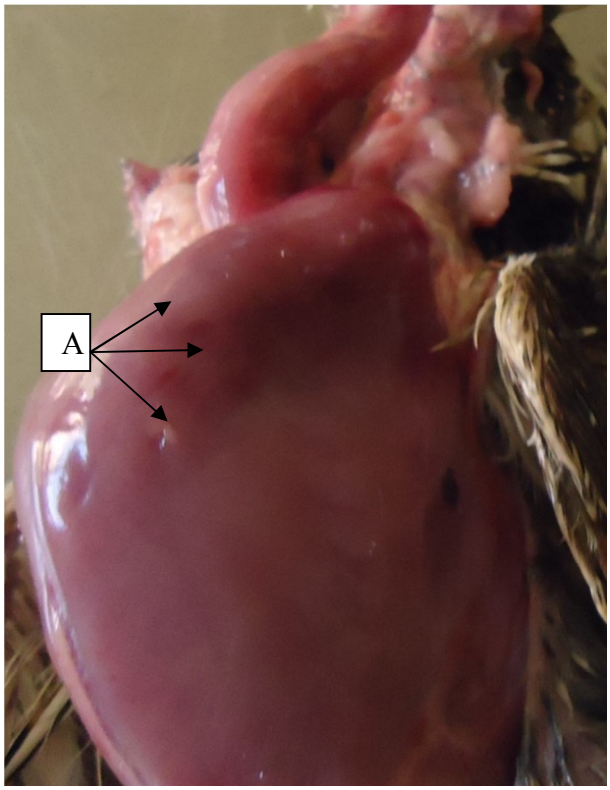


Plate IX: A - Haemorrhages in breast musle of a Japanese quail inoculated with *Pasteurella multocida*. A: 3.

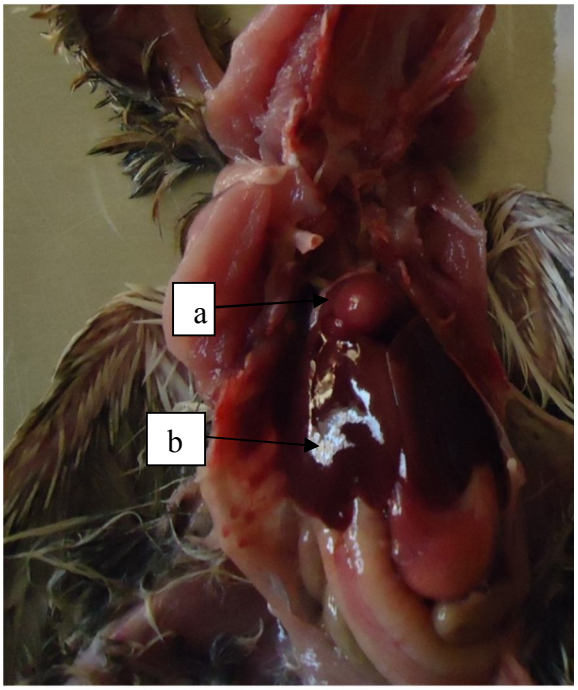


Plate X: a - Lesions of congested heart and b – congested liver of a Japanese quail inoculated with *Pasteurella multocida* serotype A: 4..

4.7.3 Respones of mice inoculated with *Pasteurella multocida* serotypes A: 1, A: 3 and A: 4

All the 18 Swiss-Webster mice inoculated with *P. multocida* serotypes: A: 1, A: 3 and A: 4 at concentrations of 10^8, 10^7 and 10^6 CFU were found dead within 24 h post inoculation (Appendix XXII). Clinical signs manifested prior to death were weakness, inappetence, dyspnea, ataxia and epitaxis (Tables 4.12 and 4.13). Postmortem findings recorded were congested heart, liver and lungs (Plate XI). Frothy exudates were noticed in the lungs of two mice inoculated with *P. multocida* of serogroup A: 1 at a concentration of 10^8 CFU. Mice in the control group and vaccine strain did not exhibit any clinical sign or mortality.

Table 4.12: Clinical signs in mice inoculated with *Pasteurella multocida* serotypes A: 1, A: 3 and A: 4

	Pasteurella multocida serotypes		
Clinical signs	A1	A3	A4
Inappetance	6/6 (100%)	6(100%)	6/6 (100%)
Weakness	6/6 (100%)	6/6(100%)	6/6 (100%)
Ataxia	0/6 (0%)	3/6 (50%)	0/6 (0%)
Epitaxis	0/6 (0%)	3/6 (50%)	0/6(0%)

Table 4.13: Gross lesions in mice inoculated with *P. multocida* serotypes A: 1, A: 3 and A: 4

	Pasteurella multocida serotypes		
Gross lesions	A1	A3	A4
Congested heart	6/6(100%)	3/6(50%)	3/6(50%)
Congested liver	6/6(100%)	6/6(100%)	6/6 (100%)
Congested lungs	6/6(100%)	6/6(100%)	3/6(50%)
Frothy exudates	6/6 (100%)	0/6(0%)	0/6(0%)

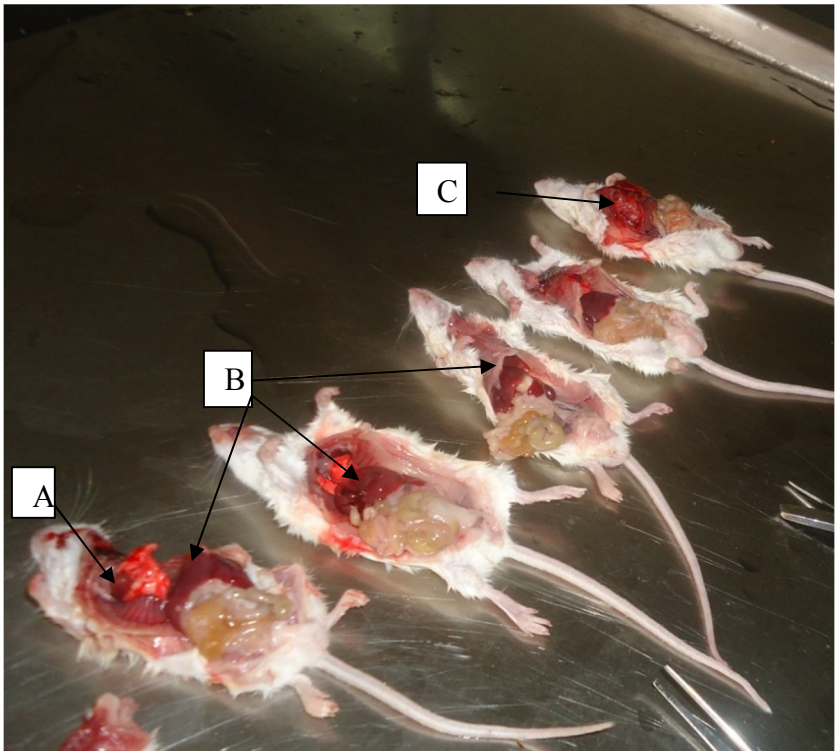


Plate XI: A - Congested heart, B - liver and C - lungs of Swiss-Webster mice inoculated with *P. multocida* serotypes A:1, A:3 and A:4.

4.7.4 HISTOPATHOLOGY

4.7.4.1 Chicken

Lungs of chicken inoculated with *P. multocida* serotype A: 1 at a concentration of 10^8 showed lymphocytic, heterophilic and macrophage cellular infiltrates associated with severe congestion and multifocal haemorrhages (Plate XII). In the heart there were few congested vessels with severe lymphocytic and heterophilic cellular infiltrates. Lymphoid and pulp depletion (Plate XIII), hyperplasia of arterial walls as well as vascular congestion was observed in the spleen of chicken inoculated with *P. multocida* A: 3 at a concentration of 10^7.

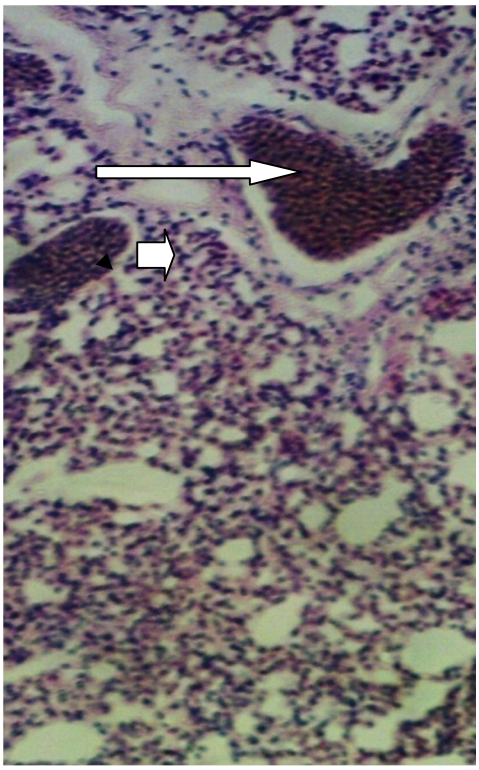
4.7.4.2 Japanese quails

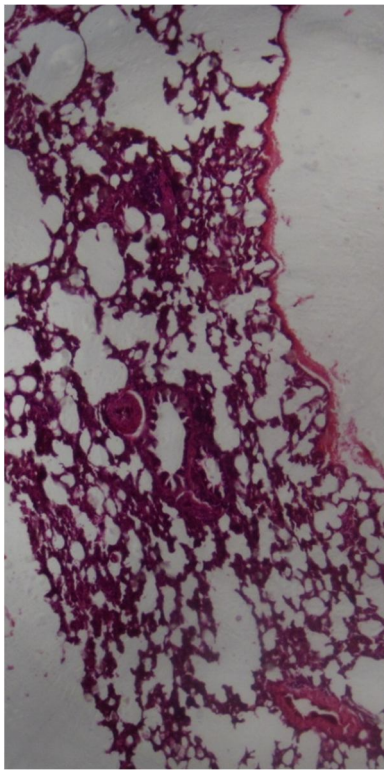
Pulmonary congestion and generalized haemorrhages resulting in the collapse of most of the alveolar spaces were the notable histopathologic lesions observed in the lungs of Japanese quail inoculated with *P. multocida* serotype A: 1 at a concentration of 10^8. Severe and generalized mononuclear cellular infiltration which aggregated around the duct system of the liver of Japanese quails inoculated with *P. multocida* serotype A: 3 at a concentration of 10^8 There was inflammatory cells infiltration of the entire myocardium; areas of congestion as well as haemorrhages were also noticed in the heart of Japanese quail inoculated with *P. multocida* serotype A: 4 at a concentration of 10^7 (Plates XIV and XV).

4.7.4.3 Mice

There was moderate vascular congestion as well as severe lymphocytic, heterophilic and macrophage cellular infiltration along the lining of bronchial walls of the lungs of mice

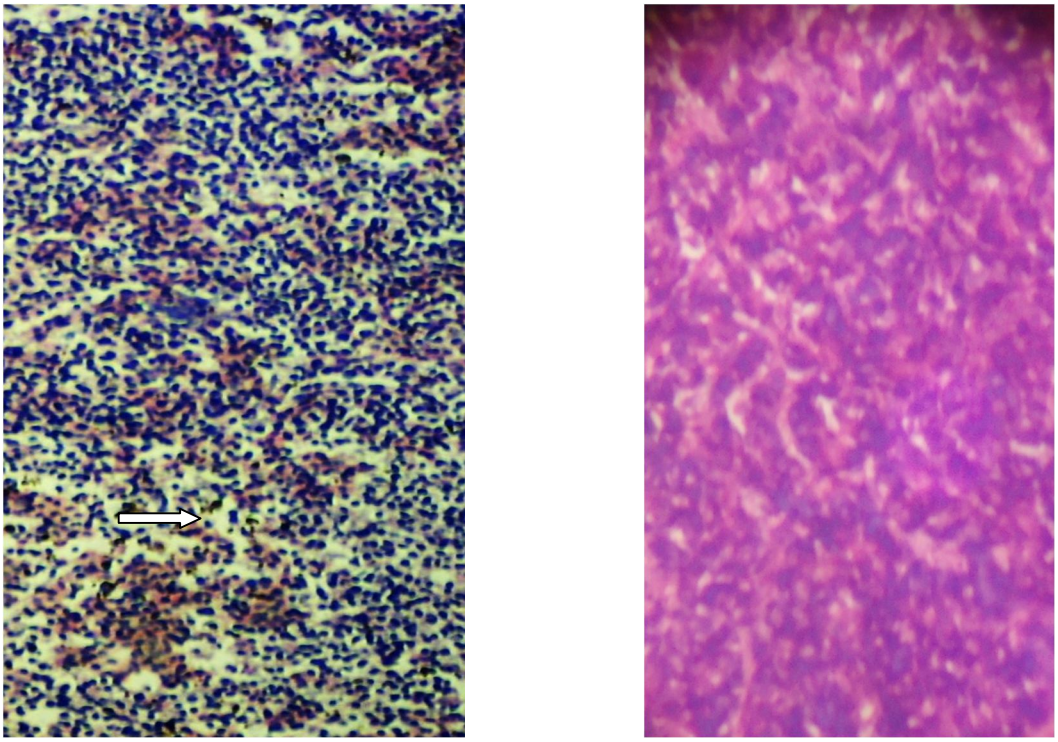
inoculated with *P. multocida* serotype A: 3 at a concentration of 10^8 (Plate XVI). The liver showed congestion and mild mononuclear cells infiltration.





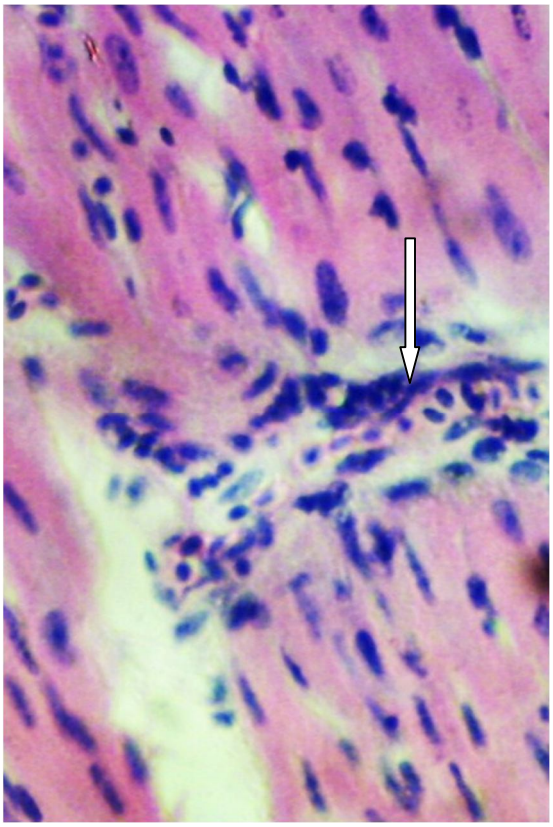
a b

Plate XII a: Photomicrograph of a lung from a chicken infected with *Pasteurella multocida* A:3=10^8. Note the congested blood vessels (arrow), the areas with obliterated alveoli and infiltrated with inflammatory cells (arrow head) H and E Stain. X 200. b: normal lung of a chicken at X 400.

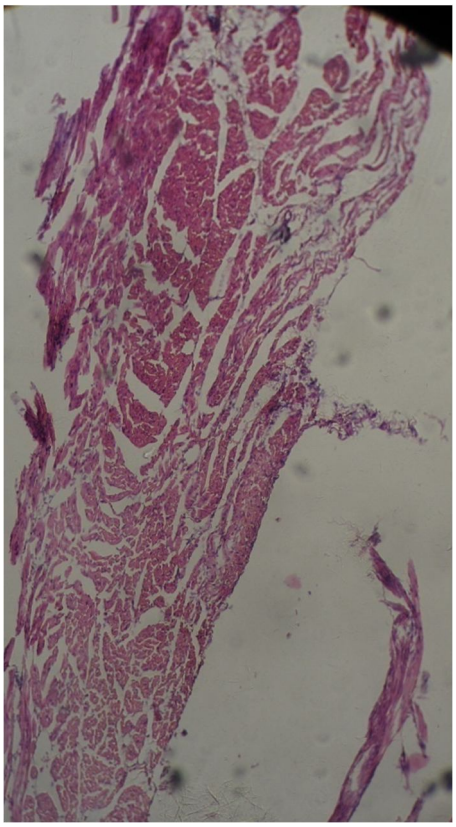


a b

Plate XIII: a - Photomicrograph of spleen from a chicken infected with *Pasteurella multocida* A: 1 = 10^7. There was mild depletion of white pulps (arrows). H and E Stain. X 200. b: Photomicrograph of a normal spleen from a chicken. X 100.

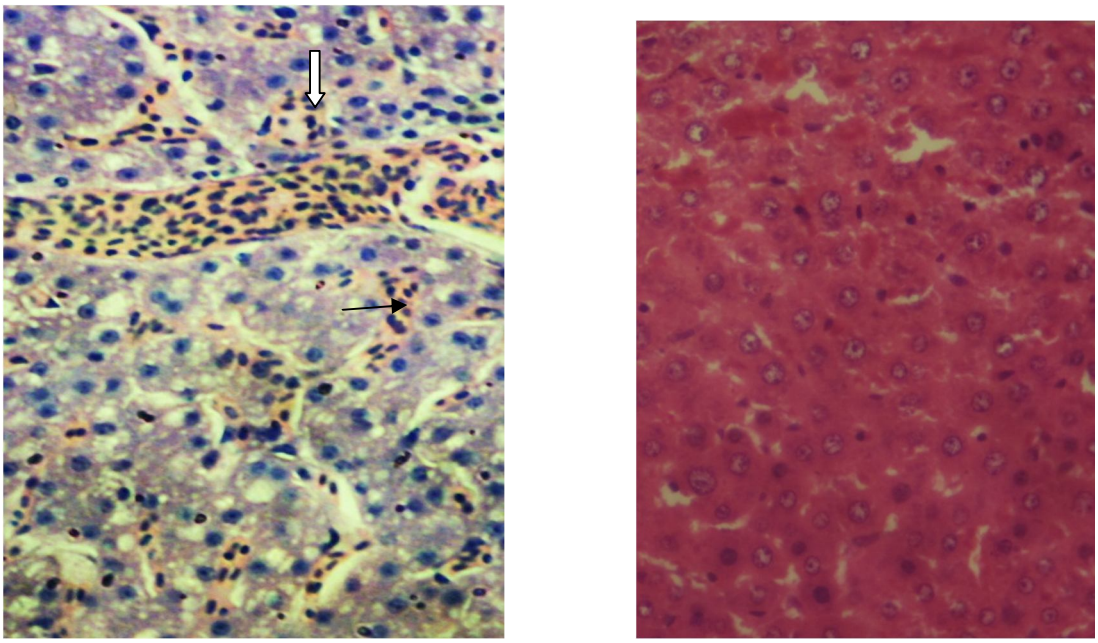


a



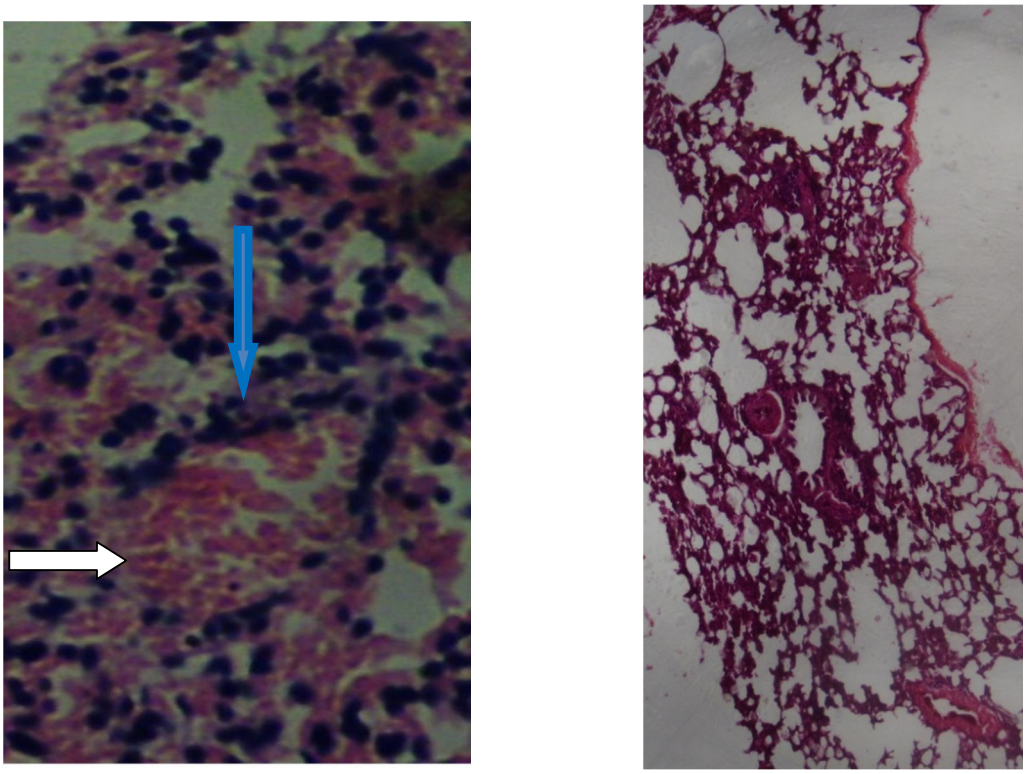
b

Plate XIV a: Photomicrograph of cardiac muscle of a Japanese quail inoculated with *Pasteurella multocida* A: 4= 10^8 CFU. Note the infiltrated inflammatory cells in an area of myocardial necrosis (arrow). H and E Stain. X 400. b: Photomicrograph of a normal cardiac muscle of a quail X 200.



a b

Plate XV a: Photomicrograph of liver from a Japanese quail inoculated with *Pasteurella multocida* A: 4 = 10^8. Note the infiltrated inflammatory cells (white arrow), congested sinusoids (black arrow) and mild vacuolar degeneration in majority of hepatic cells. H and E Stain. X 200. b: Photomicrograph of a normal liver from a quail X 200.



a b

Plate XVI a: Photomicrograph of lung from a mouse inoculated with *Pasteurella multocida* A: 3 =10^7. Note the haemorrhage (white arrows) and infiltrated inflammatory cells (blue arrow). H and E Stain. X 400. b: Photomicrograph of a normal lung from a mouse, X 400.

CHAPTER 5

DISCUSSION.

The findings of this study showed that *Pasteurella multocida* were found to be widely distributed in all the tissue samples of clinically sick chickens. This could possibly explain the debilitating nature of fowl cholera in its chronic form. It was also observed that the spleen had the lowest isolation rate of *P. multocida*, which agrees with the previous report of Peterson *et al*. (2001) who showed that *P. multocida* was eliminated from the spleen of chickens. The frequency of isolation of *P. multocida* (1.0 %) from clinically sick chickens was low. This could be linked to the fact that poultry farmers engage in injudicious use of antibiotics when they see any clinical sign of a disease. It is therefore possible that the use of antibiotic agents would have contributed in reducing the isolation of *P. multocida* in the clinically sick chickens.

The monthly distribution showed that *P. multocida* was encountered throughout the year, but the infection may have been precipitated by rainfall, as more cases were recorded during the raining season (July-October). It could be assumed that the rather more auspicious climate in Nigeria between July and November that is characterized by adverse cold experienced by poultry could also favour the proliferation of the organism in the environment and increase susceptibility. Similar pattern of high prevalence of avian pasteurellosis between April and August in Zaria has been reported by Raji *et al.* (2010). Similarly, Ambali *et al*. (2003) reported that fowl cholera was more prevalent in the months of January, June and July. Blackall *et al*. (1995) asserted that common factors such as stress, over-crowdiness, wild birds and rodents were considered to be responsible for the precipitation of fowl cholera.

The isolation of *P. multocida* from oro-pharyx of apparently healthy chickens suggests that healthy poultry may serve as reservoirs for this pathogen. The study has also revealed that apparently healthy chickens can be carriers of *P. multocida*. This agrees with the previous reports of Oladele *et al*. (1999) and Muhairwa *et al*. (2000) who isolated *P. multocida* from wild birds, free ranging chickens and contact animals.

The Microbact analysis showed that there was a minor variation between Microbact and conventional biochemical testing as some of the bacteria identified as *P. multocida* isolates by biochemical tests gave cut-off points of less than 75% by Microbact test. In a study conducted by Ekundayo *et al*. (2008) in Vom, all the *P. multocida* isolates were found to be Gram negative, oxidase and catalase positive, but differed in their ability to ferment dulcitol, sorbitol, xylose, production of indole and H_2S on Triple Sugar Iron agar. Avian strains of *P. multocida* in particular have been found to exhibit considerable phenotypic variability (Blackall *et al*., 1998; Butt *et al*., 2003). This finding differs from the report of Madsen *et al*. (1985) and Kamp *et al*. (1990) who reported that the *P. multocida* isolates were indole negative. Microbact test uses a computer based analytical programme which is incorporated to enhance precision; however, we observed certain discrepancies in this study with regard to differences in reaction to various biochemical agents. This could presumably be attributed to the phenotypic variations that exist among *P. multocida* organisms, since test results are based on colour change due to enzyme-substrate reaction.

The *P. multocida* species-specific PCR (Pm-PCR) assay employed in this study did not identify every isolate as positive. Thirteen isolates from clinically sick and apparently healthy

chikens were not positive by PCR, while 12 showed corresponding amplicons of 460 base pairs which is specific for *P. multocida* (Townsend *et al*., 1998). It was noticed that there was homogeneity in the distinct banding patterns of all the 12 *P. multocida* isolates that were amplified.

The findings of this work showed that all the 12 isolates of *P. multocida* gave a uniform amplicon size corresponding to 1,044 base pairs indicating that they all belong to capsular group A. Similar results have been observed by Chung *et al.* (1998) and Jaglic *et al.* (2005). This report has lent credence to the reports of Glisson *et al*. (2003) and Kumar *et al*. (2004) that fowl cholera is mainly caused by *P. multocida* belonging to capsular group A. The present finding differs from reports of Kumar *et al*. (1996) and Chawak *et al*. (2000) who documented that fowl cholera in avian species, such as chickens, ducks, turkeys, quails, geese, pigeons and cage birds are caused by capsular group D:3 and F:3.

One of the ardent and recurrent challenges encountered in controlling the unprecedented epornitics of fowl cholera is the existence of many serotypes of capsular group A. The finding of this study revealed that serotypes A: 1, A: 3 and A: 4 were found to occur in the study area and all were capable of causing disease in chickens, quails and mice. This report is similar to that of Yunusa (2008) who reported that serotypes A: 1, A: 3, A: 4 and A: 12 were prevalent in local chickens in Sokoto State. However, the result of this work differs slightly from his report because serotype A: 12 was not isolated in this work. This finding is an indication that in Nigeria, serotypes of *P. multocida* may differ from one state to another. The findings have presented the fact that the current challenge experienced in the production

of safe and immunogenic vaccine against the fowl cholera is attributed to the presence of diverse serotypes of *P. multocida*; bearing in mind that control of this disease will always remain dependent on elucidation of these serotypes.

The results of this study as well as that of Shivachandra *et al*. (2004) indicate that ciprofloxacin and gentamicin were highly effective against all the *P. multocida* isolates followed by streptomycin, enrofloxacin, pefloxacin, furasol and oxytetracycline. The results of this study differ remarkably from the reports of Gutierrez *et al*. (1993) in Spain and Yoshimura *et al*. (2001) in Japan who documented that aminoglycosides, such as streptomycin and gentamicin showed mild activity against some isolates of *P. multocida*. Effective treatment of fowl cholera in Jos and environs may be achieved by using any of the seven antibiotics listed above.

It was observed that most *P. multocida* isolates were resistant to ampicillin, amoxicillin/clavulanate acid, trimethoprim-sulfamethoxazole, erythromycin, anicillin, and tylosin. Kulkarni *et al*. (1990) in India also recorded 73.7% resistance to trimethoprim/sulfamethoxazole and 77.8% to ampicillin. The apparent inabilities of these conventional drugs to be effective against *P. multocida* isolates portend grave consequence to poultry farmers and clinicians because this will severely undermine the effective control of fowl cholera. The exhibition of high percentage resistance of *P. multocida* isolates to ampicillin, augmentin, trimethoprim/sulfamethoxazole, erythromycin, anicillin and tylosin has shown that prevention and therapeutic effect on avian *P. multocida* strains in Jos should no longer be expected from these antibiotics. The resistance of *P. multocida* to these drugs is

presumably attributed to the use of antibiotics as additives in poultry feed, extensive and pervasive use of antimicrobial agents by poultry farmers and veterinary practitioners (Shivachandra *et al.*, 2004).

The emergence of resistant *P. multocida* strains could also be linked to conjugative R-plasmid which is commonly responsible for interspecies and inter-generic spread of multidrug resistance and transfer of such plasmids among pathogenic strains may give rise to epidemic spread of infection (Stone, 1975)

.

If the multi-drug resistance observed in this study continues unabated, soon there will be no effective antibiotics against fowl cholera. Similar reports on the emergence of multidrug-resistant *P. multocida* strains have been documented by Shivachandra *et al.* (2004), Arora *et al*. (2005) and Zahoor and Siddique (2006). Therefore, antibiotic sensitivity should be incorporated on a routine bases as part of measure to control fowl cholera and minimize the emergence of resistance.

The present study has shown that serotypes A: 1, A: 3 and A: 4 were identified in the study area. These serotypes were able to cause clinical disease in mice, quails and chickens with high morbidity and mortality in experimental studies. Odugbo *et al*. (2004) reported an outbreak of fowl cholera due to *P. multocida* serotype A: 4 in quails in Vom. The mortality caused by this serotype was very high. In a similar study conducted in India by Kumar *et al*. (2004), they found majority of *P. multocida* belonging to serotypes A: 1, A: 3 and A: 4 were associated with fowl cholera. The findings in this study differ from that of Kumar and others

in that the A: 4 serotype in this study did not cause mortality in chickens. Snipes *et al*. (1988) asserted that not all *P. multocida* isolates are pathogenic to birds. Previous studies conducted on fowl cholera by different workers indicated that *P. multocida* serotypes 1, 3 and 4 were isolated from England (Curtis and Ollerhead, 1981); 1, 3, 4, 8, 12 and 14 in Vietnam (Gunawardana *et al*., 2000); 1, 2, 3, 4, and 13 in Australia (Gunawardana *et al*., 2000); 1, 3, 4, 8, 11, 12 and 13 in Indonesia (Mariana and Hirst, 2000; Jonas *et al*., 2001); 1, 3, 4 and 12 in Pakistan (Muhammad *et al*., 2003). In a similar study in Central Saudi Arabia, Elfak *et al*. (2002) reported the involvement of *P. multocida* serotypes1, 3, 4 and 5 in an outbreak of fowl cholera in ostriches. These reports are indications that the production of universal fowl cholera vaccine will be difficult considering the fact that *P. multocida* serotypes vary from one country to the other and immunity is known to be serotype specific (Glisson *et al*., 2003).

In experimentally challenged chickens, congested liver, spleen, lungs as well as petechial and ecchymotic haemorrhages were observed at postmortem. The result of this study also support the findings of Visut *et al*. (2010) that chickens inoculated with *P. multocida* showed various pathological lesions such as congested heart, liver, lungs and haemorrhages in coronary fat. The observed clinical signs and gross lesions in affected experimental birds in this study were not pathognomonic for fowl cholera. However, these signs and lesions could be best described as that of general septicaemia. Some of the clinical and postmortem lesions observed in this study were very close to those seen in cases of colibacillosis, fowl typhoid, infectious bursal disease, among others. It is most likely that this could account for the many cases of fowl cholera mis-diagnosed by clinicians. In the pathogenicity studies, all the experimental chickens inoculated with serotypes A: 1 and A: 3 came dawn with the clinical

signs of the disease. This finding is similar to the report of Rimler and Glisson (1997) who documented that adult chickens above 16 weeks of age are susceptible to fowl cholera. The host variation in the susceptibility to *P. multocida* serotypes was noted in that different clinical signs and gross lesions were manifested by birds inoculated by the same pathogenic agent. Botzler (1991) also observed that clinical signs of fowl cholera in birds vary depending on the age, species, dose of strain, route of entry of the bacterium and form of the disease. Some of the experimental Japanese quails were found dead without any premonitory signs, while others manifested some clinical signs; this could possibly be due to the fact that inoculated serotypes of *P. multocida* were highly pathogenic to have manifested in the per acute form of fowl cholera. Glisson *et al*. (2003) reported that in the per acute form of the disease, birds might be found dead without premonitory signs. In a similar pathogenicity study by Akpavi *et al*. (2011), they observed that some of the Japanese quails inoculated with *P. multocida* serotype A: 4 died on day one and this lasted for 5 days with highest mortality rate one day post inoculation. The differences in host susceptibility could be attributed to an interaction between a number of factors, such as burden of infections, individual defense mechanism and immune level as previously reported by Botzler (1991). The fatty degeneration seen at necropsy could be linked to loss of critical host cell protein sequel to prolonged anorexia. All the field isolates of *P. multocida* recovered in this study were found to be pathogenic to Swiss-Webster mice. They exhibited high pathogenicity by causing 100% mortality in mice within 24 h post inoculation. The high susceptibility of mice to *P. multocida* poses a major epidemiological challenge in the control of fowl cholera because there is a tendency of mice serving as reservoirs or long-term carriers that periodically shed *P. multocida* to the environment which will contribute to the spread of infection within

flocks. In a study by Mwankon *et al*. (2009) they reported that that the species and serotypes of *P. multocida* isolated from recurrent outbreaks of fowl cholera in a commercial quail farm were the same with the *P. multocida* species found in rats co-habiting quail houses. The susceptibility of mice to *P. multocida* as observed in this study has further re-emphasized the need to adequately control rodents around poultry houses in order to curtail the spread of fowl cholera to uninfected flock. Naz *et al*. (2012) also reported that isolates of *P. multocida* were able to cause mortality to BALB/c mice, when experimentally inoculated through intra-peritoneal route, and overwhelming septicaemia was observed within 24 h post infection.

Histopathological findings in this study indicated a moderate to severe lymphocytic, heterophilic and macrophagic cellular infiltration in lungs and heart of experimental chickens, Japanese quails and mice. This could possibly signify that the birds and mice were responding to the inciting bacterial agent, which is a common characteristic in acute phase of fowl cholera. The severe involvement of visceral organs such as congestion noticed in lungs, heart and spleen of chickens, Japanese quails and mice clearly demonstrate the septicaemic nature of fowl cholera. Lymphoid depletion in spleen, hepatitis and multifocal haemorrhages in lungs of chickens were the notable characteristic histopathologic lesions. This finding conforms to the report of Shilpa and Verma (2006) who observed similar histopathological changes. There were collapsed alveoli in the lungs of Japanese quail. Occasionally focal areas of necrosis with bacterial colonies involving groups of alveoli were also noticed. Some of the observed histopathological lesions consisted of interstitial pneumonia characterized by severe thickening of alveolar walls and hyperplasia of arterial walls. The widespread lesions

observed in visceral organs of birds inoculated with *P. multocida* could possibly explain the massive flock morbidity and mortality experienced in cases of fowl cholera outbreaks.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMENDATIONS

6.1 SUMMARY

This study provides information on serotypes, capsular group, antibiotic profiles, and molecular identification of the species and capsular serogroups of *P. multocida*. Pathogenicity of *P. multocida* isolated from chickens in Jos, Nigeria was also evaluated using chickens, Japanese quails and mice. Field isolates were identified based on cultural, morphological, biochemical, microbact test and *P. multocida* Specific-PCR. Most of the observations in this study are accompanied by data which are presented in forms of tables, plates and bar charts. The findings of the present study are summarized follows: The isolation rate of *P. multocida* in clinically sick chickens was 1.0% and 1.3% in apparently healthy ones. The monthly frequency of isolation of *P. multocida* showed that the bacterium was isolated most commonly between the months of July and October.There was no significant difference ($p>0.05$) in the isolation rate of *P. multocida* and breed of chickens sampled. It was however observed that a significant association ($p<0.05$) exists between the isolation rate of *P. multocida* and ages of sampled chickens as all the *P. multocida* isolates were recovered from chickens above 16 weeks old.

Biochemical profiles of *P. multocida* indicated that all were Gram negative rods, indole positive and reduced nitrate to nitrite. Microbact test identified 25 *P. multocida* isolates and all were found to ferment glucose, mannitol, ONGP and sorbitol, while 96% were indole positive, 64% lactose negative, 68% produced H_2S and 80% were ornithine positive. *Pasteurella multocida* serotypes A: 1, A: 3 and A: 4 were found to occur in Jos, Nigeria.The

highest isolation rate of *P. multocida* was from the heart blood, followed by liver, bone marrow, lung and spleen.

Polymerase Chain Reaction recognized only 12 of the 25 *P. multocida* isolates. The subjection of these 12 isolates to capsular multiplex PCR showed that all belonged to capsular group A.

The drug sensitivity profiles revealed that ciprofloxacin and gentamicin were highly effective (100%) against the twelve *P. multocida* isolates followed by streptomycin (91.7%), enrofloxacin, pefloxacin, furasol and oxytetracycline (83.3%). High resistance of *P. multocida* was recorded for ampicillin (91.7%) followed by augmentin (83.3%), septrin (66.7%), erythromycin and anicillin (58.3%), and tylosin (33.3%).

The Pathogenicity studies indicated that *P. multocida* serotypes A: 1, A: 3 and A: 4 caused 100% mortality in mice. *Pasteurella multocida* serotype A: 1 caused 83.3% mortality in chickens, A: 3 was responsible for 50.0% mortality, while A: 4 did not cause any mortality in chickens. In Japanese quails the following mortalities were recorded: A: 1 caused 83.3%, A: 3 caused 66.7% and in A: 4 mortality was 33.3%.

Histopatologically, moderate to severe cellular infiltration were noticed in the lungs and heart of infected chickens, Japanese quails and mice.

6.2 CONCLUSIONS

Based on the study conducted, the following conclusions were arrived at:

Effective control of fowl cholera in Jos, Nigeria may be achieved through the production of a heterologous fowl cholera vaccine consisting of *P. multocida* serotypes A: 1, A: 3 and A: 4.

Fowl cholera disease in Jos, Nigeria may be caused by *P. multocida* capsular group A and serotypes I, 3 and 4, since all *P. multocida* isolates recovered from apparently healthy and clinically sick chickens gave amplicons of 460bp by PM-specific PCR and 1,044bp by capsular serogroup specific multiplex PCR.

Fowl cholera in Jos, Nigeria may be treated by using the following antibiotics: ciprofloxacin, gentamicin, enrofloxacin, streptomycin, pefloxacin, tetracycline and furasol.

The widespread and injudicious use of antibiotics by poultry farmers in Jos, Nigeria may result in multi-drug resisrance of *P. multocida*.

6.2 RECOMMENDATIONS

Based on the results of this study, the following are recommended:

1. A nationwide surveillance should be conducted to elucidate the true status of prevalent serotypes of *P. multocida* for the purpose of vaccine production. Concurrently the antimicrobial susceptibilities of isolates from nationwide survey should be undertaken.

2. Antibiotic sensitivity should be carried out before the institution of therapy for the treatment of fowl cholera.

3. Rational use of antibiotics in poultry should be enforced to reduce the emergence and spread of resistant *P. multocida* strains.

4. The pathogenicity study showed that Japanese quails were highly susceptible to experimental infection with *P. multocida*; it is therefore recommended that these species should be vaccinated against fowl cholera.

5. Fowl cholera vaccine should be re-validated by incorporating the prevailing *P. multocida* serotypes.

REFERENCES

Abdu, P. A., George, J. B. and Umoh, J. U. (1985). A study of poultry diseases diagnosed at Zaria from 1981-1984. *Nigerian Veterinary Medical Journal*, 14: 63-65.

Abdu, P. A. (1990). Fowl cholera in layers. *Zariya Veterinarian*, 5: 83-84.

Adene, D. F. and Oguntade, A. E. (2006). The structure and importance of the commercial and village based poultry in Nigeria. A document of Food and Agricultural Organization, Rome, Pp 1-4

Aiello, S. E. (2008). *The Merck Veterinary Manual*. 8th.ED. Merck and Co. Inc. N. J., USA. Pp. 573.

Akpavi, V., Abdu, P. A., Mamman, P. H. and Sa'idu, L. (2011). Clinicopathological features in Japanese quails (*Coturnix coturnix japonica*) infected with *Pasteurella multocida multocida*. *Sahel Journal of Veterinary Science*, 19: 15-20.

Andreason, C. B. (2003). Staphylococcosis. In: Saif, Y. M., Barnes, H. J., Glison, J. R., Fadly, A. M., McDougald, L. R. and Swayne, D. E. (Eds), *Diseases of poultry*, 11th ED. Iowa State University Press, Ames, Iowa, USA, Pp. 798-804.

Ambali, A. G., Abubakar, N. B. and James, T. E. (2003). An assessment of poultry health problems in Maiduguri, Borno State, Nigeria. *Tropical Veterinarian,* 21: 138-145.

Arashima, Y. and Kumasaka, K. (2005). Pasteurellosis as zoonosis. *Internal Medicine,* 44: 692-693.

Arora, A. K., Virmani, S. K. J. and Oberoi, M. S. (2005). Isolation, characterization and antibiogram of *Pasteurella multocida* isolates from different animal species. *Indian Journal of Animal Science*, 75: 749–752.

Aye, P. P., Angrick, T. Y., Morishita, T. Y. and Harr, B. S. (2001). Prevalence and characteristics of *Pasteurella multocida* in commercial turkeys. *Avian Diseases*, 41: 182-190.

Bauer, A. W., Kirby, M. W., Sherris, J. C. and Turek, M. (1966). Antimicrobial susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*, 45: 493-498.

Bierer, B. W. and Derieux, T.W. (1972). Immunologic response of turkeys to an avirulent *Pasteurella multocida* vaccine in drinking water. *Journal of Poultry Science*, 51: 408-416.

Birdlife International (2006). Birdlife International statement on avian influenza http://www.birdlife.org. *Accessed 28/2/2006, 2.00 am.* Pp 490-496.

Bisgaard, M. (1993). Ecology and significance of *Pasteurellaceae* in animals. *Zentralblatt Fur Bakteriologie,* 279: 7-26.

Blackall, P. J. and Morrow, C. J. (1990). Epidemiologic studies on infectious coryza outbreaks in northern New South Wales, Australia, using serotyping, biotyping and chrosomal DNA restriction endonuclease analysis. *Avian Diseases*, 34: 267-276.

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

Blackall, P. J., Fegan, N., Chew, G. T. and Thompson, D. J. (1998). Population structure and diversity of avian isolates of *Pasteurella multocida* from Australia. *Journal of Microbiology,* 144: 279-289.

Blackwood, R. A., Rode, C. K., Reed, J. S., Law, I. H. and Bloch, C. A. (1996). Genomic fingerprinting by pulsed field gel electrophoresis to identify the source the of *Pasteurella multocida* sepsis. *Paediatric Infectious Diseases Journal*, 15: 831-833.

Botzler, R. G. (1991). Epidemiology of avian cholera in wildfowl. *Journal of Wildlife Diseases*, 27: 367-395.

Bronzwaer, S. L., Cars, O., Buchholz, U., Molstad, S., Goettsch, W., Veldhuijzen, K. I., Kool, J. L., Sprenger, M. J. and Degener, J. E. (2002). European study on the relationship between antimicrobial use and antimicrobial resistance. *Emerging Infectious Diseases*, 8: 278-282.

Butt, A. I., Kausar, T. and Asad-Raza, G. Z. (2003). Biochemical, serological and immunological properties of *Pasteurella multocida* stains from natural outbreaks of haemorrhagic septicaemia. *Pakistan Journal of Veterinary Reseach*, 1: 1-4.

Caprioli, A., Busani, J., Martel, L. and Helmuth, R. (2000). Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. *International Journal of Antimicrobial Agents*, 14: 295-301.

Carpenter, T. E., Hirsh, D. C., Kasten, R. W., Hird, D. W., Snipes, K. P. and McCapes, R. P. (1989). *Pasteurella multocida* recovered from live turkeys: Prevalence and virulence in turkeys. *Avian Diseases*, 33: 12-17.

Chawak, M. M., Verma, K. C., Kataria, J. M., and Kumar, A. A. (2000). Characterization of indigenous isolates of *Pasteurella multocida*. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases*, 21: 111-114.

Chen, X., Song, C., Gong, Y. and Blackall, P. J. (1998). Further studies on the use of a Polymerase Chain Reaction test for the diagnosis of infectious coryza. *Avian Pathology*, 27: 618-624.

Christensen, J. P., Dietz, H. H. and Bisgaard, M. (1998). Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from backyard poultry and from two outbreaks of avian cholera in avifauna in Denmark. *Avian Pathology*, 27: 373-381.

Christensen, H., Kulnet, P., Oslen, J. E. and Bisgaard, M. (2004). Comparative phylogenies of the housekeeping genes *atpD, infB* and *rpoB* and the 16S rRNA gene within the *Pasteurellaceae. International Journal of Systematic Evolution and Microbiology*, 54: 1601-1609.

Clinical and Laboratory Standard Institute (CLSI) (2009). *Procedure Manual for Laboratory Practice.* 3rd Edition. 1400, Wayne, Pennsylvania, 1887-1898, USA.

Cowan, S. T. and Steel, K. L. (2004). *Manual for Identification of Medical Bacteria*. 2nd Edition, Cambridge University press, Cambridge, Pp. 28-106.

Chung, Y. J., Zhang, Y. and Adler, B. (1998). The capsule biosynthetic locus of *Pasteurella multocida* A:1 FEMS *Microbiology Letters,* 166**:** 289-296.

Curtis, P. E. and Ollerhead, G. F. (1981). Investigation to determine whether healthy chickens and turkey are oral carriers of *P. multocida*. *Veterinary Record*, 34: 208-220.

Davies, R. L., MacCorquodale, R. and Reilly, S. (2004). Characterization of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Journal of Veterinary Microbiology,* 99: 145-158.

Dewhirst, F. E., Paster, B. J., Oslen, I. and Fraser, G. J. (1993). Pylogeny of the *Pasteurellaceae* as determined by comparison of 16s ribosomal ribonucleic acid sequence. *International Journal of Medical Microbiology, Parasitology, Virology and Infectious Dis*eases, 279: 35-44.

Diallo, I. S., Bensink, J. C. Frost, A. J. and Spradbrow, P. B. (1995). Molecular studies on avian strains of *Pasteurella multocida* in Australia. *Journal of Veterinary Microbiology*, 46: 335-342.

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

Donnio. P. Y., Allardet-servent, A., Perrin, M., Escande, P. and Avril, J. L. (1999). Characterization of dermonecrotic toxin-producing strains of *Pasteurella multocida*

subspecie multocida isolated from man and swine. *Journal of Medical Microbiology,* 48: 125-131.

Elfak, M. G., Abbas, B., Mahmoud, M. O., Haroun, E. M. and Abdel-Magied, E. M. (2002). Septicaemic pasteurellosis in ostriches (*Stricthio camelus*) in Central Saudi Arabia. *The Veterinary Journal*, 163: 218-221.

Einum, P., Kiupel, M. and Bolin, C. (2003). An outbreak of fowl cholera in ring-necked Pheasants (*Phasianus colchicus*). *Avian Diseases*, 47: 477-480.

Ekundayo, S., Odugbo, O., Atanda, O. and Okewole, P. (2008). Phenotypic variability among strains of *Pasteurella multocida* isolated from avian, bovine, caprine, laprine and ovine origin. *African Journal of Biotechnology*, 7: 1347-1350.

Etukudo, B. O. and Adegboye, D. S. (1983). Studies of chronic respiratory diseases of poultry in Nigeria. Bacteriological examination of clinically diagnosed cases. *Nigerian Veterinary Journal*, 12: 66-69.

Ezenduka, E. V., Oboegbulem, S. I., Nwanta, J. A and Onunkwo, J. I. (2011). Prevalence of antimicrobial residues in raw table egg from farms and retail outlets in Enugu State, Nigeria. *Tropical Animal Health and Production*, 43: 557-559.

Ezealor, A. U., Polet G., Boyi, M. G. and Dutse, I. S. (1996). The survey of waterfowl and water-related bird of the Hadejia-Nguru wetlands, Nigeria. Report of HNWCP, Nguru, Nigeria. Pp. 16.

Ezealor, A. U. (2006). Migratory birds and bird flu*:* A Nigerian perspective. *Public Awareness Forum on Bird Flu*, Ahmadu Bello University, Zaria, 14th March, 2006.

Finlay, B. B. and Falkow, S. (1989). Common themes in microbial pathogenicity. *Microbiology Reviews,* 53: 210-230.

Food and Agricultural Organization (FAO) (1999). Statistic Base, www.fao.org. Food and Agriculture Organization of the United Nations, Rome, Italy. *Accessed 2009 at 8.00 Pm*

Food and Agricultural Organization (2000). Statistic Base, www.fao.org**.** Food and Agriculture Organization of the United Nations, Rome, Italy. Animal Production and Health Division Publication, No 274415, Rome. FAOSTAT. *Accessed 2010 at 3.00 pm.*

Francis, D. P., Holmes, M.A. and Brandon, G. (1975). Infections after domestic animal bites and scratches. *Journal of American Medical Association*, 233: 42-45.

Frederiksen, W. (1989). Pasteurellosis of man. In: *Pasteurella* and Pasteurellosis (C. Adlam and J. M. Rutter, Eds). Academic Press, London, Pp 303-320.

Frederiksen, W. (1993). - Ecology and significance of *Pasteurellaceae* in man - an update. *International Journal of Medical Microbiology,* 279: 27-34.

Gardner, I, A., Kasten, R., Eamens, G. J., Snipes, K. P. and Anderson, R. J. (1994). Molecular fingerprinting of *Pasteurella multocida* associated with progressive atrophic rhinitis in swine herds. *Journal of Veterinary Diagnostic Investigation*, 6: 442-447.

Gast, R. K. (2003). Paratyphoid infections. In: Saif, Y. M., Barnes, H. J., Glison, J. R., Fadly, A. M., McDougald, L. R. and Swayne, D. E. (Eds), *Diseases of Poultry*, 11th Ed. Iowa State University Press, Ames, Iowa, USA, Pp. 583-613.

Glazer, A. N. and Nikaido, H. (1995). *Microbial Biotechnology*. W. H. Freeman and Company, San Francisco. Pp. 45-60.

Glisson, J. R., Hofacre, C. L. and Christensen, J. P. (2003). Avian pasteurellosis. In: Saif, Y. M., Barnes, H. J., Glison, J. R., Fadly, A. M., McDougald, L. R. and Swayne, D. E. (Eds), *Diseases of Poultry*, 11th Ed. Iowa State University Press, Ames, Iowa, USA, Pp. 658-676.

Goering, R. V. (1993). Molecular epidemiology of nosocomial infection; analysis of chromosomal restriction fragment patterns by pulse-field gel electrophoresis. *Infection Control in Hospital Epidemiology*, 14: 595-600.

Grimont, F. and Grimont, P. A. D. (1986). Ribosomal ribonucleic acid gene restriction pattern as potential taxonomic tools. *Annals Institute of Pasteur Microbiology*, 137: 165-175.

Gueye, E. F. (1999). Ethno-veterinary medicine against poultry disease in African villages. *World Poultry Science Journal*, 55: 187-198.

Gunarwardana, G., Townsend, K. M. and Frost, A. J. (2000). Molecular characterization of avian *Pasteurella multocida* isolates from Australia and Vietnam by REP-PCR and PFGE. *Veterinary Microbiology*, 72: 97-109.

Gustafson, C. R., Cooper, G. L., Charlton, B. R. and Bickford, A. A. (1998). *Pasteurella multocida* infection involving cranial air spaces in White Leghorn chickens. *Avian Diseases*, 42: 413-417.

Gutierrez, M. C. and Rodriguez, E. F. (1993). In vitro susceptibility of *Pasteurella multocida subspecies multocida* strains isolated from swine to 42 antimicrobial agents. *Zentralblatt Fur Bakteriologie,* 279: 387-393.

Harper, M., Boyce, J. D. and Adler, B. (2006). *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *Federation European Microbiological Societies Letter*, 265: 1-10.

Hansen, L. M. and Hirsh, D. L. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida. Veterinary Microbiology,* 21: 177-184.

Heddleston, K. L., Gallagher, J. E. and Rebers, P. A. (1972). Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Diseases*, 16: 925-936.

Hopkins, B. A., Huang, T. H. M. and Oslon, L. D. (1998). Differentiating turkey post vaccination isolants of *Pasteurella multocida* using arbitrarily primed polymerase chain reaction. *Avian Diseases,* 42: 265-274.

Hunt, L. M., Alder, B. and Townsend, M. K. (2000). The molecular biology of *Pasteurella multocida*. *Veterinary Microbiology*, 72: 3-25.

Hunt, M, L., Boucher, J. D., Boyce, J. D. and Adler, B. (2001). In vivo-expressed genes of *Pasteurella multocida. Infectious Immunology,* 69: 3004-3012.

Ilaria C. and Cascio A. (2007). Please, do not forget *Pasteurella multocida. Journal of Clinical Infectious Diseases*, 45: 940-943.

Jabbari, A. R., Saharee, A. A., Bahaman, A. R., Mutalib, A. R., Ideris, A., Esmaily, F., Vasfi Marandi, M. and Esmailizadeh, M. (2002). Genetic diversity among avian *Pasteurella multocida* isolates by using repetitive element PCR. *Iranian Journal of Veterinary Research*, 3: 205-210.

Jacques, M., Belanger, M., Diarra, M. S., Dargis, M. and Malouin, F. (1994). Modulation of *Pasteurella multocida* capsular polysaccharide during growth under iron-restricted conditions. *Journal of in vivo Microbiology*, 140: 263-270.

Jaglic, Z., Kucerova. Z., Nedbalcover, K., Pavlik, I., Alexa, P. and Barbs, M. (2005). Characterization and comparison of *Pasteurella multocida* isolated from different species in the Czech Republic: Capsular PCR typing, ribotyping and dermonecrotoxin production. *Veterinary Medicine Czechoslovakia,* 50: 345-354.

Jonas, M., Morishita, T. Y., Angrick, E. J. and Jahja, J. (2001). Characterization of nine *Pasteurella multocida* isolates from aviam cholera outbreaks in indonesia. *Avian Diseases*, 45: 34-42.

Kabir, J., Umoh, V. U., Audu-Okoh, E., Umoh, J. U. and Kwaga, J. K. P. (2004).Veterinary drugs use in poultry farms and determination of antimicrobial drug residue in commercial eggs and slaughtered chickens in Kaduna State, Nigeria. *Food control*, 15: 99-105.

Kamp, E. M., Laak, T. E. A. and Jong, M. F. (1990). Atypical *Pasteurella* strains producing a toxin similar to the dermonecrotic toxin of *Pasteurella multocida subspecies multocida*. *Veterinary Record,* 126*:* 434-347.

Kasten, R. W., Carpenter, T. E., Snipes, K. P. and Hirsh, D. C. (1997). Detection of *Pasteurella multocida*-specific DNA in turkey flocks by use of the polymerase chain reaction. *Avian Diseases*, 41: 676-682.

Kehrenberg, C., Shulze-Tanzil, G., Martel, J. L., Chaslus-Dancla, E. and Schwart, S. (2001). Antimicrobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis**.** *Veterinary Research,* 32: 323-339.

Kulkarni, D. D., Karpe, A. G., Bannalikar, A. S. and Gujar, M. B. (1990). Biological observation on pasteurellosis in domestic animals and poultry. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases,* 1: 22-27.

Kumar, A. A., Harbola, P. C., Rimler, R. B. and Kumar, P. N. (1996). Studies on *Pasteurella multocida* isolates on animal and avian origin from India. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases*, 17: 120-124.

Kumar, A. A., Shivachandra, S. B., Biswas, A., Singh, V. P., Vijendra, P. and Srivastava, S. K. (2004). Prevalent serotypes of *Pasteurella multocida* isolated from different animals and avian species in India. *Veterinary Research Communication*, 28: 657-667.

Kwon, Y. K. and Kang, M. I. (2003). Outbreak of fowl cholera in Baikal teals in Korea. *Avian Diseases*, 47: 1491-1495.

Lax, A. J. and Grigoriadis, A. E. (2001). *Pasteurella multocida* toxins: the mitogenic toxin that stimulates signaling cascades to regulates growth and differentiation. *International Journal of Medical Microbiology*, 291: 261-268.

Lee, M. D., Wooley, R. E. and Glisson, J. R. (1994). Invasion of epithelial cell monolayers by turkey strains of *Pasteurella multocida. Avian Diseases,* 38: 72-77.

Lee, C. W., Wilkie, I. W., Townsend, K. M. and Frost, A. J. (2000). The demonstration of *Pasteurella multocida* in the elimentary tract of chicken after experimental oral infection. *Veterinary Microbiology*, 7: 47-55.

Lion, C., Conroy, M. C., Carpenter, A. M. and Lozniewski, A. (2006). Antimicrobial susceptibilities of *Pasteurella* strains isolated from humans**.** *International Journal of Antimicrobial Agents*, 27: 290-293.

Madsen, E. B., Bisgaard, M., Mutters, R. and Pedersen, K. B. (1985). Characterization of *Pasteurella multocida* isolated from the lungs of calves with pneumonia. *Canadian Journal of Comparative Medicine,* 49: 63-67.

Marandi, V. M. and Mittal, K. R. (1997). Role of outer membrane protein H (OmpH)- and OmpA-specific monoclonal antibodies from hybridoma tumors in protection of mice against *Pasteurella multocida*. *Infectious Immunology,* 6: 4502–4508.

Mariana, S. and Hirst, R. (2000). The immunogenicity and pathogenicity of *Pasteurella multocida* isolated from poultry in Indonesia. *Veterinary Record*, 72: 27-36.

Masdooq, A. A., Salihu, A. E., Muazu, A., Habu, A. K., Ngbede, G., Haruna, G., Sugun, M. Y. and Turaki, U. A. (2008). Pathogenic bacteria associated with respiratory diseases in poultry with reference to *Pasteurella multocida. Research Journal of Poultry Sciences,* 2: 82-83.

Mathewman, R. (1975). A survey of small livestock production at village level in derive savannah and lowland forest zones of Southwest Nigeria. *Reading, U. K, University of Reading, Department of Agriculture and Forestry Study*, 24: 40-41.

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

May, B. J., Zhang, Q., Li, L. L., Paustian, M. L., Whittam, T. S., and Kapur, V. (2001). Complete genomic sequence of Pasteurella multocida, Pm70. *Proceedings of the National Academy of Science of the United Stateof America*. 98: 3460-3465.

Molokwu, J. U., Adegboye, D. S. and Emejuawe, S. O. (1988). Bacteria isolates from cases of chronic respiratory disease of chicken in Zaria area of Nigeria. *Zariya Veterinarian*, 3: 42-44.

Morishita, T. Y., Lowenstine, J. L., Hirsh, D. C. and Brooks, L. D. (1996). *Pasteurella multocida* in raptors: prevalence and characterization. *Avian Diseases*, 40: 908-918.

Muhairwa, A. P., Christensen, J. P. and Bisgaard, M. (2000). Relationship among *pasteurellaceae* isolated from free ranging chickens and their animal contacts as determined by quantitative phenotyping, ribotyping and REA-typing. *Veterinary Microbiology*, 78: 119-137.

Muhairwa, A. P., Christensen, J. P. and Bisgaard, M. (2001). Investigation on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. *Avian Pathology*, 29: 133-142.

Muhammad, J. A., Muhammed, S. and Iftikhar, H. (2003). Somatic serotyping and pathogenicity testing of *Pasteurella multocida* strains isolated from layer chickens. *Journal of Biological Sciences*, 3: 376-379.

Musa, U., Abdu, P. A., Dafwang, I. I., Saidu, L., Edache, J. A., Ahmed, M. S. and Karsin, P. D. (2009). Cost implications and benefits of intraocular vaccination of rural chickens

with Newcastle disease vaccine strain I_2. *Livestock Research for Rural Development*, 21: 1-7.

Mutters, R., Pohl, P., Fredericksen, W. and Mannheim, W. (1985). Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis*, *Pasteurella canis*, *Pasteurella anatis*, and *Pasteurella langua*. *International Journal of Systematic Bacteriology*, 35: 309-322.

Mwankon, E. S., Odugbo, M. O., Jwander, L. D., Olabode, V., Ekundayo,, S. O. Musa, U., Spencer, T. H., Isa , S. I., Kaikabo, A. and Simon, B. S. (2009**).** Investigations on the carrier rate of *Pasteurella multocida* in black rats (*Rattus rattus*) in a commercial quail farm. *African Journal of Clinical and Experimental Microbiology*, 10: 2-9.

National Population Commission (NPC) (2006). Plateau State statistical tables, Abuja.

National Fadama Development Project (NFDP) (2009). A document of Plateau State Government. Pp. 5-10.

Naz, S., Hanif, A., Maqbool, A., Ahmed, S. and Muhammand, K. (2012). Isolation, characterization and monitoring of antibiotic resistance in *Pasteurella multocida* isolates from buffalo (*Bubalus bubalis*) herds around Lahore. *Journal of Animal and Plant Sciences*, 22: 242-245.

Nigeria location map. Svg. (2010). *htt://upload.wikipedia/commons/6/66/Nigeria. Accessed on 21/4/2011, 4.08 pm.*

NVRI Annual Reports (2002). National Veterinary Research Institute, Vom Nigeria. Pp 16

Odugbo. M. O., Muhammad, M., Musa, U., Suleiman, A. B., Ekundayo, S. O. and Ogunjumo, S. O. (2004). Pasteurellosis in Japanese quail (*Coturnix coturnix japonica*) caused by *Pasteurella multocida multocida* A: 4. *Veterinary Record*, 155: 90-91.

Office International Des Epizootics (OIE) (2008). Fowl cholera. *Terrestrial Manual* 2. 3. 9: 524-530.

Okeke, I. L., Aboderin, O. A.Opintan, J. A. (2007). Growing problem of multidrug- resistant enteric pathogens in Africa. *Emerging Infectious Diseases*, 13: 1640-1646.

Oladele, S. B. and Raji, M. A. (1997). Retrospective studies of fungal and bacterial flora of chicken in Zaria, Nigeria. *Bulletin of Animal Health and Production in Africa,* 45: 79-81.

Oladele, S. B., Raji, M. T. and Raji, M. A. (1999). Prevalence of bacterial and fungal microflora isolated from some wild and domesticated birds in Zaria, Nigeria. *Bulletin of Animal Health and Production in Africa*, 47: 127-132.

Owen, R. J. (1989). Chromosomal DNA fingerprinting- a new method of species and strains identification/ application to microbial pathogens. *Journal of Medical Microbiology*, 30: 89-99.

Pandey, V. S. (1992). Epidemiology and economic of village poultry in Africa: An overview. *Proceedings of a Workshop on Village Poultry Production in Africa*. Rabat city, Morocco, Pp. 124-128.

Patrick, B., Hans H. S., André, P., Peter K., Purita, M., Gérard P. Reto, L. and Jacques, N. (2000). Molecular identification and epidemiological tracing of *Pasteurella multocida* meningitis in a baby. *Journal of Clinical Microbiology,* 38: 1235-1237.

Petersen, K. D., Christensen, H., Bisgaard, M. and Olsen. J. E. (2001). Genetic diversity *of Pasteurella multocida* fowl cholera isolates as demonstrated by ribotyping and 16S rRNA and partial *atp*D sequence comparisons. *Molecular Microbiology*, 147: 2739-2748.

Portney, L. and Watkins, M. (2007). *Foundation in Clinical Research*. Application to practice. 3rd Ed.Pearson Company. Pp 143-159.

Poultry Farms Register (2009). An up-dated data of poultry farms register of Plateau State Ministry of Agriculture, Pp. 1-20.

Raji, M. A., Ahmed, J. S., Sa'idu, L. and Ameh, J. A. (2010). Retrospective studies on the prevalence of cholera in Zaria, Kaduna State, Nigeria. *Sokoto Journal of Veterinary Sciences,* 8: 9-13.

Ragy, S. (2006). Review: Major pathogenic components of *Pasteurella multocida* and *Mannheimia* (*Pasteurella*) *haemolytica* isolated from animal origin Pp 1-13. Bacteriology Department, Animal Health Research Institute, Nadi El-Seed St. Dokki, 12311 Cairo, Egypt.

Rama Sastry, P. and Raja Rajeswari, K. (2000). Pasteurellosis in a lioness. *Indian Veterinary Journal*, 77: 549-550.

Rhoades K. R. and Rimler R.B. (1989). Fowl cholera. In: Adlam C. and Rutters J. M., Eds. *Pasteurella* and pasteurellosis: Academic Press, London, UK, Pp. 95 -113.

Rimler, R. B. and Glisson, J. R. (1997). Fowl cholera. In: Calnek, B. W., Barnes, H. J., Beard, E. W., McDougald, L. R. and Saif, Y. M. (Eds), *Diseases of Poultry,* 10th edition, Iowa State University Press, Ames, Iowa, U.S.A, Pp. 143-159.

Rimler, R. B., Sandhu, T. S. and Glisson, J. R. (1998). In: Swayne, D. E., Glisson, J. R.,Jackwood, M. W. and Reed, M. W. (Eds.), *A laboratory manual for isolation and identification of avian pathogens*, 4th Ed. American Association of Avian Pathology, Pennsylvania, USA, Pp 17-28.

Sellyei, B., Varga, Z., Szentesi-Samu, K., Kaszanyitkt, E. and Magyar, T. (2009). Antimicrobial susceptibility of *Pasteurella multocida* isolated from swine and poultry. *Acta Veterinaria Hungarica*, 57: 357-367.

Shilpa, S. and Verma, P. C. (2006). Pathology of *Pasteurella multocida* infection in chicken. *Indian Journal of Animal Research*, 40: 15-19.

Shivachandra, S. B., Kumar, A. A., Biswas, A., Ramakrishnan, M. A., Singh, V. P. and Srivastava, S. K. (2004). Antibiotic sensitivity patterns among India strains of avian *Pasteurella multocida*. *Tropical Animal Health and Production,* 36: 743-750.

Smith, J. M., Frame, D. D., Cooper, G., Bickford, A. A., Ghazikhanian, G. Y. and Kelly, B. J. (1987). *Pasteurella anatipestifer* infection in commercial meat-type turkeys in Califonia. *Avian Diseases*, 31: 913-917.

Snipes, K. P., Carpenter, T. E., Corn, J. L., Kasten, R. W., Hirsh, D. C., Hirsh, D. C., Hird, D. W. and McCcapes, R. H. (1988). *Pasteurella multocida* in wild mammals and birds in Califonia: prevalence and vilurence for turkeys. *Avian Diseases*, 32: 9-15.

Snipes, K. P., Hirsh, D. C., Kasten, R. W., Hansen, L. M., Hird, D. W., Carpenter, T. E. and McCcapes, R. H. (1989). Use of an rRNA probe and restriction endonuclease analysis to fingerprint *Pasteurella multocida* isolated from turkey and wildlife. *Journal of Clinical Microbiology,* 27: 1847-1853.

Stone, A. B. (1975). R-factor plasmid conferring resistance of antibacterial agents. *Science Progress,* 62: 89.

Stull, T. L., Lipuma, J. J. and Edlind, T. D. (1988). A broad-spectrum probe for molecular epidemiology of bacteria ribosomal RNA. *Journal of Infectious Diseases*, 157: 280-286.

Tenover, F. C., Arbeit, R. D., Goeing, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. and Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulse-field gel electrophoresis criteria for bacterial strain typing. *Journal of Clinical Microbiology*, 33: 2233-2239.

Townsend, K. M. and Papadimetrious, J. M. (1997). REP-PCR analysis of *Pasteurella multocida* isolates that cause haemorrhagic septicaemia. *Research in Veterinary Science,* 63: 151- 155.

Townsend, K. M., Frost. A. J., Lee. C.W., Papadimitriou. J. M. and Dawkins. H. J. S., (1998). Development of PCR assays for Species and type specific identification of *P. multocida* isolates. *Journal of Clinical Microbiology*, 36: 1096-1100

Townsend, K. M., Boyce, J. D., Chung, J. Y., Frost, A. J. and Adler, B. (2001*)*. Genetic organization of *Pasteurella multocida cap loci* and development of a multiplex capsular PCR typing system*. Journal of Clinical Microbiology,* 39: 924-292.

Thrusfield, M. (1997). *Veterinary Epidemiology*. 2nd Edition. Oxford: Blackwell Science. Pp 50-58.

Visut, R., Piyarat, C. and Niwat, C. (2010). Comparison of efficacy of enrofloxacin against *Escherichia coli* or *Pasteurella multocida* infection in chickens. *Thai Journal of Veterinary Medicine*, 40: 297-301.

Walser, M. M. and Davis, R. B. (1975). *In vitro* characterization of field isolates of *Pasteurella multocida* from Georgia turkey. *Avian Diseases,* 19: 525-532.

Waller, L. J. and Underhill, L. G. (2007). Management of avian cholera *Pasteurella multocida* on Dyer Island, South Africa. *African Journal of Marine Science*, 29: 105-111.

Walsh, C. and Fanning, S. (2008). Antimicrobial resistance in foodboarne pathogens-a cause for concern? *Current Drug Target*, 9: 808-815.

White, D. G., Zhao, S., Simjee, S., Wagner, D. D. and McDermott, P. F. (2002). Antimicrobial resistance of food borne pathogens. *Journal of Microbial Infections,* 4: 405-412.

Wilson, M. A., Rimler, R. B. and Hoffman, L. J. (1992). Comparison of DNA fingerprints and somatic serotyping for identification of avian *Pasteurella multocida* isolates*. Journal of Clinical Microbiology*, 30: 1518-1524.

World Health Organization (WHO) (2006). Counterfeit medicines: an update on estimates, Geneva: WHO international medicine products anti-counterfeit task force. Pp. 35-53.

Wosu, L. O. (1992). Strategy for sustained livestock production and development in Nigeria, In: *Book of Proceedings of 29th Annual Conference of Nigerian Veterinary Medical Association*, Kaduna, Pp. 165-170.

Xibiao, T., Zhanqin, Z., Junyong, H., Bin, W., Xuwang, C., Qigai, H. and Huanchun, C. (2009). Isolation, antimicrobial resistance and virulence genes of *Pasteurella multocida* strains from swine in China. *Journal of Clinical Microbiology,* 1128: 02029-08.

Yoshimura, H., Ishimaru, Y., Endoh, S. and Kojima, A. (2001). Antimicrobial susceptibility of *Pasteurella multocida* isolated from cattle and pigs. *Journal of Veterinary Medicine,* 48: 555-560.

Yunusa, A. A. (2008). Prevalence and characterization of infectious coryza and fowl cholera agents in local chickens in Sokoto State, Nigeria. Ph.D *Thesis,*Usmanu Danfodiyo University Sokoto, Nigeria, Pp. 84-86.

Zahoor, M. A. and Siddique, M. (2006). Characteristic of *Pasteurella multocida* recovered from avian sources. *Pakistan Veterinary Journal,* 26: 41-43.

Zucker, B. and Horsch, F. (1996). Differentiation of *Pasteurella multocida* subspecies *multocida* isolates from the respiratory system of pigs by using polymerase chain reaction fingerprinting technique. *Zentralbi Vetarinamed*, 43: 585-591.

APPENDICES

Appendix 1: Biochemical profile of 36 presumptive *Pasteurella multocida* isolated from chickens in Jos metropolis

P.m isol.	Gram rxn	ind	No_3	Oxi	Ure	Hae	Cat	H_2S	ODC	Lac	Mal	Man	Glu
1	-	+	+	+	-	-	+	-	+	-	-	+	+
12	-	+	+	+	-	-	+	+	+	-	+	-	-
57	-	+	+	+	-	-	+	+	-	+	-	+	+
72	-	+	+	+	-	-	+	+	+	+	-	+	+
122	-	+	+	+	-	-	+	-	+	-	-	+	+
150	-	+	+	+	-	-	+	-	+	-	-	-	+
200	-	+	+	+	-	-	+	+	-	+	+	+	-
207	-	+	+	+	-	-	+	-	+	-	-	+	+
231	-	+	+	+	-	-	+	-	+	-	-	+	-
236	-	+	+	+	-	-	+	-	+	-	-	+	+
258*	-	+	+	+	-	-	+	-	+	-	+	+	+
354*	-	+	+	+	-	-	+	-	+	-	-	+	-
30	-	+	+	+	-	+	-	-	-	-	-	+	+
46	-	+	+	+	-	-	-	-	+	-	-	+	+
40	-	+	+	+	-	+	-	-	-	-	-	+	+
4	-	+	+	+	-	-	+	-	-	-	-	+	+
111	-	+	+	-	-	+	+	-	-	-	-	+	+
63	-	+	+	-	-	-	-	-	-	-	-	+	+
182	-	+	+	+	-	-	+	-	-	-	-	+	+
180	-	+	+	-	-	+	-	-	+	-	+	+	-
382*	-	+	+	+	-	-	+	-	-	+	-	+	+
392*	-	+	+	+	-	-	-	-	-	-	-	+	+
293*	-	+	+	+	-	+	-	-	-	-	-	-	+
398	-	+	+	+	-	+	-	-	-	+	+	+	-
251	-	+	+	+	+	-	-	-	-	+	-	+	+
29*	-	+	+	-	-	-	-	-	+	-	-	-	+

104	-	+	+	-	-	-	-	-	+	-	-	+	-
90	-	+	+	-	-	+	-	-	+	-	-	+	+
89*	-	+	+	-	-	-	-	-	+	-	-	+	+
62*	-	+	+	+	-	-	+	-	+	-	-	+	-
68	-	+	+	-	+	+	-	-	+	-	-	-	+
76	-	+	+	-	-	-	-	-	+	-	-	+	-
77*	-	+	+	-	-	-	-	-	+	-	-	+	+
79	-	+	+	+	-	-	-	-	+	-	-	+	-
82*	-	+	+	-	-	-	-	-	+	-	-	-	+
112	-	+	+	+	+	-	+	-	+	-	-	+	+

*_Pasteurella multocida_ recovered from apparently healthy chickens. P. m – _Pasteurella multocida_, isol - isolate

Appendix 2: Macrobact GNB 24 E analytical profile index of 25 *Pasteurella multocida*

LANDSCAPE

Appendix III: Breed of clinically sick chickens sampled in Jos metropolis

Breed of chickens	Number of chickens	Percentage
Isa brown	143	35.7
Hubbard	48	12
Marshall	43	10.7
Ross	33	8.3
Dominant black	66	16.5
Others	67	16.8
Total	400	100

X^2=2.19, df=3, p>0.05

Others = Chickens whose breeds were not determined.

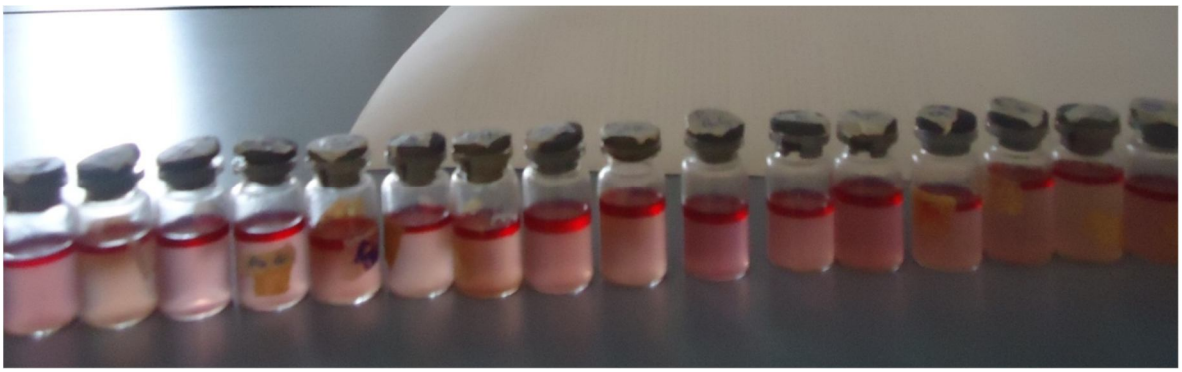
Appendix IV: Age distribution of clinically sick chickens sampled in Jos metropolis

Age (weeks)	Number of chickens	Percentage
16 weeks above	270	67.5
11-15	106	26.5
0-10	24	6
Total	400	100

X^2=6.01, df=2, p<0.005

Appendix V: Morphobiological and biochemical characterization of *Pasteurella multocida* isolated from chickens.

Test	Reaction	Percentage
Gram stain	-ve	100
Urease production	-ve	91.7
Oxidase production	+ve	69.4
Nitrate reduction	+ve	100
Indole reaction	+ve	100
Catalase reaction	+ve	50
Hemolysis	-ve	87.8
Hydrogen sulphide (H_2S)	-ve	88.9
Ornithine Decarboxylase	+ve	63.9
Lactose fermentation	-ve	83.7
Maltose fermentation	-ve	86.1
Mannose fermentation	+ve	83.3
Glucose fermentation	+v	72.2



Appendix VI**:** Positive indole reaction with pink ring layer on peptone water

Appendix VII: Antimicrobial susceptibility profile of *Pasteurella multocida* isolated from clinically and apparently healthy chickens in Jos metropolis, Nigeria.

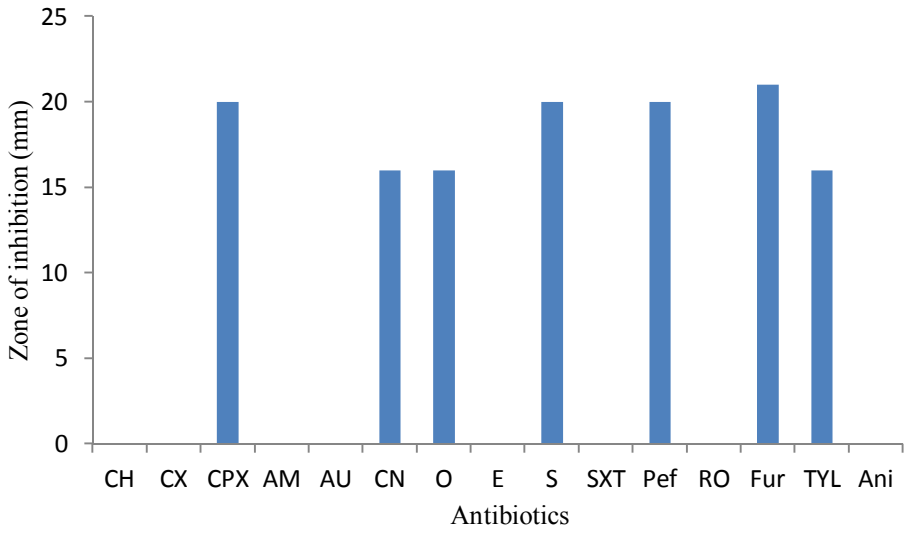
Antibiotic agent and disc concentration (μg)		No. of strains resistant	No. of strains intermediate	No. of strains sensitive	(%) sensitivity	(%) resistant	(%) Int
Chloramphenicol	30	2	3	7	58.3	16.7	25
Enrofloxacin	10	2	-	10	83.3	16.7	0.0
Ampicillin	10	11	1	-	0.0	91.7	8.3
Amoxicillin/Clavu.	30	10	2	-	0.0	83.3	16.6
Gentamicin	10	-	-	12	100	0.0	0.0
Oxytetracycline	10	1	1	10	83.3	8.3	8.3
Erythromycin	10	7	1	4	33.3	58.3	8.3
Streptomycin	10	-	1	11	91.7	0.0	8.3
Trimethoprim/Sulfa.	30	8	-	4	33.3	66.7	0.0
Ciprofloxacin	10	-	-	12	100	0.0	0.0
Pefloxacin	10	1	1	10	83.3	8.3	8.3
Rocephin	25	3	1	8	66.7	25.0	8.3
Furasol	10	2	-	10	83.3	16.7	0.0
Tylosin	10	4	4	4	33.3	33.3	33.3
Anicillin	10	7	2	3	25.0	58.3	16.6

Key

Amo/Clavu – Amoxicillin/Clavulanic potassium

Trimeth/Sulfa – Trimethoprim/Sulfamethoxazole

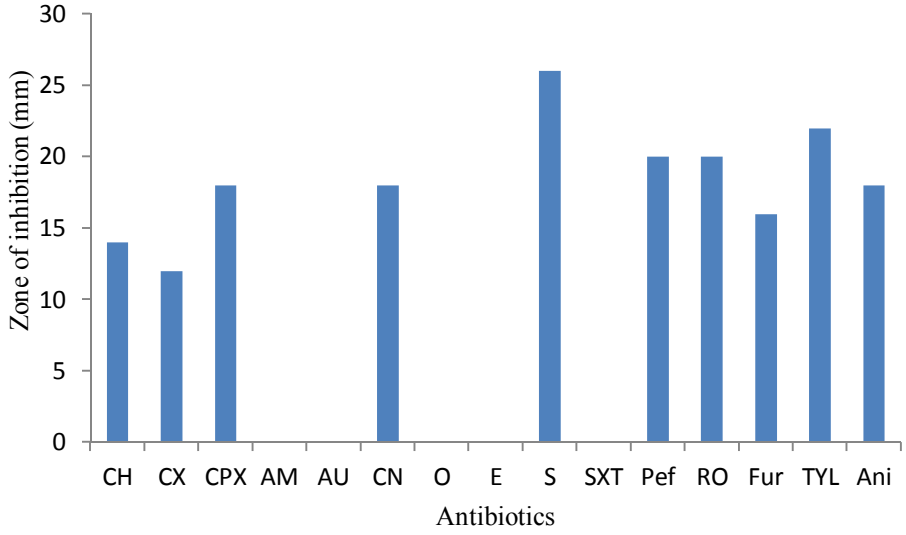
Int - Intermidiate



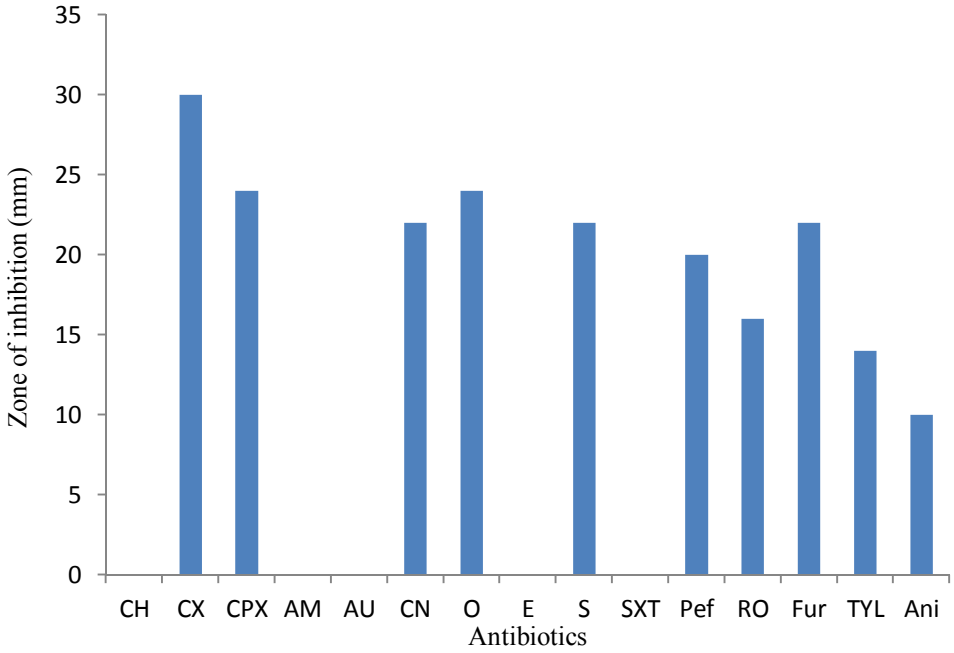
Appendix VIII: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 1recovered from chicken in Jos metroplis

KEY

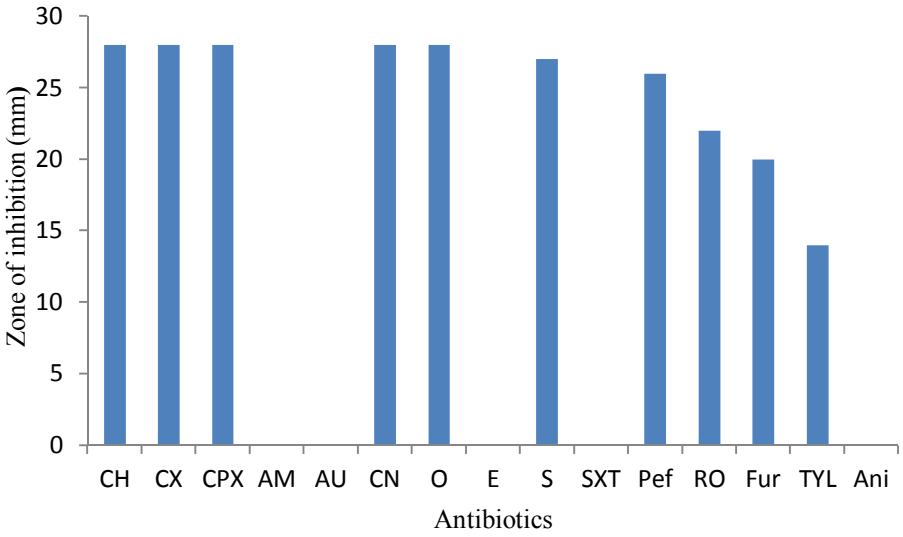
CH – chloramphenicol
CX – ennfloxacin
CPX –ciprofloxacin
AM – amoxicillin/clavulanate potassium
AU – augumentin
CN – gentamicin
O – oxytetracyclin
E – erythromicin
SXT –trimethoprime/sulfamethoxazole
Pef – perfloxacin
RO – rocephin
Fur – furasol
Tyl – Tylosin
Ani - anicillin
S – streptomycin



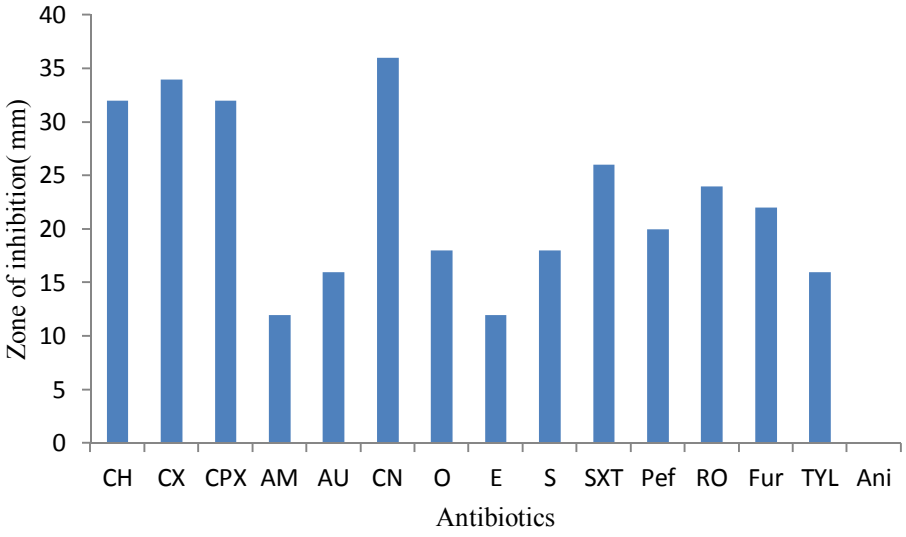
Appendix IX: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 12 recovered from chicken in Jos metropolis.



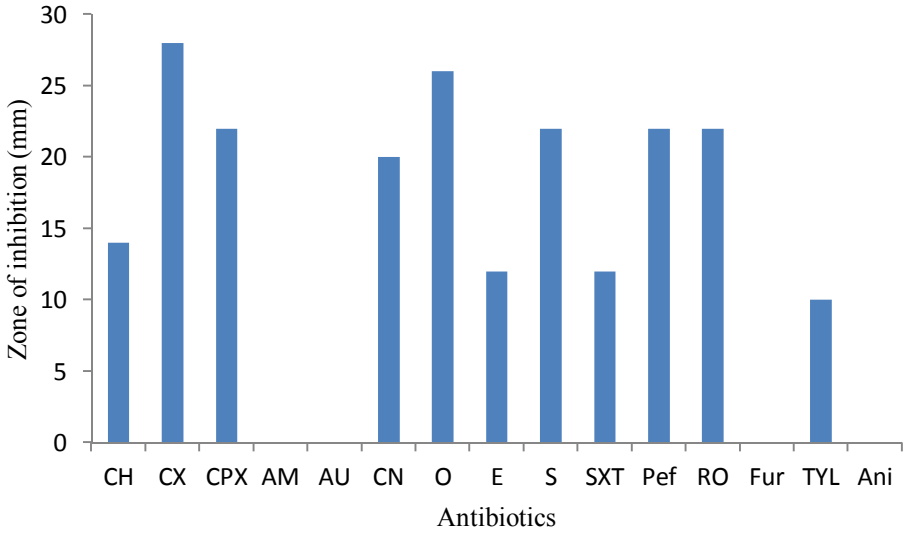
Appendix X: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 57 recovered from chicken in Jos metropolis.



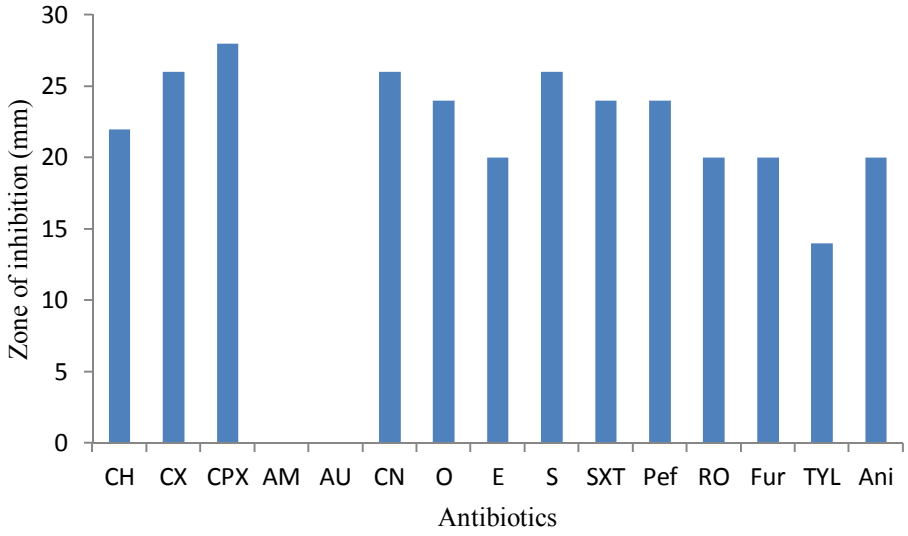
Appendix XI: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 72 recovered from chicken in Jos metroplis



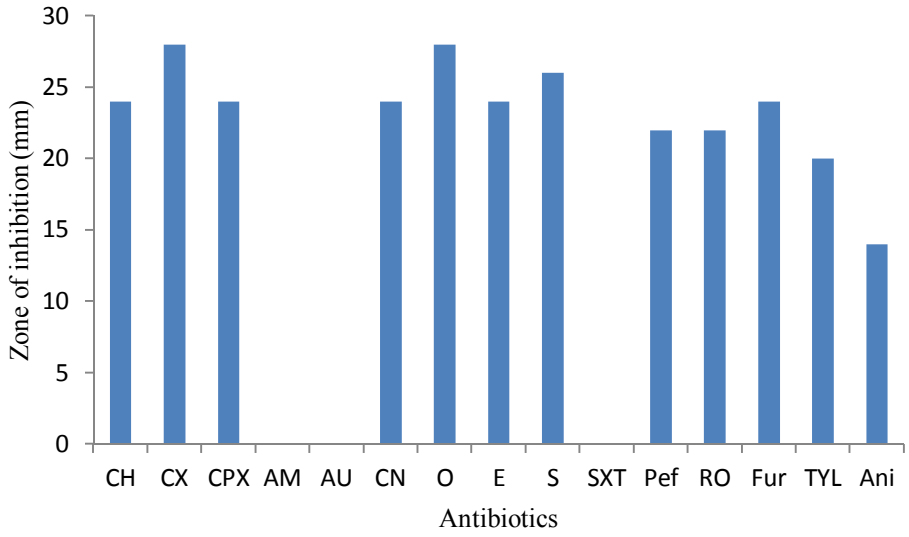
Appendix XII: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 122 recovered from chicken in Jos metroplis



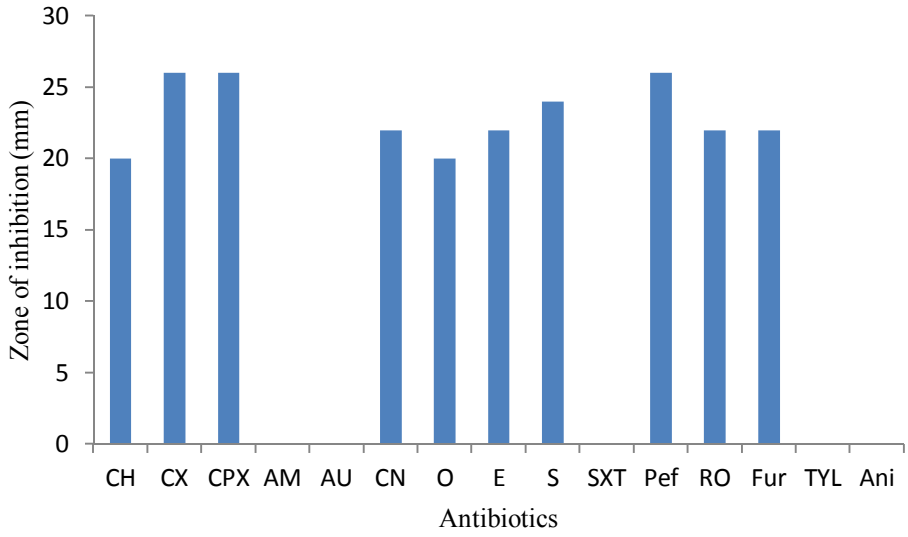
Appendix XIII: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 150 recovered from chicken in Jos metropolis.



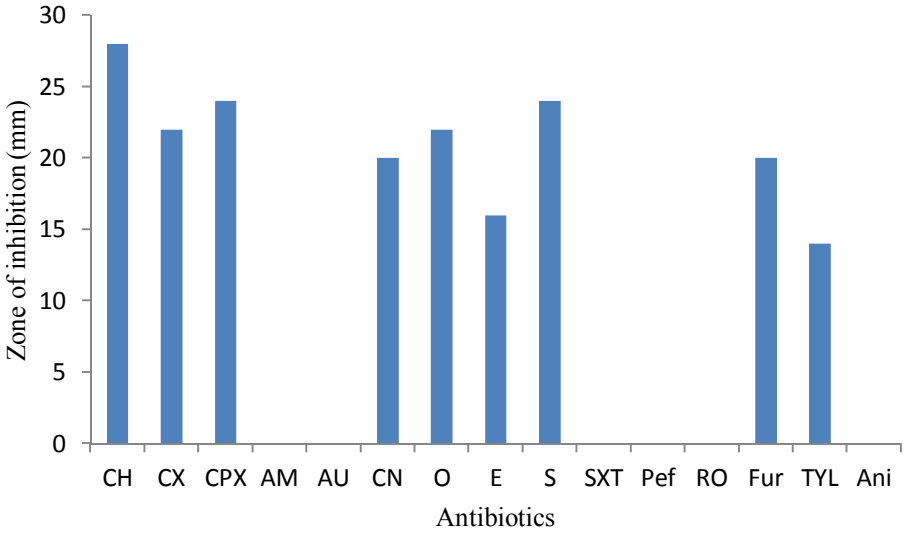
Appendix XIV: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 200 recovered from chicken in Jos metropolis.



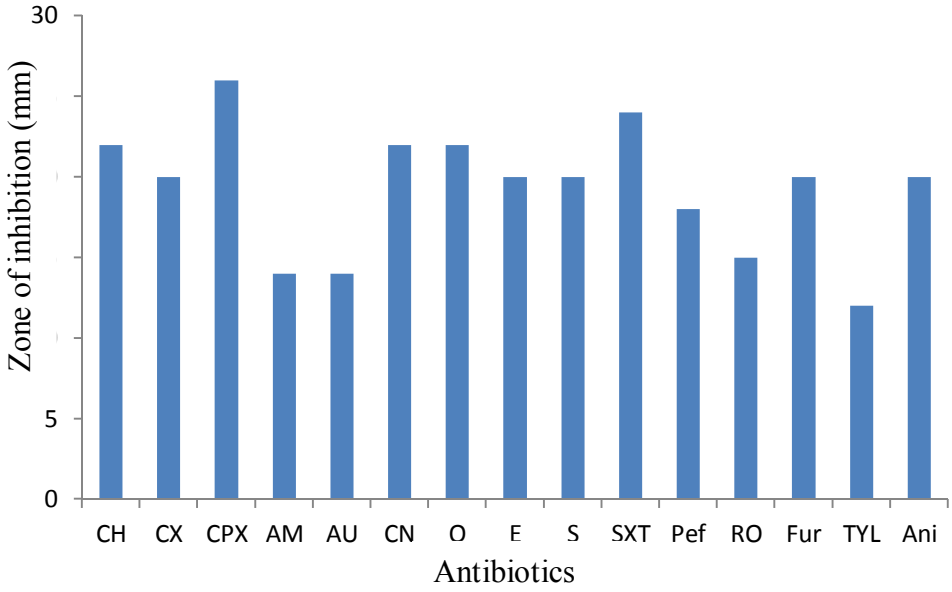
Appendix XV: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 207 recovered from chicken in Jos metropolis.



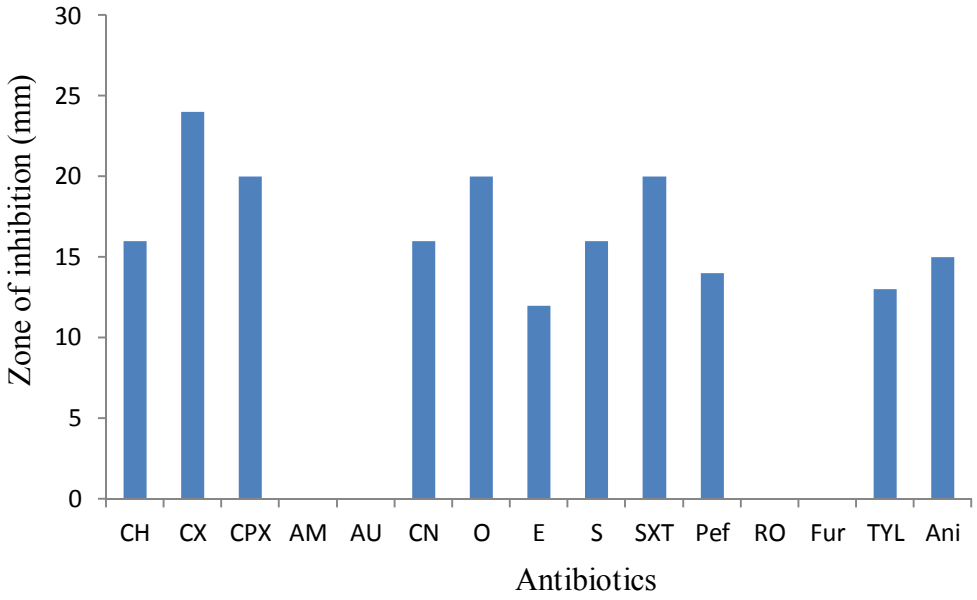
Appendix XVI: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 231 recovered from chicken in Jos metropolis.



Appendix XVII: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 236 recovered from chicken in Jos metropolis.



Appendix XVIII: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 258 recovered from chicken in Jos metropolis



Appendix XIX: Bar chart showing the susceptibility of *Pasteurella multocida* isolate 354 recovered from chicken in Jos metropolis.

Appendix XX: Daily mortality in chickens inoculated with *P. multocida* serotypes A: 1 A: 3 and A: 4

Specie of poultry	Route of inoc	*Pasteurella multocida* serotype	Dose (ml)	Conc. of inoc.	Num. Inoc.	Mortality per day					
						1	2	3	4	5	9
Chicken	(im)	A: 1	0.1	10^8	2					2/2(100%)	
				10^7	2					2/2(100%)	
				10^6	2					1/2(50%)	
		A: 3	0.1	10^8	2					2/2(100%)	
				10^7	2					1/2(100%)	
				10^6	2					(0%)	
		A: 4	0.1	10^8	2					(0%)	
				10^7	2					(0%)	
				10^6	2					(0%)	
		Vaccine strain	0.1	10^8	2					(0%)	
				10^7	2					(0%)	
				10^6	2					(0%)	
		Control (Normal Saline)	0.1	-	6					(0%)	

Appendix XXI: Daily mortality in Japanese quails inoculated with *P. multocida* serotypes A: 1 A: 3 and A: 4

Specie of poultry	Route of inocul.	*Pastenrella multocida* serotype	Dose (ml)	Conc. of inocul	Numb. Inocul.	Day mortality
						Day 7
Japanese quail	(im)	A: 1	0.1	10^8	2	2/2(100%)
				10^7	2	2/2(100%)
				10^6	2	1/2(50%)
		A: 3	0.1	10^8	2	2/2(100%)
				10^7	2	2/2(100%)
				10^6	2	(0%)
		A: 4	0.1	10^8	2	2/2(100%)
				10^7	2	(0%)
				10^6	2	(0%)
		Vaccine strain	0.1	10^8	2	(0%)
				10^7	2	(0%)
				10^6	2	(0%)
		Control Normal saline	0.1	-	6	(0%)

Appendix XXII: Day mortality in mice inoculated with *Pasteurella multocida* serotypes A: 1 A: 3 and A: 4.

Specie Of poultry	Route of inocu	*Pasteurella multocida* serotypes	Dose (ml)	Conc. of inocul	Numb. inocul	Day mortality				
						1	2	3	4	5
Mice	(ip)	A: 1	0.1	10^8	2	2/2(100%)				
				10^7	2	2/2(100%)				
				10^6	2	2/2(100%)				
		A: 3	0.1	10^8	2	2/2(100%)				
				10^7	2	2/2(100%)				
				10^6	2	2/2(100%)				
		A: 4		10^8	2	2/2(100%)				
				10^7	2	2/2(100%)				
				10^6	2	2/2(100%)				
		Vaccine strain		10^8	2	(0%)				
				10^7	2	(0%)				
				10^6	2	(0%)				
		Control Normal saline	0.1	-	6	(0%)				